PHENOTYPIC AND FUNCTIONAL ASPECTS OF CD8+ T CELLS IN ATOPY

from populations to cells

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“To give less than your best is to sacrifice the gift”

Steve Prefontaine

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Resumo Alargado

DOENÇA ALÉRGICA

A prevalência das doenças alérgicas tem vindo a aumentar nas últimas décadas, especialmente nos países industrializados. Estas doenças surgem em indivíduos geneticamente predispostos, sob influência de factores ambientais. A "hipótese higiénica" procurou explicar este aumento de prevalência, relacionando-o com uma diminuição do contacto com microrganismos e do número de infecções, características do modo de vida ocidental. Inicialmente, pensou-se que a redução do contacto com microrganismos impedia o desvio imune de respostas do tipo T auxiliar 2 ("*T helper 2*" ou Th2) para respostas do tipo Th1, que ocorre geralmente com a idade (de acordo com o paradigma Th1/Th2). A inflamação, que ocorre particularmente ao nível das vias aéreas na asma e na rinite seria resultado de uma perda de equilíbrio entre respostas do tipo Th1, importantes nos mecanismos de defesa intracelular e respostas do tipo Th2, que caracterizam a inflamação alérgica. No entanto, estudos epidemiológicos posteriores puseram esta interpretação em causa. Foi então sugerido que, por diminuição da actividade reguladora/supressora dos linfócitos T, ocorre não um desvio imune, mas sim um desenvolvimento de respostas inadequadas face a estímulos antigénicos, resultando quer em doenças alérgicas quer em doenças auto-imunes.

A atopia consiste numa predisposição genética para produzir anticorpos IgE contra antigénios ambientais comuns (alergénios) e correlaciona-se com o desenvolvimento de uma ou mais doenças alérgicas. Estas caracterizam-se pela infiltração ao nível do órgão-alvo de células inflamatórias activadas, das quais se destacam os eosinófilos, os mastócitos e os linfócitos T.

De facto, as células T, especialmente as células T CD4+ do tipo Th2, são consideradas as grandes orquestradoras da resposta alérgica. As suas funções são mediadas principalmente pela secreção de citocinas do tipo 2 (interleucina (IL)-4, IL-5, IL-9 e IL-13). Posteriormente, foi mostrado que também células T CD8+ eram capazes de sintetizar estas citocinas (padrão "*T cytotoxic 2*" ou Tc2). No entanto, o papel desempenhado pelas células T CD8+ na doença alérgica ainda não está bem esclarecido. Diversos estudos,

nomeadamente em modelos animais, referem resultados contraditórios, atribuindo às células T CD8+ quer funções reguladoras, quer funções promotoras da inflamação.

A existência de subpopulações de células T CD8+ com características fenotípicas e funcionais distintas poderá ser um dos motivos para esta disparidade de resultados.

Estudos efectuados em indivíduos atópicos sugeriram que as células T CD8+ poderiam estar envolvidas no desenvolvimento de asma alérgica. Células T CD8+ estavam presentes nos infiltrados celulares no órgão-alvo, sendo que esta presença se correlacionava com asma severa e morte por asma brônquica. No entanto, estudos noutras patologias relacionaram a existência de células T do tipo CD8+ com funções reguladoras. A evidência é mais relevante em casos de transplantes de órgão sólido, onde a presença de células T supressoras do tipo CD8+CD28− se correlaciona com aumento de tolerância ao transplante e menor necessidade de medicação imunossupressora.

Tendo em atenção estes e outros estudos prévios, procurámos, neste estudo, clarificar o papel das células T CD8+ na atopia, nomeadamente no que diz respeito ao seu fenótipo e função na asma e rinite alérgicas. Definimos cinco objectivos: avaliar a proliferação alergénio-específica e a síntese de citocinas por células T CD8+; verificar se as células T CD8+ isoladas do sangue periférico têm propriedades supressoras face a respostas alergénio-específicas; verificar se as células T CD8+ estão presentes ao nível do órgão-alvo e se se encontram activadas; observar se existe correlação entre a expressão dos marcadores de activação expressos por células T CD8+ e a severidade da asma ou o seu controlo e por último, pretendemos caracterizar demograficamente a nossa população de estudo.

De modo a contextualizar os resultados a nível celular, decidimos iniciar o nosso estudo pela caracterização epidemiológica da população alvo (doentes com rinite alérgica e doentes com asma brônquica alérgica). Apesar do número de publicações sobre prevalência, diagnóstico e tratamento das doenças alérgicas ter aumentado nos últimos anos, dados relativos aos perfis de sensibilização continuam escassos. Dados relativos a prevalência em crianças foram obtidos maioritariamente através do estudo ISAAC ("*International Study on Asthma and Allergy in Children*") nas suas diversas fases. Neste estudo, os dados relativos a Portugal mostram um aumento da prevalência na rinoconjuntivite alérgica e na asma brônquica em ambos os grupos etários de crianças (6-7 anos e 13-14 anos) entre as fases I e III, separadas no tempo por sete anos.

Relativamente aos dados na população adulta, Portugal participou no estudo ECHRS ("*European Community Respiratory Health Survey*"), que avaliou, através de questionário,

adultos entre os 20 e os 44 anos. Dados relativos a este estudo estimam uma prevalência de asma de cerca de 5% e de rinite de 17% para a população avaliada.

Apesar da região da Cova da Beira ter dos mais altos níveis polínicos a nível nacional (como descrito no *Mapa Polínico de Portugal* (1998-1999), neste momento não está incluída na Rede Portuguesa de Aerobiologia, nem existe nenhuma estação de monitorização de pólenes na região. Os dados sobre doença alérgica, especificamente relativos à região da Cova da Beira são muito escassos, revelando esforços mais ou menos individuais para clarificar a situação em termos de prevalência, sensibilizações e severidade de patologia.

Pareceu-nos, pois, essencial caracterizar a doença alérgica na Cova da Beira de um modo mais sistematizado, como complemento importante dos nossos estudos de Imunologia básica da Alergia.

Recolhemos dados relativos aos doentes da consulta de Imunoalergologia do Hospital da Cova da Beira, centrando-nos especificamente em doentes com asma brônquica e rinite alérgica. Para o estudo da asma brônquica foram incluídos doentes admitidos à Consulta de Imunoalergologia entre 2003 e 2006 (1078 doentes), enquanto o estudo da rinite incluiu doentes admitidos à Consulta de Imunoalergologia entre 2003 e 2007 (1092 doentes). Para a análise posterior foram excluídos doentes com menos de 18 anos, doentes não residentes no concelho e todos aqueles com resultados laboratoriais discordantes em termos de avaliação de alergia.

A asma brônquica é uma doença inflamatória crónica, caracterizada por obstrução intermitente, reversível espontaneamente ou mediante tratamento e por hiper-reactividade brônquica. A rinite é também uma doença com características inflamatórias, caracterizada clinicamente por rinorreia, obstrução nasal e crises esternutatórias. Apesar dos avanços no conhecimento da sua fisiopatologia e terapêutica, a asma e a rinite mantém-se como duas das doenças crónicas mais frequentes em crianças e adultos.

O diagnóstico da asma e da rinite foi feito com base numa história clínica positiva e no exame objectivo (incluindo rinoscopia anterior), suportado por testes específicos de alergia (testes cutâneos por picada e determinação de IgE específica sérica), bem como por provas funcionais ventilatórias, no caso da asma brônquica. De acordo com a presença ou não de processos mediados pela IgE, os doentes foram divididos em duas grandes categorias: alérgicos e não alérgicos. Para a classificação de aspectos ligados à severidade da doença e ao seu controlo foram aplicadas "*guidelines"* internacionais ("*Global Initiative for Asthma*" para a asma e "*Allergic Rhinitis and its Impact on Asthma*" para a rinite).

Foram também avaliadas as principais co-morbilidades da asma e da rinite, nomeadamente no que diz respeito ao conceito actual de "*one airway one disease*" que considera asma e rinite manifestações de uma mesma entidade. Especificamente, doentes com rinite foram avaliados quanto à presença de asma pela história clínica, exame objectivo do tórax e avaliação de obstrução e, do mesmo modo, doentes com asma foram avaliados quanto à presença de rinite pela história clínica e rinoscopia anterior.

Trinta por cento dos doentes asmáticos e vinte e oito por cento dos doentes com rinite eram não alérgicos. Globalmente, os doentes não alérgicos eram mais velhos, predominantemente do sexo feminino e com uma maior prevalência de alergia/intolerância medicamentosa. Como descrito atrás e de acordo com as "*guidelines"* do projecto ARIA ("*Allergic Rhninitis and its Impact on Asthma*"), verificámos a associação de asma e rinite. Na população em estudo, a rinite estava mais frequentemente associada com a asma não alérgica do que com a asma alérgica. Pelo contrário, a asma brônquica estava preferentemente associada com rinite alérgica. No primeiro caso, estes resultados podem dever-se ao facto da rinite alérgica geralmente preceder o desenvolvimento de asma alérgica (marcha atópica). De facto, a imunoterapia específica parece atrasar ou mesmo impedir a progressão da rinite alérgica para asma. Pelo contrário, não existe esta progressão temporal quando se fala de doença não alérgica, em que geralmente existem desencadeantes comuns.

Os dois tipos de asma e de rinite (alérgica e não alérgica) não puderam ser distinguidos em termos de severidade da doença, nem em termos de sintomatologia clínica. Este facto era inesperado, uma vez que estudos anteriores mostravam que asma e rinite não alérgicas eram mais severas do que quando se deviam a alergia. Contudo, não podemos descurar que esta conclusão possa ter tido origem num viés na análise: geralmente a severidade da doença aumenta com a idade e os doentes com patologia não alérgica são mais velhos, pelo que, em estudos de amostras não emparelhadas quanto à idade, a patologia não alérgica vai parecer mais severa.

Os alergénios mais frequentes na região são os pólenes de gramíneas (*Poaceae*), sejam elas cultivadas ou não, os ácaros e os pólenes de oliveira. A monosensibilização não é comum e, nos casos em que existe, refere-se unicamente a alergia a ácaros. Um dado interessante prende-se com o facto de os pólenes das gramíneas não serem apenas responsáveis pela maioria das sensibilizações, mas serem também os responsáveis pelos níveis mais elevados de IgE específica. No entanto, não foi possível estabelecer uma correlação entre os níveis de IgE específica e a severidade da asma ou da rinite.

Estudos semelhantes levados a cabo noutras regiões mostraram uma associação significativa entre os níveis de poluição atmosférica e o aumento da sintomatologia ligada à asma e à rinite. A prevalência de atopia e doença alérgica era maior nas zonas urbanas do que nas zonas rurais, devido quer a um aumento da poluição atmosférica nas zonas urbanas, quer a um efeito protector do ambiente rural. Nas áreas urbanas, os grãos de pólen podem ficar revestidos com resíduos de combustível e com produtos da combustão, sendo que esta ligação a partículas de exaustão de diesel pode alterar os epítopos alergénicos e mesmo aumentar a sua alergenicidade. Convém salientar que irritantes não específicos como a poluição atmosférica são capazes de agravar ou mesmo induzir sintomas em doentes asmáticos.

No nosso estudo, asmáticos residentes em ambiente rural ou urbano apresentavam perfis de sensibilização distintos, tendo, no entanto severidades da doença semelhantes. Doentes com rinite alérgica apresentavam perfis de sensibilização semelhantes. Estes resultados podem ser explicados pelo tamanho relativo dos grãos de pólen que condiciona a sua passagem pelas vias aéreas. Geralmente, grãos de pólen intactos são incapazes de penetrar as regiões mais distais das vias aéreas, sendo a maioria dos sintomas localizados ao nível dos olhos, nariz e faringe. No entanto, alergénios derivados dos grãos de pólen podem depositar-se em partículas sub-micrónicas e desencadear asma, tal como ocorre durante as trovoadas.

Os nossos estudos epidemiológicos forneceram uma base de trabalho extensa e pormenorizada da nossa população, permitindo delinear melhor os estudos celulares subsequentes. Por outro lado, estes estudos vieram reforçar a necessidade de tratar em conjunto doenças das vias aéreas superiores e inferiores usando uma estratégia simultaneamente mais eficaz e mais segura. No entanto, estes estudos apresentam uma importante limitação, visto analisarem amostras provenientes de uma consulta da especialidade. De um modo geral, a maioria dos doentes seguidos em consultas da especialidade são aqueles em que a severidade da doença é maior. Outro ponto a considerar prende-se com a classificação da severidade da doença. Apesar de esta ter sido efectuada na primeira consulta, alguns dos doentes já se encontravam sob medicação, o que poderia enviesar a classificação.

De qualquer forma, trata-se, na nossa opinião, de um estudo muito interessante uma vez que inclui parâmetros clínicos, contrastando com a maioria dos estudos anteriores que apenas se baseavam em questionários.

CÉLULAS T CD8+ NA ALERGIA

A asma e a rinite alérgicas caracterizam-se pela presença de uma inflamação crónica mediada por múltiplas proteínas inflamatórias, incluindo citocinas, quimiocinas e enzimas. Em ambas as patologias ocorrem exacerbações quando a intensidade da inflamação aumenta. Diversos estudos sugeriram que as células T desempenham um papel fundamental nesta inflamação. Através da síntese e secreção de citocinas do tipo 2, as células T promovem a diferenciação de células Th2 e a síntese de IgE pelas células B (IL-4 e IL-13); promovem a diferenciação, a activação e a sobrevivência dos eosinófilos (IL-5) e aumentam a diferenciação de mastócitos e a produção de muco (IL-9). Tal como foi referido, a grande maioria dos estudos avaliou fenotípica e funcionalmente as células T CD4+, sendo escassos os resultados relativos às células T CD8+.

Uma vez que a atopia se caracteriza por elevados níveis de IgE específica sérica, muito provavelmente alterações no órgão-alvo vão reflectir-se ao nível do sangue periférico. O estudo das células T CD8+ do sangue periférico de doentes alérgicos constituiu o nosso primeiro projecto ao nível da imunologia básica da doença alérgica. Uma vez que a expressão da molécula co-receptora CD28 permite definir duas subpopulações distintas ao nível das células T CD8+, procurámos, ao longo do estudo, aprofundar a sua caracterização fenotípica e funcional.

As células T foram isoladas a partir de sangue periférico de doentes com asma e/ou rinite por centrifugação em gradiente de densidade. Em seguida, as células T CD8+CD28+ e CD28− foram isoladas por separação imunomagnética num sistema semi-automático (MACS®) e usadas, posteriormente, para os estudos funcionais. Para avaliar a proliferação e síntese de citocinas em resposta a aeroalergénios usámos extracto purificado de *Dermatophagoides pteronyssinus*. Para avaliar a capacidade supressora das células T CD8+ face às respostas alergénio-específicas desenvolvemos um sistema de co-culturas com células mononucleares do sangue periférico tratadas com mitomicina. A pureza das fracções usadas foi avaliada por citometria de fluxo.

Observámos que a atopia não estava relacionada com alterações nas percentagens ou fenótipo das células T CD8+; no entanto, células T CD8+CD28+ eram distintas em termos fenotípicos de células T CD8+CD28−. Esta alteração fenotípica era acompanhada

por uma resposta proliferativa distinta relativamente a mitogénios e alergénios, se bem que semelhante do ponto de vista da secreção de citocinas.

Ambas as subpopulações apresentavam uma capacidade proliferativa semelhante em doentes atópicos e não atópicos e não apresentavam características supressoras.

Estes resultados devem, no entanto, ser analisados com cuidado, uma vez que as culturas *in vitro* não conseguem imitar o estímulo repetido e prolongado que ocorre *in vivo*. Além disso, as células T do sangue periférico não partilham o mesmo ambiente das células T dos órgãos-alvo, não estando sujeitas ao mesmo tipo de interacções e contacto com alergénio.

Para clarificar se o que ocorre ao nível do sangue periférico reflecte o que se passa no órgão-alvo, procurámos estudar células T CD8+ provenientes deste. Procurando uma abordagem menos invasiva do que os lavados broncoalveolares ou as biópsias, realizámos o estudo em células T provenientes da expectoração induzida de doentes asmáticos alérgicos.

Foram incluídos no estudo asmáticos alérgicos com asma intermitente ou moderada persistente com diversos graus de controlo.

A expectoração induzida foi obtida por inalação de uma solução hipertónica salina (NaCl a 5%). A expectoração foi seguidamente homogeneizada e incubada com ditiotreitol, sendo posteriormente filtrada através de um filtro celular de 0.40μm de diâmetro de poro. A viabilidade da suspensão celular obtida foi avaliada pelo método de exclusão de Azul Tripano em câmara de Neubauer.

Neste estudo mostramos claramente que, quer as células T CD4+, quer as células T CD8+ apresentam um fenótipo activado na expectoração induzida, o que sugere que ambas as subpopulações celulares participam no processo inflamatório ao nível do órgão-alvo. Uma vez que esta inflamação se relaciona com a severidade da doença, procurámos avaliar a correlação existente entre a activação celular e o grau de severidade da asma.

Apesar da escolha criteriosa de voluntários, emparelhados quanto a sexo, idade e controlo da doença, não observámos correlação entre a percentagem relativa de expressão de marcadores de activação (CD25 e CD69) e o grau de severidade da doença.

Atendendo a que o controlo é um dos objectivos mais determinantes do tratamento da asma, avaliámos também a correlação existente entre os nossos marcadores de activação em estudo e o controlo da doença. Optámos aqui por incluir apenas asmáticos alérgicos com asma moderada persistente, sob tratamento mais ou menos equivalente. Tal como se verificou com a severidade, também não se observou correlação entre a percentagem relativa dos marcadores de activação e o controlo da doença.

Para clarificar o efeito dos corticosteróides inalados na expressão dos marcadores de activação, comparámos a percentagem relativa dos marcadoress de activação entre asmáticos com asma intermitente controlados e não controlados. Não observámos correlação entre a percentagem relativa dos marcadores e o grau de controlo dos doentes, o que parece indicar um efeito mínimo dos corticosteróides inalados em termos de avaliação de controlo da asma.

Em resumo, nesta Tese clarificámos as características demográficas da população de asmáticos e doentes com rinite da consulta de especialidade do Hospital Pêro da Covilhã e caracterizámos as células T, especialmente da população T CD8+ nestes indivíduos em termos fenotípicos e funcionais. Finalmente, procurámos novas estratégias para isolar células T do sangue periférico e diferenciar linhas celulares T alergénio-específicas.
Relativamente às nossas hipóteses iniciais, verificámos que as células T CD8+ isoladas a partir do sangue periférico não apresentavam características supressoras das respostas alergénio-específicas. Em segundo lugar, as células T CD8+ da expectoração induzida de doentes asmáticos expressavam marcadores de activação, sendo a percentagem relativa de expressão maior do que a encontrada ao nível do sangue periférico. No entanto, embora apresentando um fenótipo de células activadas, a percentagem relativa dos marcadores de activação não se correlacionou com a severidade da doença nem com o seu grau de controlo.

Abstract

The prevalence of allergic diseases has been increasing during the last decades. It is believed that the development of allergic diseases in susceptible individuals is dependent on T cells, especially type 2 CD4+ T cells. CD8+ T cells may also be involved in the pathophysiology of allergic diseases, albeit studies in animal models of asthma have found confounding results.

With our study we attempted to clarify the phenotypic and functional properties of CD8+ T cells in allergic disease settings. The specific aims were to assess allergen-specific proliferation and cytokine synthesis of CD8+ T cells and evaluate their suppressor function on antigen-specific responses. In addition, it was also our goal to assess the presence of CD8+ T cells at the target organ and their activation status, correlating our findings with disease severity and control. In order to contextualize our cellular findings, we initially characterised our study population from an epidemiological point of view.

We enrolled asthma and rhinitis patients attending the Allergy Clinic of the Cova da Beira Hospital. Asthma and rhinitis were diagnosed by a positive clinical history and specific diagnostic tests, and severity was assessed by current guidelines. We also identified the main co-morbidities in both disease settings and evaluated asthma symptoms in rhinitis patients and rhinitis symptoms in asthma patients. In the allergic groups we investigated the sensitisation profiles to aeroallergens and concluded that major allergens included grass and cereal pollen, mites and olive tree pollen. Sensitisation profiles and severity were also compared between rural and urban-based allergic patients. In this regard, we found contrasting results between asthmatic patients and patients with rhinitis.

In the second part of our study we clarified the role of CD8+ T cells in allergic inflammation through basic cellular systems. We assessed proliferation capacity and cytokine synthesis in response to *Dermatophagoides pteronyssinus* extract, and devised a co-culture system in order to evaluate the suppressor function of CD8+ T cells. CD8+CD28+ and CD28− T cells isolated from the peripheral blood had distinct phenotypes and proliferated at different levels to common stimuli, although sharing similar cytokine production patterns. A potential suppressor activity was not found in the co-culture systems, either for CD8+CD28+ or CD28− T cells.

We then studied CD8+ T cells at the target-organ, assessing activation phenotype in cells from the induced sputum of asthmatic patients. We showed that CD8+ T cells are

activated at the target-organ, although we were unable to demonstrate a relationship between activation of this T cell subset and severity or control of asthma.

Abbreviations

ACT	Asthma Control Test
AHR	Airway hyperresponsiveness
AICD	Activation induced cell death
APC	Antigen presenting cell
-APC	Allophycocyanin conjugated
AR	Allergic rhinitis
ARIA	Allergic Rhinitis and its Impact on Asthma
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
BW	Bronchial washing
CBA	Cytometric bead array
CD	Cluster of differentiation
CFSE	Carboxylfluorescein diacetate succinimidyl ester
COPD	Chronic obstructive pulmonary disease
cpm	Cintilations per minute
CRA	Cockroach antigen
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DC	Dendritic cell
DC2	Plasmacytoid dendritic cell
Der p	*Dermatophagoides pteronyssinus*
DTT	Dithiothreitol
ECRHS	European Community Respiratory Health Survey
ELISA	Enzyme linked imuno-sorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FcεRI	High affinity IgE receptor
FcεRII	Low affinity IgE receptor (CD23)
FEV1	Forced expiratory volume in one second
-FITC	Fluoresceine isothiocyanate conjugated
FOXP3	Forkhead box p3
GINA	Global Initiative for Asthma
GITR	Gluocorticoid-induced TNF receptor
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILT	Immunoglobulin-like transcript
ISAAC	International Study of Asthma and Allergies in Childhood
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
NAR	Non-allergic rhinitis
NFκB	Nuclear factor kappa-B
NK	Natural killer
OVA	Ovalbumin
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
-PE	Phycoerythrin conjugated
PEF	Peak expiratory flow
-PerCP	Peridinin chlorophyll protein conjugated
PHA	Phytohaemagglutinin
PMA	Phorbol myristate acetate
sem	Standard error of the mean
Tc	T cytotoxic cells
TCC	T cell clones
TCL	T cell lines
T_{CM}	Central memory CD8+ T cell
TCR	T cell receptor
T_{EFF}	Effector memory CD8+ T cell
Th	T helper cells
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	T regulatory (cells)
Ts	T suppressor (cells)

List of Publications

Related to this Thesis

Functional and phenotypic characterization of CD8+CD28+ and CD28− T cells in atopic individuals sensitized to *Dermatophagoides pteronyssinus*.
Lourenço O, Fonseca AM, Arosa FA, Taborda-Barata L
Allergol. et Immunopathol. 2006; 34 (6): 234-41

Demographic, laboratory and clinical characterisation of adult Portuguese asthmatic patients.
Lourenço O, Fonseca AM, Taborda-Barata L
Allergol. et Immunopathol. 2007; 35 (5): 177-83

Non-allergic rhinitis is more frequently associated with asthma than allergic rhinitis in adult Portuguese patients.
Lourenço O, Fonseca AM, Taborda-Barata L
Rhinology (Accepted for publication)

T cells in Sputum of asthmatic patients are activated independently of disease severity or control.
Lourenço O, Fonseca AM, Taborda-Barata L
Submitted to Respiration

Unrelated to this Thesis

Respostas celulares T aos alergénios do látex.
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Thesis Overview

This Thesis is divided into five chapters.

The first chapter consists of a brief literature review running from atopy and allergic diseases to specific issues regarding CD8+ T cells in allergy settings.

The second chapter includes an overview of the methods used.

Subsequently, the third chapter is based on original research papers published or submitted during the Ph.D. course, and directly related to this Thesis.

The fourth chapter comprises the general discussion and conclusion remarks.

Finally, the fifth chapter refers to unpublished work developed during the course and directly related to this Thesis.

1. General Introduction

1.1. Atopy and Allergic Diseases

Atopy is an individual predisposition for excessive immunoglobulin (Ig)-E production against harmless environmental allergens. It is a genetically and environmentally determined condition [1, 2], which may lead to allergic diseases such as asthma, allergic rhinitis, conjunctivitis, and atopic dermatitis [3]. It is estimated that as many as 300 million people of all ages, and all ethnic backgrounds, suffer from asthma and the burden of this disease to governments, health care systems, families, and patients is increasing worldwide [4].

The prevalence of allergic diseases has been increasing during the last decades, especially in developed countries. The "hygiene hypothesis" attempts to explain this increase on the basis of reduced exposure to infectious agents, especially during early life. Early studies suggested that a skewing of the T cell response towards type 2 CD4+ T cells could explain the subsequent development of allergy [5]. Inflammation, particularly in the airways in asthma and rhinitis would represent a loss of balance between T-helper (Th)-1 cells, critical for intracellular defence mechanisms, and Th2 cells, which mediate allergic inflammation. However, several observations argue against this. Firstly, epidemiological studies showed that both Th2-related allergies and Th1-related autoimmune diseases could co-exist in the same populations [6, 7]. Secondly, high parasite burdens, which are associated with strong Th2 responses, seem to protect against allergic disease [8, 9]. Thirdly, in asthma, IFN-γ levels are enhanced, suggesting that Th1 cytokines contribute to the pathogenesis of the disease, instead of protecting from it [10, 11].

The first interpretation of the "hygiene hypothesis" states that a reduced microbial burden impairs the shift of allergen-specific responses from the Th2 to the Th1 phenotype that usually takes place with age (thereby failing to undergo immune deviation). The more recent interpretation states that the lower microbial burden reduces the activity of regulatory T cells leading to allergy and autoimmunity (reduced immune suppression).

(For review on the "hygiene hypothesis" see [12, 13, 14]).

It is generally believed that the development of allergic disease in susceptible individuals first requires repeated and persistent exposure to allergens, leading to aberrant activation and recruitment of T cells with subsequent differentiation of increased numbers of allergen-specific Th2 cells. These T cells provide interleukin (IL)-4 and contact-mediated

signals that promote differentiation of B cells and class-switching to the production of IgE. The produced IgE binds to high affinity IgE receptors (FcεRI) on the surface of circulating basophils and on mast cells in various tissues. Following such sensitisation, subsequent exposure to the specific allergen initiates a secondary immune response. The allergen binds to cell-associated IgE and cross-links the IgE molecules and the FcεRI receptors to which they are bound. Cross-linking of the IgE-FcεRI complexes on basophils and mast cells signals these cells to release preformed and newly generated mediators, including biogenic amines such as histamine, lipid mediators such as leukotrienes, and cytokines (early phase response) [15]. Biogenic amines and lipid mediators create the signs and symptoms of allergic disease that typically begin within one hour of allergen exposure. These include rapid vascular leakage of plasma fluid and protein, and vasodilatation. Six to twenty-four hours later, a second round of symptoms (late phase reaction) may develop, mainly driven by the release of cytokines and chemokines. Specifically, in terms of target-organs, in the nose, this late reaction is characterised by an inflammatory infiltrate including activated eosinophils, basophils, neutrophils, and lymphocytes; resulting in major congestion, itching and rhinorrhea [2]. Furthermore, in the lung, repeated episodes of this late phase reaction can lead to tissue damage and remodelling.

Antigen presenting cells (APC) including monocytes, macrophages, B cells, dendritic and Langerhans cells are important in IgE-mediated allergic responses. Both the high affinity, FcεRI, and the low affinity FcεRII (CD23) IgE receptors are expressed on these APC and are important in facilitating allergen presentation to allergen-specific T cells. Triggering of the CD23 receptor by IgE on B cells not only enhances antigen presentation, but also increases synthesis of IgE.

The priming of allergen-specific CD4+ T cells and their differentiation towards a Th2 phenotype are key events for the development of allergic diseases. Th2 cells orchestrate atopic inflammation through the secretion of a subset of cytokines, mainly IL-4, IL-5, IL-9 and IL-13. Similarly, CD8+ T cells from atopic patients have the capacity to produce type-2 cytokines (T cytotoxic type 2 or Tc2 phenotype) [16].

1.2. CD8+ T Cells

CD8+ T cells are major histocompatibility complex (MHC) class I-restricted. As MHC class I molecules contain mainly peptides derived from the cytosol, this is an effective mechanism for killing cells infected with viruses or other intracellular pathogens. Antigen recognition by naïve CD8+ T cells triggers a programme of proliferation and differentiation that leads to the production of effector lymphocytes (cytotoxic T lymphocytes or CTL) directly able to lyse antigen-bearing cells. The lytic mechanism primarily involves release of cytoplasmic granules loaded with perforin (a pore-forming protein) and granzyme B (a serine protease) at the contact site between the CTL and the target cell [17], resulting in specific killing without bystander cell damage. The nonsecretory mechanism is initiated by receptor-mediated triggering of apoptosis-inducing target cell surface molecules (Fas-FasL)[17].

However, CD8+ T cells have also been associated with suppression of immune responses, and their ability to produce various cytokines, such as IFN-γ and TNF-α, suggests an additional function.

Generally, the peptides bound in the grooves of the MHC class I molecules are derived from proteins synthesised within the cell that bears the class I molecules (self-proteins and viral proteins); in contrast, allergic responses are thought to occur when CD4+ T cells react to exogenous antigen presented on MHC class II molecules. It was believed that CD8+ T cells were unable to respond to allergens. However, both macrophages and dendritic cells (DC) have been reported to cross-present exogenous antigen to CD8+ T cells in some circumstances, by at least two different mechanisms (the phagosome-to-cytosol pathway and the vacuolar pathway)(for review see [18]).

Altough most allergens are proteins, some are lipids or carbohydrates. Peptides are not the only target of T cell responses, as T cells can recognize lipid antigens through a CD1-dependent pathway. In fact, lipids are important in pollen grain structure and function, and may be relevant in enhancing grain capture by DC, which is likely to be required for their subsequent recruitment of pollen-specific T cells in sensitised subjects [19]. So, a potential pathogenic role can be envisioned for CD1-restricted T cells, as lipid components of allergens can be presented by epithelial DC to T cells [20]. T cell clones specific for pollen lipids secrete both type 1 and type 2 cytokines and display helper

function for IgE production [19]. CD1-restricted T cells can be TCR-αβ, -γδ, or NKT cells, but the majority of lipid-specific clones decribed to date are CD4+ [19, 21].

Memory and Naïve CD8+ T cells

Immune responses to viral infections involve the proliferation and differentiation of CD8+ T cells into an effector population that controls viral replication. The expansion of the effector cells is accompanied by an increased sensitivity to apoptosis, which regulates proliferation and maintains lymphocyte homeostasis. A small proportion of activated CD8+ T cells survive, forming the memory populations. Memory CD8+ T cell subsets have been broadly divided into central memory CD8+ T cells (T_{CM}) and effector memory CD8+ T cells (T_{EFF}) distinguishable by phenotype and function. T_{CM} reside in lymphoid organs, do not express immediate lytic functions, are $CD62^{hi}CCR7^{hi}$ and can be generated following culture with IL-15 after a short stimulation with allergen [22]. T_{EFF} are found in non-lymphoid tissues, express lytic activity, are $CD62^{lo}CCR7^{lo}$ and are generated in culture with IL-2 [22]. T_{EFF} can play a critical role in the development of airway hyperresponsiveness (AHR) and allergic inflammation by producing IL-13 in the lung.

When activated under optimal conditions, these cells will emerge as functionally competent CTL that are armed with various effector mechanisms to eliminate abnormal cells. There are two mechanisms of cytotoxicity: a membranolytic one, depending on the formation of pores in the target cell membrane, and mediated by perforin and granzymes; and a nonsecretory one, receptor-mediated (Fas-FasL) [17]. Helper CD4+ T cells may not be required to initiate CTL activity but are critical for preserving antigen-specific CD8+ T cell responsiveness during chronic infection. Since some pathogens are able to directly stimulate APC to properly present antigen to $CD8^+$ T cells, helper cells may not be required to activate APC during the early stages of infection. During persistent infection, where antigen may be minimal and thus unable to effectively condition professional APC, CD4+ T cells play a critical role in sustaining CD8+ T cell immunity by activating the APC to properly stimulate CD8+ T cells [23, 24].

In humans, the CD45RA and CD45R0 surface antigens have been used to separate T cells into naïve and memory pools. However, discrimination based only on CD45 isoform expression is insufficient since, with time, some CD45R0 T cells may revert to CD45RA [25]. Furthermore, naïve CD8+ T cells are responsive to IL-15 in an antigen-independent way,

with concomitant phenotypic changes and acquisition of functional properties (IFN-γ and TNF-α synthesis, and cytotoxic function) that are difficult to distinguish from antigen-primed T cells [26].

Tc1 and Tc2 cells

The current model of naïve T cell activation postulates that full activation requires two signals. The first signal is antigen displayed by the APC in the form of peptides bound to histocompatibility molecules; the recognition of antigen by the T cell receptor (TCR) provides specificity to the response. The second signal, called the costimulatory signal, is provided by molecules on the APC that engage particular costimulatory receptors on T cells [27]. It can dictate the fate of the response and provides a mechanism for regulating the appropriateness of a given response. A third signal or polarization directs T cell differentiation into various effector types, such as Tc1 and Tc2; it can be derived from cytokines or from inflammatory signals supplied by Toll-like receptor (TLR) ligands [28, 22].

CD8+ T cells can differentiate into two effector phenotypes, Tc1 and Tc2, secreting different cytokine patterns. Tc1 cells are defined as CD8+ T cells that secrete IFN-γ but not IL-4 or IL-5, and Tc2 cells are CD8+ cells that secrete IL-4, IL-5, and low amounts of IFN-γ [29], [30]. The synthesis of IL-2 by mouse Tc1 cells is subjected to further regulation, and IL-6 and IL-10 are preferentially, but not exclusively, synthesized by Tc2 cells [29]. Overall, CD8+ T cells are able to produce the same range of type 1 and type 2 cytokines as CD4+ T cells [31].

Naïve CD8+ T cells show a strong preference for differentiating into Tc1 cells. Tc2 differentiation requires substantial amounts of IL-4 and is very much dependent upon the APC [29], whereas IFN-γ and IL-12 encourage differentiation to Tc1 cells [30].

Human Tc1 CD8+ T cell clones express little CD28, CD30 or CD40L, while Tc2 cells express significant amounts of these ligands, which may facilitate their interaction with other immune cells [32].

Both subsets are cytotoxic via the perforin and the Fas pathways, and both are able to kill resting and activated B cells [29, 30]. Furthermore, both subsets induce inflammation with similar cellular infiltrates [29].

The CTL response to acute infection can generally be divided into four phases. During the effector phase, naïve CTL precursors are primed, undergo dramatic expansion, acquire effector function, travel to sites of infection, and mediate pathogen clearance by killing infected cells and secreting effector cytokines. During the contraction phase, most effector CTL die, leaving behind 5-10% of the original burst size as long-lived memory cells. During the memory maintenance phase, memory CTLs are maintained at stable levels throughout the mouse lifespan and for many years in humans. The rapid recall response of memory CTL following re-exposure to the pathogen provides enhanced protection to the host.

It is unlikely that CD8+ T cells provide help for B cells, since, via the regular antigen-processing pathways, foreign antigens would be presented on MHC class II in the B cells leading to help by CD4+ T cells. In contrast, if the B cells were infected, the antigen would be presented in MHC class I leading to killing of these cells by CD8+ T cells [29].

CD8+CD28+ and CD8+CD28− T cells

CD8+ T cells can be subdivided into two subsets with different biological properties according to the surface expression of CD28.

At birth, the great majority of T cells express CD28 (less than 1% of CD28− T cells in cord blood [33]). Aging is characterised by a progressive exhaustion of naïve T cells involving both CD4 and CD8 subsets [34] which is accompanied by a progressive expansion of CD28− T cells, that is particularly evident in CD8+ T lymphocytes [35, 36]. The CD28+ T cell population is predominant in young healthy individuals and expands during primary viral infection, whereas CD28− T cells are more common in elderly healthy individuals [37] and increase dramatically in human immunodeficiency virus (HIV) patients [38, 25]. In these patients, specific cytotoxic activity is mainly mediated by CD8+CD28− T cells, while inhibition of HIV replication is controlled by CD8+CD28+ T cells [39].

CD8+CD28− T cells have been characterised by oligoclonal expansions [40, 41], impaired proliferative responses, but preserved cytotoxicity and reduced telomeres [42]. Some authors suggested that the CD28− subset has a longer replicative history (more rounds of cell division), and consistent with the loss of CD28 might have reached a state of replicative senescence [42]. The average telomere length shortens between 50 to 100 base pairs with each

round of replication, and thus, telomere lengths can be used to assess the replicative history of cell populations.

It has been proposed that CD8+CD28− T cells derive from CD8+CD28+ precursors [43], because the CD28 negative phenotype can be induced with *in vitro* stimulation of cord blood lymphocytes which typically contain only CD8+CD28+ T positive cells [44]. BrdU labelling showed that CD8+CD28− T cells derive from CD8+CD28+ precursors *in vitro* [45]. IL-4 was able to prevent, but not reverse, the switch in CD28 expression in one study [43], but in another study, treatment of CD8+ T cells with IL-4 decreased the levels of both CD28 surface expression and message and increased CD8 expression [46].

Signalling through CD28 is the major co-stimulatory signal for activation of naïve T cells. Its interaction with CD80/CD86 expressed on B cells, macrophages, and dendritic cells stimulates IL-2 mRNA production [47], and stabilises IL-2 mRNA, thereby enabling T lymphocytes to proliferate in response to antigen. It also inhibits T cell receptor-induced apoptosis during primary T cell responses [48, 49]. Once established, the proliferative response appears to be less dependent on CD28 co-stimulation, since exogenous growth factors such as IL-2 can restore mitotic progression. Normally, most CD8+ T cells do not produce enough IL-2 to support their own expansion [43].

Freshly-isolated peripheral blood CD8+CD28− T cells are enriched for large and granular lymphocytes as assessed by flow cytometry [33, 50], and have a limited Vβ repertoire [51]. This oligoclonality is common in healthy subjects as well as in pathological conditions [40]. Loss of CD28 expression is also associated with a phenotype of antigen-experienced CD8+ T cells: CD57+CD11b+CD27−CD49+ with variable expression of CD45RO/RA [33, 37, 52]. Co-expression of chronic activation antigens such as CD38 and HLA-DR can occur, particularly in HIV-infected patients [53], but acute activation antigens, such as, CD25, CD69 and CD71 are not usually expressed [33, 37, 52, 54].

When phenotypically compared with their CD28 positive counterparts, CD8+CD28− T cells have a higher expression of CD11b, CD29, CD57, and CD94 and lower expression of CD25 [55]. Affymetrix gene chip analysis of CD8+CD28− and CD28+ T cells from five different human T cell lines (TCL) showed differences in the level of expression of genes that encode cell surface molecules, signal transduction molecules, chemokines, cytokines, apoptosis-related proteins, cell growth regulators and metabolic enzymes [56]. Natural killer (NK)-related receptors (CD16, CD56, CD57, CD85j, CD94, CD161, CD244, EB6, gl183, NKG2A, NKB1) were preferentially expressed on the CD28 negative subset, both in

healthy donors and also in melanoma patients [57]. It is possible that the specific up-regulation of some inhibitory NK cell-related receptors on CD8+CD28− T cells can block their cytotoxic capacity, accounting for the inability of these cells to kill their target cells [58].

In our previous work, we evaluated the expression of several phenotypic markers on CD3+CD8+CD28+ and CD28− subpopulations in whole blood from ten atopic and ten non-atopic volunteers. Although the phenotypic expression was similar in both atopic and non-atopic volunteers, overall the phenotypic expression in CD8+CD28+ T cells was different from that in CD8+CD28− T cells, as previously described by other authors [33, 37, 52].

The functional response of the CD8+CD28− T cells is distinct from that of the CD8+CD28+ T cells. After stimulation of T cells with immobilised anti-CD3, freshly isolated peripheral blood CD8+CD28− T cells proliferated poorly, compared to the vigorous response of the CD8+CD28+ T cells [33]. Increased anti-CD3 can compensate for the loss of co-stimulatory signal caused by reduced CD28 levels but fails to significantly increase the responsiveness of the CD28− population [46]. This hyporesponsiveness could not be overcome by addition of exogenous IL-2, excluding the lack of IL-2 as the cause for this anergic response [33, 46]. Proliferation of these cells was impaired, even when activated with mitogens that bypass TCR signalling [55]. Similarly, T cell clones also demonstrated modest growth in response to stimulation with anti-CD3 plus IL-2, whereas CD8+CD28+ clones proliferated vigorously [33]. Both CD8+CD28+ and CD28− T cell lines were observed to expand when cultured in the presence of IL-2, IL-7 or IL-15, and survived without significant expansion in the presence of IL-4, suggesting that proliferation might be important in inducing the loss of CD28 [59]. The reduction in proliferation might be due to increased RNA and protein levels of the cyclin dependent kinase inhibitor p16, as described by Scheuring *et al.* in CD8+CD28− T cells from aged adults [55].

Overall, CD8+CD28− T cells are a heterogeneous subset that contains both memory and effector cells [60]. The most established view considers CD8+CD28− T cells as terminally differentiated or end stage/senescent cytotoxic T cells [45, 52], although these cells were first described as suppressors of B and T cell function. Interestingly enough, in a number of situations, ranging from chronic inflammatory conditions [61] and infectious diseases [53, 62] to aging [52, 63], immunodeficiency [64], iron overload [65] and heavy alcohol intake [66], an increase in the percentages of CD8+ T cells lacking CD28 expression was found. It is currently not

known whether this increase in CD8+CD28− T cells is a cause for the pathology or an immunological response to it [50], but this association with disease suggests that they are an effector population.

Suppressor CD8+ T cells

A central finding from experimental models and human studies shows that allergic diseases are due to an aberrant immune response mediated through Th2 cells, characterised by a specific cytokine pattern. To avoid chronic cell activation and inflammation against non-pathogenic antigens, and control harmful T cell responses, the immune system has evolved a variety of regulatory mechanisms mediated by distinct T cell subsets. The regulation mediated by these suppressor subsets is superimposed on intrinsic regulatory mechanisms (such as deletion and anergy). The cellular basis of suppression was initially proposed in the early 1970s by Gershon and Kondo, who demonstrated that spleen cells from mice tolerant to sheep red blood cells could transfer the unresponsive state to naïve syngeneic mice [67]. However, for many years, their existence was questioned until animal model studies of multiple sclerosis demonstrated a role for suppressor cells in protection against disease recurrence and exacerbation (for review see [68]).

Although, in the beginning, suppressor activity was restricted to the CD8+ T cell subset, nowadays the most extensively characterised population of regulatory/suppressor cells is that of natural CD4+CD25+ T regulatory (Treg) cells. These cells suppress immune responses via cell-to-cell interactions and/or the production of IL-10 and TGF-β.

Three major subsets of CD8+ T suppressor (Ts) cells have been identified. The first type acts in an antigen-dependent manner via the transfer of inhibitory signals to APC by direct cell-to-cell contact [69, 70, 71]. The second type does not require antigen recognition and acts via cytokine secretion [72]. The third type is antigen-specific but acts through IL-10 secretion [73]. Several studies have shown that Ts cells can act in inflammation models in chronic infections, organ transplantation, and autoimmunity.

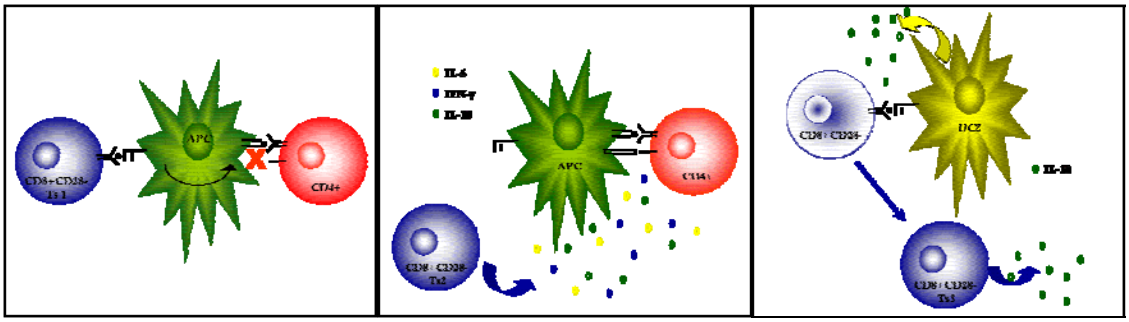

Figure 1 – Schematic representation of the mode of action of different suppressor T cells. From left to right: type1, type 2, and type 3 CD8+ T suppressor cells. (Adapted from Filaci and Suciu-Foca, 2002)

Type 1 Ts are represented by CD8+CD28− T cells which inhibit allo-antigen, xeno-antigen and nominal antigen-specific CD4+ T cell responses. Type 1 CD8+CD28− Ts cells can be generated *in vitro* by multiple rounds of stimulation of peripheral blood mononuclear cells (PBMC) with allogeneic [69], xenogeneic [70] or autologous (antigen-pulsed) APC [71]. These Ts cell lines express specific molecular markers related to natural CD4+CD25+ regulatory T cells, namely FOXP3 [56, 74], have a limited Vβ usage [51, 70], and are MHC-class-I-restricted [69]. Unprimed CD8+CD28− T cells from fresh peripheral blood, which have no regulatory activity, do not express FOXP3, GITR, OX40, CD25, CD62L or 4-1BB [56]. Inhibition of Th cell proliferation is not caused by killing either the APC or the Th cell, nor is the suppressor effect mediated by the production of cytokines. Instead, it requires direct interactions between the suppressor cell and the APC used for priming [69, 70, 75]. The consequence of this interaction is the inhibition of the CD40-mediated up-regulation of other costimulatory molecules (CD80 and CD86) [69, 76] and the up-regulation of expression of the immunoglobulin-like transcript (ILT)3 and ILT4 on the surface of the APC [77, 78]. The suppressed APC are rendered unable to induce and sustain the full programme of CD4+ Th cell activation due to the inhibition of the NFκB activation and transcription of costimulatory molecules in the APC [76, 78]. Of note is the fact that Ts have a low proliferating rate at first but reach a sizable population upon repeated stimulation with antigen [79]. Type 1 Ts can also be generated *in vitro* by allo-stimulation of PBMC from the peripheral blood of baboons or ACI rats. These Ts suppressed the proliferative response of CD4+ T cells from the same T cell line to allogeneic APC in a dose-dependent manner [80].

Non-antigen-specific Ts, or Type 2 Ts, as well as Type 1 Ts are phenotypically characterised by the lack of expression of the CD28 costimulatory molecule. Type 2 Ts can be generated *in vitro* from purified circulating CD8+CD28− lymphocytes incubated for 1

week with IL-2 and IL-10 [72]. These Ts inhibit the proliferative response of T cells stimulated with specific antigens, anti-CD3 monoclonal antibodies or mitogens in a non-antigen specific way. They also seem to suppress the lysis mediated by cytotoxic cells [72]. The suppressor effect is mediated by the secretion of soluble factors, namely IFN-γ, IL-6 and IL-10, without direct interaction with APC [72, 81]. They have a phenotype (CD45RA+CD27−CCR7−) and TCR repertoire suggesting that they are pre-activated and expanded clones [72] (for review see [82]). Type 2 Ts seem to be involved in the pathogenic mechanisms of diseases, since failure to generate non-antigen-specific Ts is associated with relapse in patients with multiple sclerosis and systemic lupus erythematosus [81, 83], and in HIV- or hepatitis C virus (HCV)-infected patients [84]. These findings may reflect two opposite conditions: the physical elimination of CD8+ Ts cell precursors or the compartmentalization of these cells in tissues. In fact, in autoimmune thyroiditis and cancer, type 2 Ts were found to infiltrate affected organs leading to different outcomes: a protective function in thyroiditis and a pathogenic role in cancer [84, 85].

A third Ts subpopulation was also identified. These cells are generated by stimulating purified, naïve CD8+ T lymphocytes with plasmacytoid dendritic cells (DC2). Their generation depends on antigen presentation and secretion of IL-10 by DC2. DC2 primed CD8+ T cells develop anergy to further stimulation with the same antigen and start secreting IL-10 themselves. These DC2 cells induce a population of CD8+ regulatory T cells that are anergic, non-cytolitic and capable of inhibiting primary T cell responses through the production of IL-10. Their inhibitory activity affects naïve but not pre-activated CD8+ T cells and is directly mediated by IL-10. IL-10 producing CD8+ T suppressor cells are directly induced via antigen presentation by DC2 and require antigen-specific re-stimulation to deliver their immunosuppression through the production of IL-10 [73].

CD8+ T suppressor cells may be involved in the pathogenic processes of chronic inflammatory diseases with immunological involvement, either due to an impairment of their function (autoimmune diseases) or due to an unregulated function (tumours). The presence of Type 1 suppressor T cells has been associated with the absence of acute rejection in transplanted patients [86, 87] and is responsible for immunosuppression in animal models of transplantation [88]. The failure to generate Type 2 Ts has been associated with relapse or disease progression in autoimmune diseases [81, 83], but in other settings (tumours),

it is the presence of these cells that allows the disease to go unchecked [82, 84]. However, a great deal of uncertainty remains about differentiation factors, antigen specificity, and mechanisms of action of Ts cells.

1.3. CD8+ T Cells in Allergy

Animal models of allergy

CD8+ T cells which are pivotal in tumour cell killing and protection during viral infection through secretion of IFN-γ and cytolytic factors have been considered to be much less important or even negative regulators of the development of allergic inflammation.

A number of animal studies reported conflicting effects of CD8+ T cells in allergic airway disease.

In a high IgE-responder rat model of asthma (Brown Norway rat), the depletion of CD8+ T cells with the monoclonal antibody (mAb) OX8 (a mouse anti-rat anti-CD8α monoclonal cytotoxic antibody) enhanced allergen-induced airway hyperresponsiveness (AHR) and eosinophil numbers in bronchoalveolar lavage (BAL) fluid. There was also a reduction in gene expression of Th1 cytokines in the lungs (IL-2 and IFN-γ), while the levels of IL-4 and IL-5 remained unchanged [89].

Using the same approach in a low IgE-responder rat (Sprague-Dawley rat), Olivenstein *et al.* observed an increased magnitude of the late airway response to inhaled allergen. This was accompanied by a significant increase in antigen-specific IgE and airway inflammation characterised by an influx of total leucocytes, macrophages, neutrophils and lymphocytes into the airway lumen [90].

In an attempt to clarify the role of CD8+ T cells in the regulation of IgE and CD4+ T cell responses to ovalbumin (OVA), Holmes and co-workers not only depleted but also reconstituted sensitised rats with purified CD8+ T cells. Depletion of CD8+ T cells *in vivo* was time-dependent: depletion, 7 days after immunisation, failed to enhance IgE production, while depletion on days 12-18 greatly enhanced it. Reconstitution of CD8-depleted rats on day 12 with purified CD8+ T cells completely inhibited the IgE response. This effect was antigen-specific since CD8+ T cells from OVA-primed animals had little effect on the IgE response of bovine serum albumin immunised rats [91].

The mechanism of this IgE regulation by CD8+ T cells was further investigated in wild-type and in IL-12 and IFN-γ knock-out mice. Contrary to common knowledge, the authors concluded that IFN-γ was required for inhibition, but had an origin other than the

CD8+ T cell (indirect suppression). The immunoregulatory potential of the CD8+ T cells was dependent on their ability to stimulate IL-12 production by the APC, leading to the activation of Th1 cells. In turn, the generation of OVA-specific Th1 cells inhibited Th2-dependent IgE class switching via production of IFN-γ [92]. Surprisingly, both Tc1 and Tc2 cells promoted Th1 cell development and inhibited the generation of Th2 cells [92].
Finally, Yang *et al.* examined the cellular basis for the poor IgE production in SJL/J mice upon OVA immunisation. The authors found that increased IFN-γ production by CD8+ T cells was responsible for their failure to make IgE [93].

However, other studies suggested that CD8+ T cells are important contributors and might be necessary for AHR and eosinophilic airway inflammation.
In fact, Euthymic Hooded Lister rats depleted of CD8+ cells by repeated injection of OX8 mAb did not mount a greater IgE response when immunised with antigen alone, or antigen plus ricin, suggesting that CD8+ in this setting might have a "helper" function [94]. Moreover, CD8−/− mice develop significantly lower AHR and airway inflammation compared with wild type mice after sensitisation and challenge with OVA. Reconstitution of the CD8−/− mice with magnetically isolated, OVA-primed CD8+ T cells fully restored the development of AHR and lung inflammation to levels comparable to those seen in wild type mice [95, 96]. In another murine OVA model of asthma, if CD8+ T cells were removed prior to sensitisation, a significant decrease in AHR was observed. This was correlated with a decrease in the levels of IL-5 and eosinophil numbers in the lungs of these mice [97].
Schaller *et al.* investigated CD8+ T cell responses in a murine cockroach antigen (CRA) model of asthma. Their results showed that depletion of CD8+ T cells after allergen sensitisation to CRA significantly reduced airway hyperreactivity, airway eosinophilia and pulmonary type 2 cytokine levels. CD8+ T cells from CRA-sensitised mice produced type 2 cytokines IL-4, IL-5, and IL-13 upon antigen challenge, and the transfer of these cells into naïve mice caused airway hyperreactivity when exposed to CRA. This transferred airway response was dependent both on IL-4 and IL-13 [98]. The author's data further suggests that both CD4+ and CD8+ T cells can mediate an airway response, and that when both cells are transferred into naïve mice from CRA-sensitised mice, the airway response is much greater than when one population alone is transferred [98].

Direct comparison between these studies is difficult because of the different protocols used: different species (rat vs. mice) and strain models, differences in recipient status at the

time of reconstitution (naïve/sensitised), different routes of sensitisation, different adjuvants; all these factors can play a role in the final effect. In addition, different subpopulations of CD8+ T cells do exist and can be at different functional stages of differentiation, some of which suppress and others enhance Th2-responses. It is noteworthy that it is being recognised that the cell and cytokine requirements during the initiation/sensitisation phase differ considerably from those that might be necessary during the challenge/maintenance/progression phase [99, 100]. CD8+ T cells could play an important inhibitory role early on during the sensitisation phase on the allergen-induced response, but once the systemic sensitisation is established, CD8+ T cells may play a bystander or pro-inflammatory role [101].

Human allergic disease

As we have seen, CD8+ T cells are important immunoregulatory cells in some animal models of allergic disease, but proinflammatory in others. Dichotomous findings with respect to CD8+ cells led to attempts to further characterise CD8+ cells in human allergic immune responses.

According to the "Th2 hypothesis for asthma" asthmatic subjects have a relative deficiency in IFN-γ production or an imbalance towards type 2 cytokine production [102]. Because Th1 cells antagonise Th2 cell functions, it was proposed that immune deviation towards a Th1 pattern could protect against asthma. It was then suggested that CD8+ T cells might play a protective role in the pathogenesis of allergic inflammation via their ability to produce IFN-γ. This IFN-γ could inhibit the development of Th2 cells directly and inhibit IgE synthesis by B cells.

Several authors investigated the secretion of IFN-γ by CD8+ T cells from patients with allergic asthma and healthy controls.
One study showed that PMA (phorbol myristate acetate) and calcium-ionophore-induced IFN-γ secretion by isolated CD8+ T cells in patients with asthma was lower than that of healthy controls, but was significantly increased after stimulation with anti-CD3 antibodies [103]. Using a bronchial explant system without any stimulation, the mRNA expression for INF-γ was higher in cultured bronchial biopsies from control subjects compared with that

from asthmatic subjects [104]. Several other studies observed an increased rather than a decreased synthesis of IFN-γ by CD8+ T cells in allergic patients, when compared with healthy controls. This increased IFN-γ production was observed when imunomagnetically isolated CD8+ T cells from the peripheral blood were stimulated with *Der p* 1 [105] or total PBMC were stimulated with PMA and ionomycin [31, 106]. Interestingly enough, the number of IFN-γ-producing CD8+ T cells correlated with asthma severity, bronchial hyperresponsiveness and blood eosinophilia in a group of atopic asthmatic patients [106].

In allergic patients, there is evidence that Tc2 CD8+ T cells are present both at the site of allergic inflammation and in the peripheral blood. Using double immunohistochemistry and *in situ* hybridisation, Ying *et al.* showed that in atopic and non-atopic asthma 70% of IL-4 and IL-5 mRNA signals co-localized to CD3+ T cells, the majority of which were CD4+ although CD8+ T cells also expressed IL-4 and IL-5 mRNA [107]. Similar results were obtained for CD8+ T cell from the peripheral blood of asthmatic children [108].

Both activated CD4+ and CD8+ T cells were shown to encode mRNA for IL-4 and IL-5 in atopic and non-atopic asthmatic adults [107] and children [108]. It is of note that, after treatment with inhaled corticosteroids, there was a clinical improvement in the asthmatic patients associated with reduced T cell activation and cytokine mRNA expression [108].

CD8+ T cells isolated from the peripheral blood of patients with mild atopic asthma contain higher levels of intracellular IL-4 than those from healthy non-atopic subjects. Additional *ex vivo* stimulation with Concanavalin A does not enhance IL-4 production, suggesting that in asthma CD8+ T cells are conditioned *in vivo* to produce greater amounts of this Th2-type cytokine [109].

The Th2 cytokine medium found at the asthmatic lung can transform CD8+ cytotoxic T cells into non-cytotoxic IL-5 producing cells *in vivo*. This IL-4 dependent switch to CD8+ T cells that secrete IL-5 is thought not only to promote eosinophilia, but also to lead to impaired viral clearance owing to a reduction in IFN-γ production [110].

However, CD8+ T cells are not the principal source of lung IL-5; Till *et al.* even doubt that they are relevant. In freshly isolated PBMC, IL-5 but not IFN-γ production by T cells was elevated in atopic patients with asthma and in atopic patients with rhinitis in response to challenge with dust-mite allergen. This IL-5 production correlated with bronchial hyperreactivity and was abrogated by depletion of CD4+, but not CD8+ T cells[111].

Finally, it was suggested that CD8+ T cells can contribute to the pathology of asthma death due to an enhancement of the pre-existing airways inflammation in response to viral

infection [112, 113]. In fact CD8+ T cells are more sequestrated than CD4+ T cells in the airway during an acute asthma attack [114].

Recently, Seneviratne *et al.* showed that *Dermatophagoides pteronyssinus* (*Der p*) 1-specific CD8+ T cells exist in humans and that it is possible to map the precise class I epitopes and characterise patterns of cytokine production. The authors' data demonstrate that as in the case of allergen-specific IgE, allergen-specific CD8+ T cells do exist, are restricted to the atopic group, and have impairment in IL-10 production [16]. This is an unexpected finding as it proves that CD8+ T cells are able to respond to exogenous antigens and associates diminished production of a regulatory cytokine, IL-10, to disease severity.

In summary, CD8+ T cells do not exhibit a fixed phenotype but can differentiate into functionally distinct subsets that might suppress, enhance, or divert immunopathologic processes in response to the environment.

In order to contextualize our cellular findings, we decided to initially characterise our study populations (patients with allergic rhinitis and patients with bronchial asthma) from an epidemiological point of view.

HYPOTHESES

Many authors believe that atopy and allergic diseases are the result of inadequate or impaired inhibition of allergen-specific T helper-type responses by regulatory T cells (Treg) [115], or even a consequence of decreased frequencies of those cells in atopic individuals [116]. In other disease settings (transplantation, autoimmunity), CD8+ T cells were proven to have a suppressor function and contribute to the maintenance of peripheral tolerance. Papers on the mechanisms of asthma and allergic inflammation were unable to assign a single function for CD8+ T cells in these settings. This fact can be due to the existence of more than one CD8+ subpopulation with relevance for the development and regulation of immediate hypersensitivity. Bearing in mind all the research on the subject, it was our firm belief that the present study could enhance the knowledge and shed some light on the functional properties, rather cytotoxic or suppressor, of CD8+ T cells in allergic diseases.
In this sense, we put forward the following hypotheses:

1. Peripheral blood CD8+ T cells inhibit proliferation and cytokine synthesis in CD4+ effector T cells induced by exposure to allergen.
2. Sputum CD8+ T cells from asthmatic patients are activated.
3. CD8+ T cell activation in sputum from asthmatic patients correlates with disease severity and control.

AIMS OF THE THESIS

Despite the growing interest in T cells observed in the last few years in various fields, studies addressing the function of CD8+ T cells in allergy have been scarce. The main aim of this thesis was to study the role played by CD8+ T cells in the development of allergic diseases. The specific aims were:

1. To assess allergen-specific proliferation and cytokine synthesis of CD8+ T cells.
2. To evaluate the suppressor function of CD8+ cells on antigen-specific responses.
3. To assess the presence of CD8+ T cells at the target organ and their activation status.
4. To assess the correlation between the expression of the activation markers on CD8+ T cells and asthma severity and control.
5. To demographically characterise samples of patients used for the cellular studies.

2. Background on the Methods

2.1. Collection of Biological Material

Peripheral Blood

Peripheral blood offers an indirect way to analyse inflammation at the target-organ. It has the advantage of being an easy access source and generator of minimal discomfort to the volunteers. Prior to using freshly drawn peripheral blood, we tried an alternative method of obtaining leucocytes from leucocyte depletion filters (for further information see Chapter 5).

Induced Sputum

Induced sputum offers a more accurate assessment of airway inflammation and cellular infiltration than other non-invasive techniques that analyse markers in peripheral blood such as blood eosinophil count, serum ECP or in exhaled air, such as nitric oxide.
Although sputum is derived mainly from the central, more proximal airways, and BAL fluid and bronchial washings (BW) samples from more peripheral airways and the alveolar compartment, the cellular composition of sputum correlates well with that found both in BW and BAL fluid and, to a lesser extent, with the cellular influx seen in bronchial biopsies. In addition, sputum induction is less invasive than BAL or bronchial biopsies, and has been shown to be safe even in severe asthma.
Sputum is composed of fluid and cellular components, including macrophages, bronchial epithelial cells and inflammatory cells. When it is expectorated, it becomes mixed with saliva composed mainly of fluid, squamous epithelial cells, and oropharyngeal bacteria.
The method of sputum induction has many advantages, but it has also a few major drawbacks. These include the need for qualified technicians and the time consuming nature of the technique. The total time required for induction, processing and performing a differential cell count will amount to approximately 100min. The need to process sputum within two hours after induction restricts the use of the method.

Finally, a very important aspect of sputum obtained by standardised techniques is that it can yield sufficient amounts of cells that can be analysed using different techniques. Above all, it is now possible to use flow cytometry for the analysis of cellular subtypes and cellular activation.

2.2. Cell Isolation

Immunomagnetic cell sorting

The ability to fractionate lymphocytes on the basis of surface phenotype has been a major technical advance in the study of the functional diversity of these cells. Many of the methods currently in use exploit antibody specificity to separate cells of one type from a mixed population. Cell lysis with antibody and complement is useful only for cell elimination. Separation by affinity column chromatography, lymphocyte panning or fluorescence-activated cell sorting offers the advantage of positive or negative selection by allowing the recovery of both enriched and depleted cell populations.

In recent years, several companies have been working on methods for increasing the purity and recovery of rare cell types. Pre-enrichment steps usually start with "debulking" of the sample based on differences in cell density or cell size, followed by a more selective method that can yield a higher number of purified cells. A balance must be maintained during the process as higher purity can result in low yield.

Differences in size and density have led to the use of density gradients for the removal of mature red blood cells from haematopoietic cell suspensions. In the present study, we used Lymphoprep (Axis-Shield). For further purification of our cell fraction, we used magnetic cell sorting as available from Miltenyi Biotec (MACS system).

In magnetic cell sorting, cells are incubated with an antibody (or an antibody mixture) specific for a particular cell that is covalently linked to a magnetic bead. After magnetic labelling, cells are passed through a separation column which is placed in a magnetic field; unbound cells flow through the magnetic phase while the bead-bound cells are retained in the column. Once the column is removed from the magnetic field, the bead-bound cells are eluted.

Cells can also be labelled indirectly by first being incubated with a primary unconjugated or fluorochrome-conjugated antibody followed by magnetic labelling by using anti-immunoglobulin or anti-fluorochorme MicroBeads. Indirect labelling is compatible with simultaneous fluorescent staining for subsequent flow cytometric or microscopic analysis. Indirect labelling is useful to amplify the magnetic label, which may be important if dimly expressed markers are used for magnetic separation.

There are two main strategies for isolating cells: positive selection or enrichment and negative selection or depletion. With positive selection, the target cells are labelled with the magnetic beads and are retained in the column. The binding of MACS MicroBeads to the cell surface will rarely affect its function or viability. By positively selecting we take advantage of the speed and specificity of a monoclonal antibody and, in most situations, this strategy saves time and money. If a positive selection strategy is not feasible (no available antibody or if cell activation may be induced by the existing antibody) then a depletion strategy should be used. The method of choice will depend on the antibodies available and the subsequent use of the cells.

MicroBeads are an average 50nm in diameter (which avoids mechanical stress) and are made of biodegradable iron oxide and polysaccharide. Removal of the bead is therefore not necessary for further analysis and the cells retain their function. The beads form a colloidal suspension, do not sediment or aggregate in magnetic fields, and can be used for positive selection of cells with frequencies as low as 10^{-8}. As only a certain percentage of the cell epitopes are bound by antibody-bead complexes, it is possible to use antibodies with the same specificity for magnetic and fluorescent labelling (this general rule cannot be applied to dimly expressed antigens).

MACS technology can separate 10^5 to 10^{11} cells using the same technique with different size columns with a typical purity and recovery greater than 90 percent. Cells eluted from the column are ready for cell culture, flow cytometry, PCR (polimerase chain reaction), fluorescent microscopy or FISH (fluorescence in situ hybridisation).

2.3. T Cell Phenotype

Flow Cytometry

Flow cytometry is a technique which evaluates cellular parameters, either morphological or phenotypical at a single cell level. Cells under study are incubated with monoclonal antibodies conjugated with fluorochromes that specifically bind cellular components.
The system measures biophysical properties of the cells that are related to biochemical and biological properties. Without the use of fluorochromes it is possible to determine size (by forward scatter) and complexity (by side scatter) of cells (or other particles) under study. The fluorochromes are excited by the laser beam and emit light in longer wavelengths. It is this emitted light that is captured by the detectors and transformed into digital signals that can be stored and analysed.
The relative amount of a particular molecule in different cell subpopulations can be studied by determining the amount of fluorescence that is emitted.
The majority of the conjugated monoclonal antibodies are specific for components on the cellular surface; however, it is possible to mark intracellular components by permeabilizing the cells temporarily, which permits the entry of the antibody. It is also possible to use fluorescent probes that indicate ionic cytoplasmic concentrations and oxidation-reduction potential.
This method evaluates different parameters (number-dependent on cytometer) at a single cell level and it is possible to study the heterogeneity of a sample and the presence of contaminants or rare populations. It is a very fast technique with measurements being made at a rate of 1000 events per second on average. There are simple protocols for whole blood which eliminate the need for previous cell isolation. The sensitivity is high as well as the reproducibility, but laser usage and compensation settings should be carefully monitored as they might influence these parameters. As a setback, the equipment is rather expensive and all the procedures must be performed by trained personnel.

2.4. Cytokine Synthesis

Cytometric Bead Array

The Cytometric Bead Array (CBA) by Becton Dickinson employs a series of particles with distinct fluorescence intensities to simultaneously detect multiple soluble analytes in a particle-based immunoassay. Each bead provides a capture surface for a specific protein and is analogous to an individual coated well in an ELISA plate. The bead mixture is in suspension to allow for the detection of multiple analytes in a small volume sample. The CBA Human Th1/Th2 kit we used can quantitatively measure IL-2, IL-4, IL-5, IL-10, TNF-α, and IFN-γ protein levels in a single sample. This kit performance is optimised for analysis in tissue culture supernatants, plasma and serum samples.
CBA provides several advantages when compared with conventional ELISA: the required sample volume is approximately one-sixth of the quantity necessary for conventional ELISA assays due to detection of six analytes in a single sample; a single set of diluted standards is used to generate standard curves for all 6 analytes; the experiment takes less time and is cheaper when compared with six conventional ELISA tests. However, due to the complexity and kinetics of a multi-analyte assay, sensitivity is lower than in some ELISA tests (microELISA).

2.5. T Cell Proliferation

Tritiated Thymidine ([^{3}H]-thymidine) Incorporation

The measurement of tritiated thymidine ([^{3}H]-thymidine) incorporation is one of the most familiar and widely used methods for quantifying cell proliferation. Cells incorporate the labelled DNA precursor into newly synthesised DNA, such that the amount of the incorporation, measured by liquid scintillation counting, is a relative measure of cellular

proliferation. However, this technique is time-consuming and labour intensive, and exposes the researcher to scintillation fluid and tritium, both of which are toxic.

CFSE Fluorescence Loss

T cells can be labelled with chemically reactive lypophilic fluorescent esters, which enter cells and form covalent bonds with cytoplasmic proteins and then cannot leave the cells. These dyes become markers of T cells. Every time a T cell divides, its dye content is halved. By analysing their dye content in a flow cytometer, it is possible to estimate the number of doubling processes each T cell has gone through.

Carboxy-fluorescein diacetate succinimidyl ester (CFSE or CFDA-SE) is a fluorescein derivative which is cell permeant and non-fluorescent. After entering the cell, cellular esterases cleave the acetate groups, rendering the molecule fluorescent and cell impermeant. The succinimidyl ester binds to free amines resulting in long lived fluorescent adducts that are equally diluted into daughter cells after mitosis. After each division, halving of CFSE fluorescence is observed. Fluorochromes compatible with fluorescein can be used to assess other cellular properties.

When combined with staining with fluorescent-labelled monoclonal antibodies, CFSE fluorescence loss has advantages over [^{3}H]-thymidine incorporation in the assessment of T cells proliferation, as it allows simultaneous identification of the cells, the number of proliferating cells and the number of cell divisions undergone by the proliferating cells.

3. Results

3.1. Demographic, Laboratory and Clinical Characterisation of Adult Asthmatic Patients

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3.1.1. Abstract

Background

Asthma is a heterogeneous chronic inflammatory condition characterised by reversible airway obstruction and hyperresponsiveness associated with underlying bronchial inflammation and structural changes. It represents an increasing health problem and is a huge burden on patients, their families and society. The aim of the present study was to characterise the adult asthmatic population attending a Hospital Allergy Clinic between the years of 2003 and 2006.

Methods

Clinical files from the Allergy Outpatient Clinic of the Cova da Beira Hospital were sequentially studied. The total population analysed included 335 female and 130 male asthmatic patients. Bronchial asthma was characterised by clinical history, skin prick testing to aeroallergens, determination of total and specific IgE and lung function testing, and classified according to international guidelines.

Results

Of the patients studied, 70% had allergic asthma and 30% had non-allergic asthma. When compared to allergic asthma, non-allergic asthma was more frequently associated with older age, perennial symptoms and female gender. More allergic than non-allergic asthma patients also had rhinitis and the reverse was true regarding drug allergy and oesophageal reflux. Grass pollen and mites were the major sensitisers for allergic asthmatic patients. The sensitisation profile was significantly different between urban- and rural-based asthmatic patients regarding tree pollen, fungi and moulds.

Conclusions

In this population, rhinitis was more frequently associated with allergic than with non-allergic asthma. The two types of asthma did not differ in clinical severity or changes in lung function. Sensitisation profiles were different between the urban and rural patients.

3.1.2. Introduction

Asthma is a chronic, complex, obstructive lung disease characterised by acute symptomatic episodes of varying bronchial constriction that occur in response to allergens or other triggers such as viral infections and exercise. Asthma is a major cause for work and school absenteeism with repercussions on quality of life and high socio-economic impact [117], [118].

The majority of bronchial asthma cases generally start in childhood or adolescence in individuals responding to common aeroallergens and are mediated by immunologic mechanisms (allergic asthma). Other patients develop asthma later in life, often as a consequence of viral respiratory infections and without history or symptoms characteristic of atopy or allergic diseases (non-allergic asthma) [3]. Most patients with allergic asthma have other concurrent allergic diseases, namely rhinitis, which should be treated in order to improve asthma symptoms [119]. In addition to phenotypes, asthma can also be classified according to its severity ranging from intermittent, mild transient episodes to severe, chronic, life-threatening bronchial obstruction [120, 121].

Asthma is a worldwide disease that has been recognised for centuries, but prevalence figures vary, in part, because of differences in definition and methods of case finding. In order to know the prevalence of allergic disease worldwide, the *International Study of Asthma and Allergies in Childhood* (ISAAC) has been developing a research for the last 15 years, involving more than 50 countries. Portuguese data estimate a prevalence of actual asthma of 12.9% in 6-7 year olds and of 21.8% in 13-14 year olds [122, 123]. The data from the *European Community Respiratory Health Survey* (ECRHS), which involved surveys of asthma and allergic rhinitis prevalence in adults aged 20-44 years, estimated that 5% of the Portuguese adult population has bronchial asthma [124]. However, in spite of these aspects, studies describing the features of Portuguese asthma patients are lacking in the literature.

To increase the current knowledge about asthma patients in Portugal we aimed at characterising the adult asthmatic population attending the Allergy Clinic of the Cova da Beira Hospital between the years of 2003 and 2006.

3.1.3. Materials and Methods

Clinical files from patients attending the Allergy Outpatient Clinic of the Cova da Beira Hospital between 2003 and 2006 were sequentially studied.

The study protocol was approved by the Hospital Ethics Committee.

The diagnosis of bronchial asthma was based upon clinical history, physical examination, pulmonary function tests, and response to inhaled β-adrenergic agents according to international guidelines [120]. Skin prick testing to aeroallergens and determination of total and specific IgE were also performed on the asthmatic patients.

Assessment of severity was based on daytime symptoms, night-time symptoms, frequency and intensity of attacks, impact on daily activities, asthma treatment used and predicted percentages of forced expiratory volume in one second (FEV1) and peak expiratory flow (PEF). All patients were also examined by anterior rhinoscopy and filled in a questionnaire regarding nasal symptoms. Smoking habits were recorded as pack-years.

The designation of "non-allergic" was applied when the history, skin prick testing, and serum specific IgE measurements included in the allergy examination were all negative.

All the data were analysed using non-parametric tests. Results are expressed as medians and range. Wilcoxon signed rank test was used for comparisons within groups and Mann Whitney U test, chi-square test and Kruskal-Wallis test were used for comparisons between groups. The association of various clinical parameters was analysed using the Spearman rank correlation test. A p value of less than 0.05 was considered significant. All analyses were performed using Minitab 14 for Windows.

3.1.4. Results

Demographic characterisation

The total population analysed included 1078 clinical files. From those, we excluded all patients under 18 years and patients not currently living in the area. In addition, 25 patients were excluded because of discordance between skin prick tests and specific IgE tests.

Four hundred and sixty five clinical files, from adult Caucasian asthmatic patients (335 females) living in the Cova da Beira area were included for further study.

Allergic versus non-allergic asthmatic patients

Seventy percent (327) of the asthmatic patients had a diagnosis of allergic asthma and thirty percent had non-allergic asthma. Demographically, there were significant differences between the two groups. There were more women among non-allergic asthmatic patients than among allergic asthmatic patients, with female: male ratios of 5.3 and 2.0, respectively ($p<0.001$). In addition, allergic patients were significantly younger than non-allergic asthmatic patients (median age 35 (18-85) vs. 51 (18-84) years, $p<0.001$). Smoking habits were not different between allergic and non-allergic patients.

Bronchial asthma was classified according to the old practice that divided it into seasonal and perennial. In this regard, we observed that seasonal symptoms were more common in allergic than in non-allergic patients (40.0% versus 18.2%, $\chi^2= 8.992$, $p= 0.003$).

Patients were divided into three age groups and severity was assessed according to the *Global Initiative for Asthma* guidelines [121]. With increasing age, there was an increase in the severity of asthma both in allergic and non-allergic asthmatic patients ($\chi^2= 12.305$ for allergic and 12.937 for non-allergic, $p=0.015$ and 0.012 respectively). However, there were no differences in terms of severity between allergic and non-allergic asthmatic patients ($p>0.05$) (Figure 2).

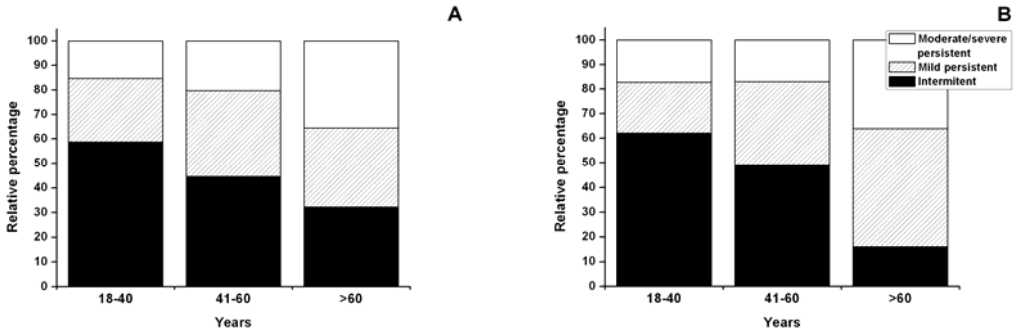

Figure 2 – Relationship between severity of asthma and age in allergic asthmatic (Panel A) and non-allergic asthmatic (Panel B) patients.
Patients were grouped into three age groups and severity was assessed according to GINA guidelines. Severity increased with age but was not statistically different between the two groups.

Some patients with asthma have chronic cough as their main, if not only, symptom, and others have physical activity as the single cause for asthma. More non-allergic than allergic asthmatic patients had cough-variant asthma (15.2% vs. 6.4%; $\chi^2= 9.137$; $p=0.003$), which could not be attributed to differences in work problems or exposure. Exercise-induced asthma was equally prevalent in both groups (Figure 3).

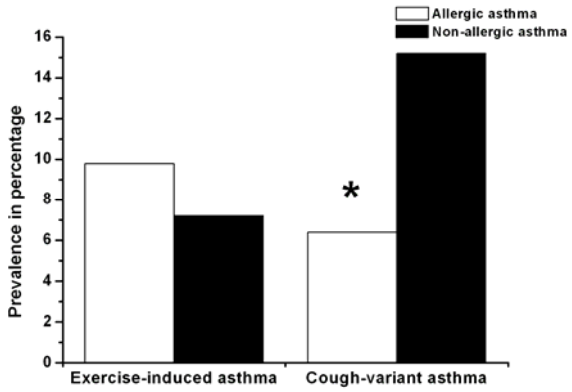

Figure 3 – Prevalence of exercise-induced and cough-variant asthma in allergic and non-allergic asthmatic patients.
Non-allergic asthmatic patients had a higher prevalence of cough-variant asthma compared to allergic asthmatic patients.

Measurements of lung function provide an assessment of the severity, reversibility, and variability of airflow limitation, and help to confirm the diagnosis of asthma. Pulmonary function test data was expressed in terms of percentage of predicted values.
Lung function testing values were not significantly different between allergic and non-allergic asthmatic patients (Table 1).

Table 1 – Lung function testing values in allergic and non-allergic asthmatic patients.

	Allergic asthma	Non-allergic asthma	
% FEV1	101.9 ± 1.1 102.0 [51-148]	101.9 ± 1.8 103.0 [49-143]	p=0.6979
% FVC	105.8 ± 1.0 106.5 [50-159]	107.7 ± 1.7 108.0 [56-145]	p=0.1811
FEV1/FVC	0.82 ± 0.01 0.82 [0.46-1.1]	0.80 ± 0.01 0.81 [0.51-1.2]	p=0.3034
% PEF	92.7 ± 1.2 92.0 [41-151]	95.8 ± 2.0 94.5 [30-155]	p=0.1126
% FEF_{25-75}	80.9 ± 1.9 81.0 [15-180]	78.4 ± 3.0 83.0 [13-150]	p=0.7321

Data are represented as mean ± standard error of the mean and median and range.
Comparisons between both groups were evaluated by the Mann-Whitney U test.

Bronchial asthma is frequently associated with rhinosinusal involvement (even in non-allergic patients) and with other allergic diseases. We studied the main co-morbidities

present in our asthmatic population and observed that the great majority of the asthmatic patients had rhinitis and conjunctivitis. There were differences between both groups, with non-allergic asthmatic patients having a lower prevalence of rhinitis, but a higher prevalence of drug allergy and oesophageal reflux as compared to allergic asthmatic patients (Figure 4).

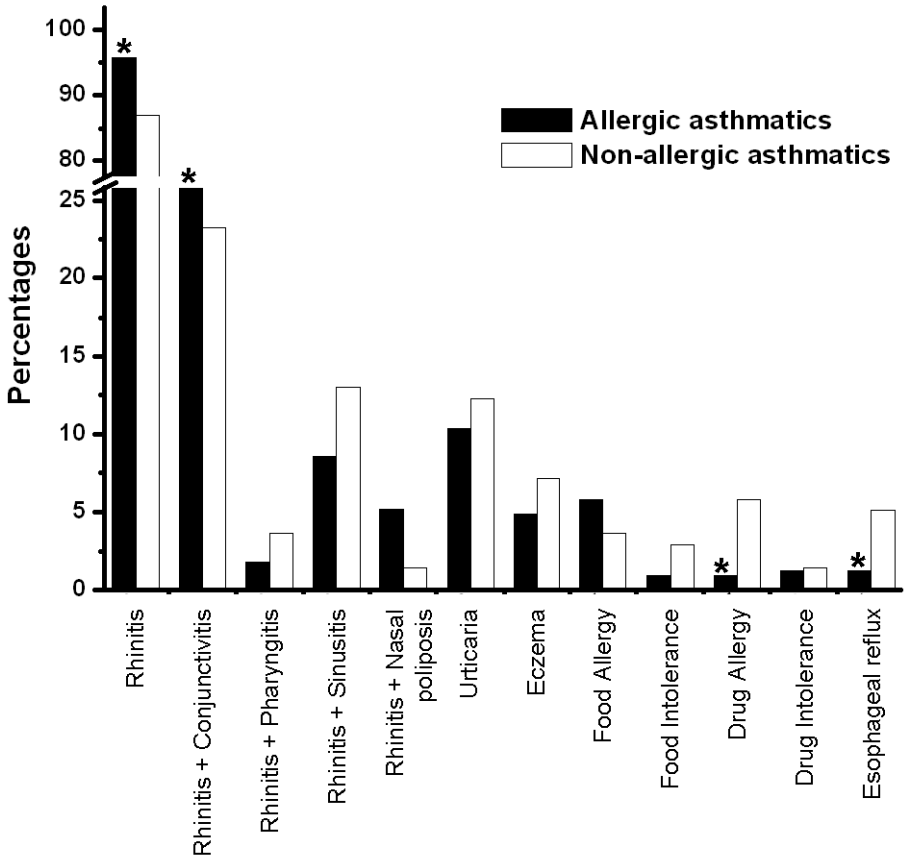

Figure 4 – Main co-morbidities associated with bronchial asthma.
Allergic asthmatic patients (light bars) had a higher prevalence of rhinitis, whereas non-allergic asthmatic patients (dark bars) had a higher prevalence of drug allergy and oesophageal reflux.

As expected, total serum IgE was significantly higher in allergic asthmatic patients than in non-allergic asthmatic patients (median 205 (3-5000 IU/ml) vs. 27 (<2-653 IU/ml), $p<0.001$). There was no correlation between total IgE levels and features of allergic sensitisations (wheal size on the skin prick tests or specific IgE levels).

Sensitisations in allergic asthma

In allergic asthmatic patients, the frequency of sensitisations evaluated by skin prick testing was 65.5% for grass pollen, 62.9% for mites, 62.6% for cereal pollen, 49.3% for tree pollen, 49.3% for weed pollen, 30% for dog dander, 21.9% for moulds and fungi, and 15.3% for

cat dander (Figure 5). The percentage of monosensitised allergic asthmatic patients was 12%, with 6.5% of asthmatic patients being monosensitised to mites, 2.5% to grass pollen and 2.2% to weed pollen. There were no asthmatic patients monosensitised to cereal, dog dander or moulds. The frequency of sensitisation evaluated by specific IgE was slightly different but was directly correlated with that from skin prick tests (Table 2).

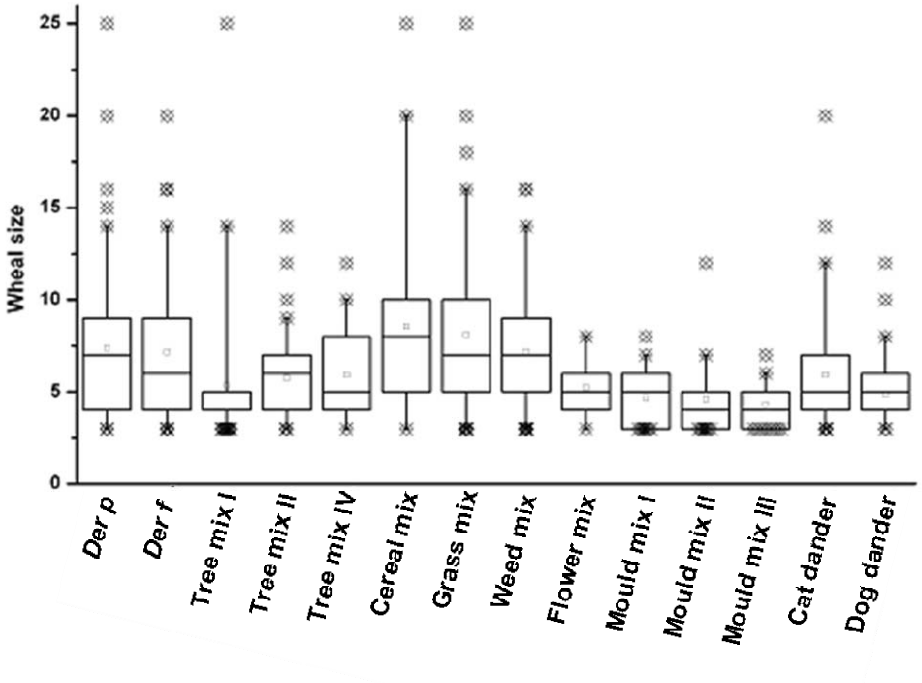

Figure 5 – Cutaneous reactivity to aeroallergens in allergic asthmatic patients.
Allergen sensitisation was evaluated by skin prick test with a battery of the most common aeroallergens in the local flora, as well as mites, moulds and animal dander.

Table 2 – Sensitisation profile in the allergic asthmatic patients.

Allergens	Skin prick test (n, %)	Specific IgE (n, %)
Cat dander	101 (36.3%)	32 (15.3%)
Cereal pollen	174 (62.6%)	110 (52.6%)
Dog dander	83 (30%)	27 (12.9%)
Grass pollen	182 (65.5%)	123 (58.9%)
Mites	175 (62.9%)	123 (58.9%)
Moulds and fungi	61 (21.9%)	4 (1.9%)
Tree pollen	137 (49.3%)	99 (47.4%)
Weed pollen	137 (49.3%)	116 (55.5%)

The major allergen sensitisers, as evaluated by skin prick testing and specific IgE, were grasses (*Lolium perenne, Phleum pratense, Dactylis glomerata* and *Poa pratensis*), cereals (*Secale cereale*, *Triticum sativum* and *Avena sativa*), mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), trees (*Olea europea* and *Platanus acerifolia*), the weed *Parietaria judaica* and the mould *Alternaria alternata*. A very interesting observation was that, in spite of similar prevalence of these major allergens, there were significant differences in terms of the class/level of sensitisation as measured by wheal size and specific IgE values (Figure 6). There was no correlation between the wheal size of the prick tests or the pattern of allergen sensitisation and the severity of the disease.

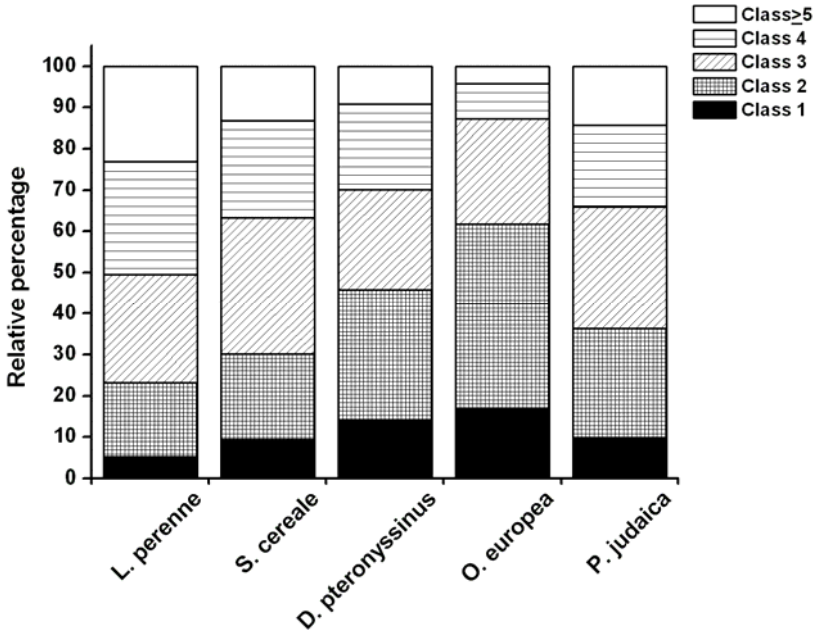

Figure 6 – Degree of sensitisation to aeroallergens in allergic asthmatic patients.
The degree of sensitisation, as analysed by specific IgE levels, was different between the major aeroallergens.

Rural versus urban asthmatic patients

As environmental factors, such as air pollution, are thought to play a part in sensitisation, we then compared several features between urban and rural asthmatic patients. Our population included 141 urban and 182 rural allergic asthmatic patients. The samples were paired for age and gender.

The sensitisation profile was significantly different between urban- and rural-based asthmatic patients, with more rural than urban asthmatic patients being sensitised to tree pollen and less to moulds and fungi (Chi-square test, 50.3% vs. 36.1% p= 0.017 and 7% vs.

22.1% $p<0.001$, respectively). However, the level of sensitisation was similar for all the allergens ($p>0.05$ Mann Whitney U test). Furthermore, there were no differences regarding severity of asthma between rural and urban allergic asthmatic patients and the same was valid for non-allergic asthmatic patients.

3.1.5. Discussion

Asthma is a major public-health problem that is increasing in prevalence in most developed countries. Data regarding prevalence and sensitisation profiles in bronchial asthma are scarce in Portugal. Given its heterogeneous nature, it is important to thoroughly characterise bronchial asthma in order to improve treatment measures.

Thirty percent of our asthmatic patients were non-allergic. This proportion is analogous to that found in similar studies with hospital-based recruitment [125], and is in accordance with the relative prevalence of non-allergic asthma, considered to vary between 10 and 30% [126].

In the present study, we observed that non-allergic asthmatic patients were older, predominantly female, and had a higher prevalence of drug allergy and oesophageal reflux, but a lower prevalence of rhinitis than allergic asthmatic patients. A previous French study aiming at describing clinical similarities and differences between allergic and non-allergic asthmatic patients also found an association between non-allergic asthma and older age, and female sex. However, in contrast to this study, we did not find differences in terms of FEV1 values between allergic and non-allergic asthma [125]. This difference may be attributed to different study designs, namely in terms of severity and treatment of the asthmatic patients included in both studies.

Asthma and rhinitis frequently occur concomitantly, with a reported prevalence of up to 100% in those patients with allergic asthma [127]. In the ECRHS, an association between asthma and rhinitis was observed even in non-atopic individuals [128]. In our study, there were significantly more allergic than non-allergic asthmatic patients with concurrent rhinitis. This may be due to the fact that many of the allergic asthmatic patients develop allergic rhinitis early in life and later develop bronchial asthma [128]. The importance of having concurrent rhinitis is highlighted by the key concept that has emerged in recent years that rhinitis and asthma should be viewed as disorders of a single airway. Having co-morbid allergic rhinitis is a marker for the presence of more-difficult-to-control asthma and worsened asthma outcomes [119].

It is possible that the prevalence of co-morbid allergic rhinitis in this retrospective study was underestimated, because the diagnosis of allergic rhinitis was restricted to that recorded in medical records. Many people with allergic rhinitis self-manage the condition with over-the-counter products, do not seek a physician's help, or indeed do not recognise allergic rhinitis as a condition needing treatment. However, it must be borne in mind that all asthmatic patients included in this study were specifically asked about symptoms of rhinitis and anterior rhinoscopy was carried out in all of them.

Some patients with asthma have chronic cough as their main, if not only, symptom. Curiously, in our study, we found significantly more non-allergic than allergic asthmatic patients with cough-variant asthma. This cannot be explained in terms of severity of the underlying asthma since there were no differences between allergic and non-allergic patients in this regard. It may be due to the fact that significantly more non-allergic asthmatic patients had gastro-oesophageal reflux. Alternatively, non-asthmatic patients may have a lower threshold for triggering cough reflex receptors in the bronchial mucosa.

In the literature, non-allergic asthmatic patients characteristically have a later onset of symptoms with a more severe clinical course of the disease than those with allergic asthma. This is in clear contrast with the present study. This difference could be related to the fact that our patients were classified by asthma severity at the first appointment but many of them were not treatment-naïve at that time.

In our population, major allergic sensitisers included the *Poaceae* family (grass and cereal pollens) and mites. This is in agreement with a previous study that analysed aeroallergen sensitisation in the paediatric population of Cova da Beira, and which observed a similar pattern of sensitisation with grasses, *Olea europea*, *Parietaria judaica* as the most representative sensitisers [129]. The Iberian study of aeroallergen sensitisation in allergic rhinitis reported similar results with common polysensitisation, involving both mites and pollens [130], without significant differences between the coastal and inland areas.

In the present study we provide for the first time data on the magnitude of sensitisations for the major allergens in Portugal. Interestingly enough, we show that there are significant differences in terms of the level of sensitisation as measured by specific IgE classes. In spite of similar prevalences within the major allergens, a particular species or family seems to be the main responsible agent for the symptoms, as happens with the grass pollen *Lolium perenne*, in contrast to *Olea europea.* Accurate identification of the specific cause of allergic asthma is important to implement avoidance measures.

In terms of allergen sensitisation in urban- and rural-based asthmatic patients, we found a higher prevalence of tree pollen allergy in the rural context and a higher prevalence of sensitisation to moulds in the city. This may be due to the fact that city dwellings are more humid and tree pollen sources (Olea trees) are more densely present in the countryside, thereby locally increasing the allergen load. In contrast, in a similar study involving patients with allergic rhinitis in the Cova da Beira area, the prevalence of sensitisation to grasses, weeds and *Olea europea* was higher in the urban group [131]. In urban areas, pollen grains can become coated with fuel residues and combustion products, and this binding of pollen to diesel exhaust particles may modulate the allergenic epitopes and increase their allergenicity [132]. Our findings, however, may be related to the relative size of sensitiser particles, as our study essentially included patients with bronchial asthma in contrast with the other study, which mainly involved patients with allergic rhinitis.

One limitation of our study is that the study sample of adults with asthma was drawn from a specialised allergy practice, and may differ significantly from the general population of asthmatic patients, since asthmatic patients treated by specialists appear to have more severe asthma than those seen by general practitioners.

In summary, in this study, rhinitis was more frequently associated with allergic than with non-allergic asthma, but drug allergy and oesophageal reflux were more associated with non-allergic asthma. In addition, the two types of asthma could not be differentiated in terms of severity of clinical symptoms.

The major allergens in the area were grass pollen, cereal pollen, mites and *Olea europea*. Monosensitisation was uncommon and it mainly included mites.

Urban- and rural-based asthmatic patients had different sensitisation profiles, but could not be differentiated in terms of severity of their disease.

Acknowledgements

The authors would like to thank all the volunteers without whom this study would have been impossible, the administrative staff from the Hospital Centre and A. Raposo for invaluable help with the data base.

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3.2. Asthma is more frequently associated with Non-allergic than Allergic Rhinitis in Portuguese Patients

Accepted for publication in Rhinology

3.2.1. Abstract

Background

The prevalence of rhinitis is increasing worldwide and is frequently associated with asthma, for which it is a risk factor. The aims of the study were to characterise the adult population with rhinitis attending the Cova da Beira Hospital Allergy Clinic, and to assess the relationship between rhinitis and asthma.

Methods

686 patients were characterised by clinical history and anterior rhinoscopy, and classified according to international guidelines. Atopy was determined by skin prick testing to aeroallergens and quantification of specific IgE.

Results

Seventy two percent of patients had allergic rhinitis (AR), and 28% had non-allergic rhinitis (NAR). NAR was more frequently associated with older age, perennial symptoms and female gender. NAR patients more frequently had bronchial asthma. In addition, more NAR than AR patients also had drug allergy, pharyngitis, sinusitis and urticaria. AR patients with nasal polyps more frequently had asthma. Grass pollen and mites were the major sensitisers for AR patients. Sensitisation profiles were not significantly different between urban- and rural-based AR patients.

Conclusions

Asthma was more frequently associated with non-allergic than with allergic rhinitis. The two types of rhinitis did not differ in clinical severity. Although sensitisation profiles were

not different between the urban and rural patients, the prevalence of allergic rhinitis was higher in urban patients.

3.2.2. Introduction

Rhinitis is a symptomatic disorder of the nose characterised by nasal blockage/congestion, rhinorrhea, itching and sneezing. Subdiagnosis is frequent as the disease is often trivialised, despite important impairment in the quality of life, increased medical costs and decreased school and work productivity.

The majority of rhinitis cases generally start in childhood or adolescence in individuals responding to common aeroallergens and are mediated by immunoglobulin E (IgE) (allergic rhinitis or AR) [133]. AR is diagnosed by history and examination backed up by specific allergy tests [134].

Other patients develop non-allergic forms of rhinitis, such as idiopathic rhinitis, hormonal rhinitis, food-induced rhinitis, or drug-induced rhinitis (non-allergic rhinitis or NAR) [135]. NAR includes a number of heterogeneous nasal conditions, in their majority poorly defined in terms of their underlying mechanisms, and with multifactorial aetiology [136]. Unlike AR, there are no specific diagnostic tests for NAR. The diagnosis is primarily made by excluding allergy-related features and infectious causes, as well as by assessing history of reactions to specific irritant/toxic triggers. In addition, the presence of nasal and blood eosinophilia as well as hormonal status (mainly estrogens) should be analysed. Furthermore, NAR can also contribute to rhinitis symptoms in atopic patients, creating a "mixed" phenotype [135].

Based on the time of exposure to allergens, allergic rhinitis has been classified as seasonal allergic rhinitis (SAR), also known as hay fever, and perennial allergic rhinitis. SAR was associated with outdoor allergens, such as pollens, and perennial rhinitis was associated with indoor allergens, such as dust mites, moulds, and animal dander. As this classification was regarded as insufficient, the revised classification adopted by *Allergic Rhinitis and its Impact on Asthma* (ARIA) divides rhinitis according to frequency and duration of symptoms into "intermittent" and "persistent", and according to severity (based on symptoms and quality of life) into "mild" or "moderate/severe" [136].

The prevalence of rhinitis is increasing worldwide [137], and the estimated prevalence in Portuguese adults aged between 20 and 44 years is 17%, as reported by the *European*

Community Respiratory Health Survey (ECRHS) [124]. However, studies describing other features of Portuguese rhinitis patients are scarce in the literature.

In order to increase the current knowledge about rhinitis patients in Portugal, we aimed at characterising the adult population with rhinitis attending the Allergy Clinic of the Cova da Beira Hospital between the years of 2003 and 2007, as well as assessing the relationship between rhinitis and asthma.

3.2.3. Materials and Methods

Patients referred by General Practitioners and ENT surgeons to the Allergy Outpatient Clinic of the Cova da Beira Hospital between 2003 and 2007 for suspected allergic rhinitis were sequentially studied.

The diagnosis of rhinitis was based upon clinical history, physical examination with anterior rhinoscopy, and response to medication. Skin prick testing to aeroallergens and determination of total and specific IgE were also performed in rhinitis patients to determine the presence of atopy. Sensitisation profiles were analysed both by skin prick tests and specific IgE. Skin prick testing included a first panel containing the 35 most prevalent aeroallergens in the region. According to results from this initial battery, as well as from clinical history, a second panel with more specific aeroallergens was also tested. For presentation of results, among the other allergens, the grass family (Poaceae) was divided into "grass" (non-cultivated Poaceae) and "cereal" (cultivated Poaceae).

Assessment of severity and frequency of disease was based on the revised classification of allergic rhinitis adopted by ARIA, even for non-allergic rhinitis [136]. All patients were specifically examined in terms of concurrent asthma by filling in a questionnaire, clinical examination and lung function testing (for those patients with a positive questionnaire). In addition, patients were also examined to assess other existing co-morbidities.

The designation of "non-allergic" was applied when the history, skin prick testing, and serum specific IgE measurements included in the allergy examination were all negative.

County administrative centres with predominantly industrial or tertiary services were considered "urban". Remaining centres were regarded as "rural".

The study protocol was approved by the Hospital Ethics Committee.

All data were analysed using non-parametric tests. Results are expressed as medians and range. Wilcoxon signed rank test was used for comparisons within groups and Mann Whitney U test (continuous variables), Chi-square test or Fisher's Exact Test (categorical

variables) for comparisons between groups. The association of various clinical parameters was analysed using the Spearman rank correlation test. A p value of less than 0.05 was considered significant. All analyses were performed using Minitab 14 for Windows.

3.2.4. Results

The total population analysed included 1092 patients, which represents 86% of all the patients in our clinic. From those, we excluded all patients under 18 years of age and patients without a permanent residence in the region. In addition, 32 patients were excluded because of discordance between clinical history, skin prick tests and specific IgE tests. Patients with hormonal, infectious or anatomic rhinitis were also excluded.

Six hundred and eighty six patients, all adult Caucasian, (473 females), living in the Cova da Beira area were included for further study.

Seventy two percent (494) of the patients had a diagnosis of AR and twenty eight percent (192), had NAR. Demographically, there were significant differences between the two groups. There were more women among NAR than among AR patients, with female: male ratios of 3.6 and 1.9, respectively ($p=0.001$). In addition, AR patients were significantly younger than NAR patients (median age 33 (18-102) vs. 47 (18-84) years, $p=0.001$). Smoking habits (recorded as pack-years) were not different between AR and NAR patients.

Rhinitis was classified according to the old practice that divided it into seasonal and perennial. As expected, we observed that seasonal symptoms were more common in AR than in NAR patients (36.7% vs.15.5%, $\chi^2= 22.354$, $p< 0.001$).

Patients were divided into three age groups; severity and duration of symptoms were classified according to the ARIA guidelines [136]. Severity did not increase with aging, either in AR or in NAR patients ($\chi^2=0.704$ for allergic and 2.844 for non-allergic, $p>0.05$). Moreover, there were no differences in terms of severity between AR and NAR ($p>0.05$) (Figure 7).

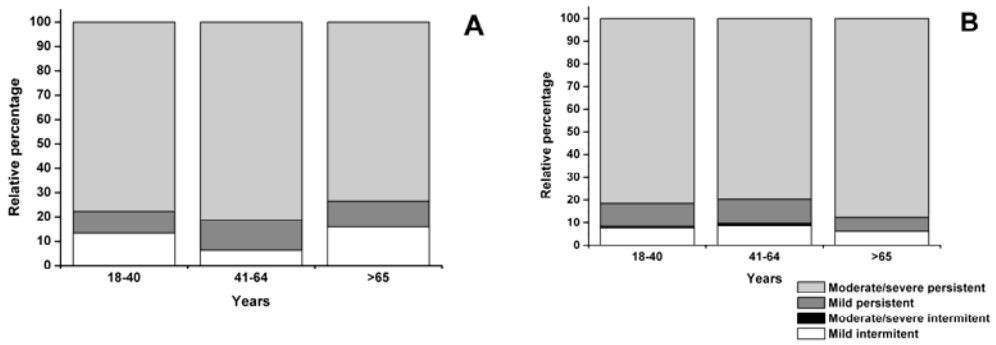

Figure 7 – Profiles of disease severity in different age groups of non-allergic rhinitis (Panel A) and allergic rhinitis (Panel B) patients.
Patients were grouped into three age groups and severity was assessed according to ARIA guidelines. Severity did not increase with age and was not statistically different between the two groups.

Rhinitis is frequently associated with bronchial involvement (even in NAR) and with other diseases. We studied clinical associations present in our population and observed that the great majority of them also had asthma. Although overall there were more allergic than non-allergic asthmatic patients, a significantly higher percentage of NAR than AR patients had concurrent asthma (87% vs 75%, respectively; $p < 0.001$; Chi-square Test). Severity of rhinitis did not correlate with that of asthma. In addition, a significantly higher percentage of NAR than AR patients had sinusitis, pharyngitis, urticaria and drug allergy. In contrast, a lower percentage of NAR than AR patients had conjunctivitis (Figure 8).

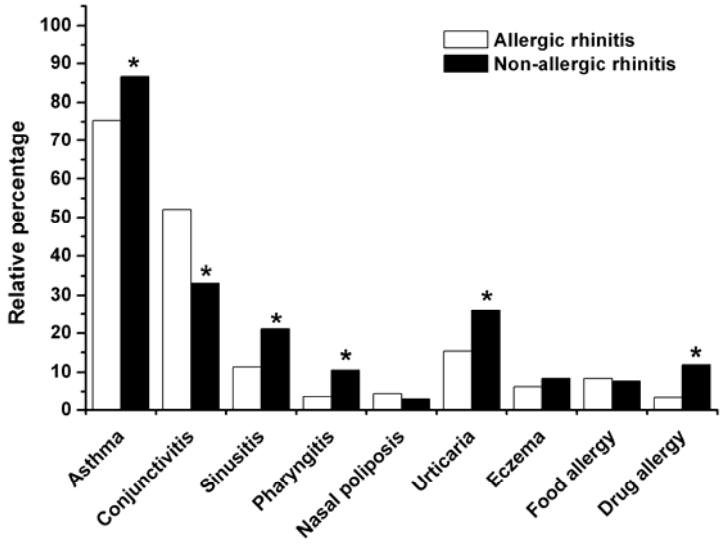

Figure 8 – Main co-morbidities associated with rhinitis.
Allergic rhinitis patients (light bars) had a higher prevalence of conjunctivitis, whereas non-allergic (dark bars) rhinitis patients had a higher prevalence of asthma, sinusitis, pharyngitis, urticaria, and drug allergy.

We used anterior rhinoscopy to detect and characterise nasal polyposis. No significant differences in terms of the percentage of patients with nasal polyps were observed between AR and NAR patients (4.4% (22 patients) vs 2.8% (5 patients); p=0.399; Fisher's Exact Test). Nasal polyposis was not associated with more severe rhinitis in either RA (p=0.11; Chi-square test) or NAR (p=1.0; Fischer's Exact Test) patients. Curiously, in AR (p=0.05; Chi-square test), but not in NAR (p=0.61; Fischer's Exact Test) patients, there was a trend for significant association of nasal polyposis with the presence of bronchial asthma. Finally, in AR patients, nasal polyposis was not significantly associated with preferential sensitisation to seasonal or perennial allergens.

As expected, total serum IgE was significantly higher in allergic than in non-allergic patients (median 177 (3-5000 kU/L) vs. 26 (<2-865 kU/L), p<0.001). There was no correlation between total IgE levels and features of allergic sensitisations (wheal size on skin prick tests or specific IgE levels).

In AR patients, the frequency of sensitisations evaluated by skin prick testing was 64.7% for grass pollen, 61.4% for mites, 59.2% for cereal pollen, 56.6% for weed pollen, 49.5% for tree pollen, 32.7% for cat dander, 29.4% for dog dander, and 23.5% for moulds and fungi. The frequency of sensitisation evaluated by specific IgE tests was slightly different, but directly correlated with that from skin prick tests: 60.3% for grass pollen, 55.33% for mites, 51.3% for cereal pollen, 55.0% for weed pollen, 45.6% for tree pollen, 13.3% for cat dander, 11.7% for dog dander, and 2.7% for moulds and fungi. The percentage of monosensitised AR patients, as assessed by skin prick testing, was 7.0%, with 4.0% of them monosensitised to mites, 0.9% to grass pollen, 0.5% to tree pollen, 1.2% to weed pollen, 0.2% to cat dander and 0.2% to dog dander. There were no AR patients monosensitised to cereal pollen or moulds.

The major allergen sensitisers, as evaluated by skin prick testing and specific IgE, were mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), grasses (*Phleum pratense, Dactylis glomerata, Lolium perenne,Festuca eliator, Poa pratensis* and *Cynodon dactylon*), cereals (*Secale cereale* and *Triticum sativum*), olive tree (*Olea europea)*, and the weed *Parietaria judaica* (Figure 9).

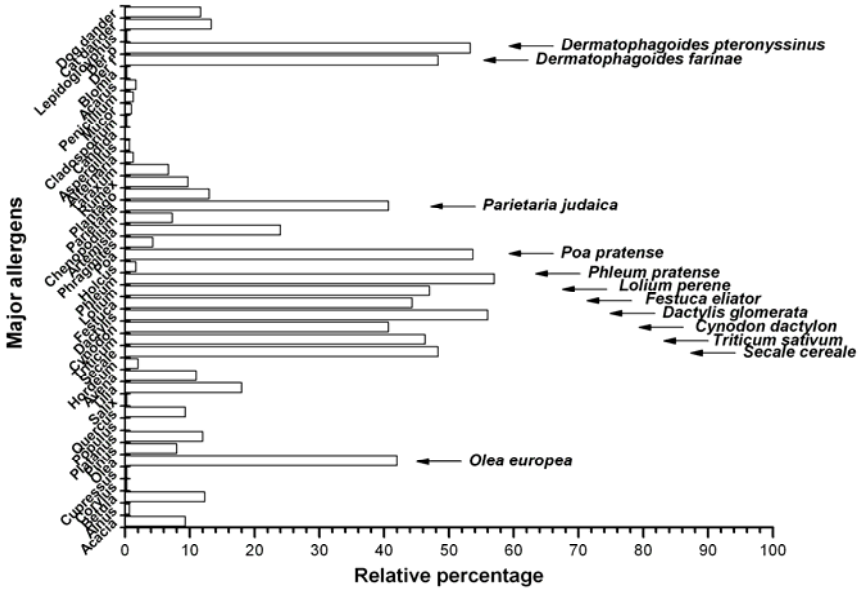

Figure 9 – Major aeroallergens in allergic rhinitis.
Allergen sensitisation was evaluated by skin prick test with a battery of the most common aeroallergens in the local flora, as well as mites, moulds and animal dander.

In spite of similar prevalence of these major allergens, there were significant differences in terms of the class/level of sensitisation as measured by wheal size and specific IgE values (χ^2=161.692, p<0.0001, Figure 10), with higher sensitisation levels, (classes 5 and 6) more prevalent with grass pollens.

We then went on to compare several features between urban and rural AR patients. Our population included 226 urban and 268 rural AR patients. The samples were paired for age and gender.

The sensitisation profile was not significantly different between urban- and rural-based rhinitis patients, and the level of sensitisation was similar for all the allergens (p>0.05).

Furthermore, there were no differences regarding severity of rhinitis between rural and urban allergic rhinitis patients and the same was valid for NAR patients.

However, AR prevalence as compared to NAR was higher in urban- than in rural-based patients (77.3 vs. 68.2; p=0.009; Chi-square test).

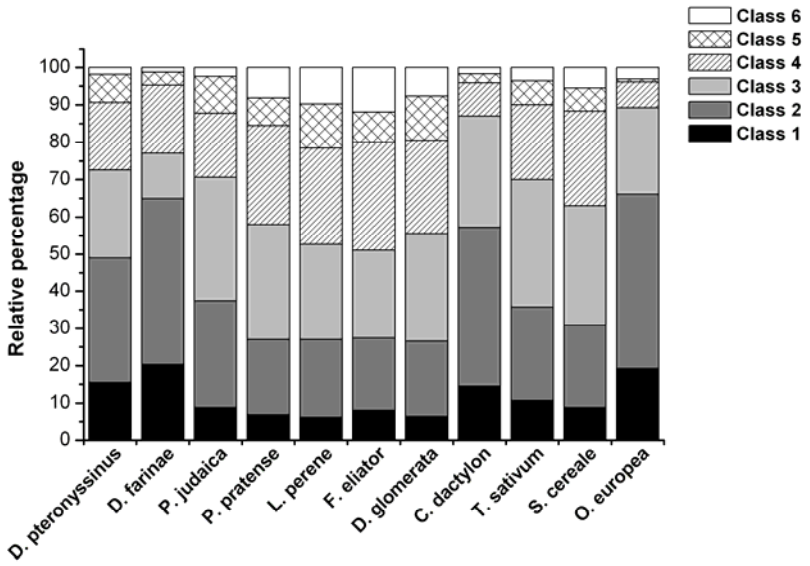

Figure 10 – Degree of sensitisation to aeroallergens in allergic rhinitis patients.
The degree of sensitisation, as analysed by specific IgE levels, was different between the major aeroallergens.

3.2.5. Discussion

Rhinitis is a major public-health problem that is increasing in prevalence in most developed countries [138]. Many people with rhinitis self-manage the condition with over-the-counter products, do not seek a physician's help, confuse it with recurrent infectious disease, or indeed do not recognise rhinitis as a condition needing treatment. This leads to subdiagnosis and impaired quality of life.

In the present study, we present data from a Portuguese patient population with rhinitis. As far as we know, this is the first full characterisation of Portuguese patients with rhinitis, involving not only epidemiological but also clinical aspects. We studied several aspects involving rhinitis in an allergy outpatient clinic. Our diagnosis of rhinitis was based upon subjective clinical symptoms as reported by the patients, but also on more objective aspects such as anterior rhinoscopy, allowing characterisation of turbinate swelling and rhinorrhea. Furthermore, the latter technique was also crucial for confirming the presence of nasal polyposis in our patients.

In this study, twenty eight percent of our rhinitis patients were non-allergic. These NAR patients were older, predominantly female, and, very interestingly, had a higher prevalence of asthma than AR patients. In line with these findings, the ECRHS reported a frequency of 25% of NAR, a higher prevalence of rhinitis among women and a stronger association

with asthma among NAR patients [139]. Overall, our patients had a high prevalence of bronchial asthma. This is in contrast with the known prevalence of asthma in patients with allergic rhinitis in the general population, which may average between 25% and 40% [136, 138] However, since we are a specialised clinic and not a general practitioner office, most patients referred to our outpatient clinic tend to have more severe and long-standing rhinitis, which increases the likelihood of having developed concurrent bronchial asthma.

In recent years, the key concept of "one airway, one disease" has emerged [136]. The majority of allergic asthma patients concomitantly have rhinitis, and some of the AR patients have asthma [136, 138]. In the ECRHS, an association between asthma and rhinitis was observed (even in non-atopic individuals), and asthma attacks were reported more often where there were high prevalences of nasal allergies [124, 128]. This association between rhinitis and asthma was not fully explained by shared risk factors, (including total IgE levels, parental history of asthma, or sensitisation to allergens), or genetic predisposition to atopic diseases [139]. Moreover, rhinitis is a significant risk factor for the occurrence of asthma, independently of allergy [140]. In a study by Leynaert *et al.*, asthma was strongly associated with rhinitis not only among atopic subjects (odds ratio (OR) =3.1; 95% confidence interval 2.4-4.0), but also among non-atopic subjects (OR=6.2; 95% confidence interval 4.3-8.8) [128]. In addition, having co-morbid allergic rhinitis is a marker for the presence of more-difficult-to-control asthma and worsened asthma outcomes [141, 142]. Concomitantly, there is considerable reduction in asthma morbidity in individuals with asthma and rhinitis, when the latter is properly treated [138].

Curiously, in our study, more NAR than AR patients had drug allergies, most of these were related to aspirin intolerance. Some NAR patients had concomitant asthma, aspirin intolerance and urticaria. Aspirin and other anti-inflammatory drugs can precipitate adverse reactions in bronchial asthma and urticaria. There is also a distinct clinical syndrome, "aspirin-induced asthma" or the aspirin triad, characterised by nasal polyposis, aspirin sensitivity and asthma [143].

Similarly, in our study, chronic pharyngitis and sinusitis were more frequent in NAR. This is most likely due to the fact that rhinitis tends to be more difficult to control in these patients and is often associated with more persistent post-nasal dripping that may contribute towards inflammation in the pharynx.

In a survey in a representative sample of the Belgian population, that evaluated both AR and NAR, the authors found a high prevalence of self-declared rhinitis, with AR being about three times more prevalent than NAR. In addition, AR patients suffered from a

greater number of co-morbidities (asthma, skin allergy and food allergy), and displayed a more severe profile than NAR patients [144]. However, no clinical evaluation was carried out to confirm the diagnosis, and no data on IgE-mediated allergy were known.
A similar study in Danish adolescents and adults showed that subjects with AR more often suffered from asthma, food allergy and conjunctivitis, whereas patients with NAR suffered more frequently from recurring headaches and sinusitis [145]. This study included clinical evaluation, spirometry and skin prick testing; however, as it is very clearly pointed out by the authors, only the 10 most common allergens were tested and no total or specific IgE was measured, leading to overlooked subjects with AR being included as NAR patients.

Nasal polyposis is a factor that can significantly decrease the quality of life of patients with rhinitis and even worsen underlying bronchial asthma. It was curious to notice that, although a relatively high percentage of our patients had moderate/severe persistent rhinitis, the prevalence of nasal polyposis was low, both in AR and NAR patients. We believe that the prevalence we found is representative of the true values within this population since all patients were also specifically asked about nasal obstruction (visual analogue score) and were analysed using anterior rhinoscopy. However, since not all patients were co-jointly observed with the ENT Department, not all patients underwent nasal endoscopy or CT scan of the nose and nasal sinuses. We therefore have to accept that some patients with clinically silent nasal polyposis may have escaped our diagnosis.
Overall, no significant differences in terms of the percentage of patients with nasal polyps were observed between AR and NAR patients in our study. This is not surprising since various reports in the literature, focusing on the relationship between allergy, rhinitis and nasal polyposis have shown discrepant results. In fact, although some authors have shown a higher prevalence of allergy in patients with nasal polyposis, varying between 54 and 64% [146, 147], others have not shown any association at all [148, 149, 150]. Differences in the methodological approach as well as different genetic populations may account for these differences.
In our study, nasal polyposis was not associated with more severe rhinitis in either RA or NAR patients. This was surprising since we expected the more relevant underlying inflammation in patients with more severe underlying rhinitis to be associated with a facilitated development of nasal polyps.
Importantly, there was a trend for AR (but not NAR) patients with nasal polyposis to have bronchial asthma. This important finding is in line with what has been described by others,

in terms of general asthma. For instance, in a population-based study, Johansson and co-workers, described that nasal polyps were more frequent in patients with bronchial asthma [151]. However, some authors have reported that non-allergic asthma is more frequently associated with nasal polyposis [152]. The discrepancy between these results and our own may be due to the low numbers of patients with nasal polyps who were not allergic in our patient population. Another possibility is that the differences may be due to the number of years with the disease, which was not similar in the two studies. Finally, and since our study tended to have a high proportion of patients with moderate/severe rhinitis, genetic differences may account for the observed differences. In fact, some genetic polymorphisms in IL-1 and IL-4 genes have been described in some populations, which increase or decrease the likelihood of developing nasal polyps [153, 154]. Such polymorphisms should also be studied in our population.

Grass pollen is the major cause of pollinosis in the Mediterranean region of Europe [155]. In our AR patients, major allergic sensitisers included the Poaceae family (grass and cereal pollens) and mites, with a low percentage of monosensitisation. There was a high degree of cross reactivity among the grass pollens. Olive tree and Parietaria were other major causes of sensitisation in this population. However, monosensitisation to olive tree pollen was not frequent. Interestingly enough, patients sensitised to Parietaria had perennial symptoms, possibly due to the long persistence of the pollen in the atmosphere, given current climatic changes [155]. We used a battery of 35 screening aeroallergen extracts some of which were mixtures of allergens. When these were positive, we further performed skin prick testing with the individual allergens. Although, theoretically, this approach may be associated with a higher rate of false positive results, we do not believe this is the case since we used highly tested commercial extracts each of which was selected by our team as the ones having the highest positive predictive value. Furthermore, clinicians performing the tests were highly trained in the technique.

This sensitisation profile is in agreement with a previous study that analysed aeroallergen sensitisation in the population of Cova da Beira, and which observed a similar pattern of sensitisation with a mixture of grasses, *Olea europea*, and *Parietaria judaica* as the most representative sensitisers [129].

The Iberian study of aeroallergen sensitisation in AR reported similar results with common polysensitisation, involving both mites and pollens, without significant differences between the coastal and inland areas [130]. This is the only report regarding Iberian populations.

In the present study, we provide, for the first time, data on the magnitude of sensitisations for the major allergens in Portuguese AR patients. We show that there are significant differences in terms of the level of sensitisation, as measured by specific IgE classes. In spite of similar prevalence within the major allergens, a particular species or family seems to be preferentially associated with high levels of specific IgE, as happens with timothy (*Lolium perenne*), in contrast to olive tree (*Olea europea*). This may suggest that those allergens are the principal determinants of clinical symptoms. In fact, a correlation between serum allergen-specific IgE and severity of perennial allergic rhinitis was found in children [156]. Accurate identification of the specific cause of allergic rhinitis is important for the implementation of avoidance measures and immunotherapy.

Epidemiological studies carried out in different geographical regions in the world have shown a significant and consistent association between levels of airborne pollutants (diesel exhaust particles, ozone, nitrogen dioxide, and sulphur dioxide) and increased asthma and rhinitis symptoms [132]. Similar data was obtained from experimental studies carried out in humans and animals [157]. In addition, rural living, especially on a farm, has been inversely associated with asthma, hay fever and atopy in children [158, 159]. Thus, the "rural protection phenomenon" may be a combination of both mechanisms.

Surprisingly enough, in terms of allergen sensitisation in urban- and rural-based AR patients, we found no differences in prevalence. This is in disagreement with a similar study which demonstrated that the prevalence of sensitisation to grasses, weeds and *Olea europea* was higher in the urban group [131]. Furthermore, prevalence of AR was found to be higher in urban than in the rural patients. This is because in urban areas, pollen grains can interact with fuel residues and combustion products, and this may modulate the allergenic epitopes and increase their allergenicity [132].

In a previous study in asthmatic patients of this region we did observe differences in the sensitisation profile with a higher prevalence of tree pollen allergy in the rural context and a higher prevalence of sensitisation to moulds in the city [160]. This fact could be related to the allergenic particles' size, and to how deep they can travel along the airways. In fact, intact pollen grains typically do not enter the lower regions of the respiratory tract, and main symptoms are located in the eyes, nose and nasopharynx [155].

One limitation of our study is that the sample of adults with rhinitis was drawn from a specialised allergy practice, and may differ significantly from the general population of

patients with rhinitis, since patients treated by specialists appear to have more severe symptoms than those seen by general practitioners. In addition, severity of rhinitis in these patients was classified at the first appointment, even though many of them were not treatment-naïve at that time. Previous studies in Portugal obtained discrepant results from those presented here, but different methodological approaches were used, as the majority of them were only questionnaire-based, whereas our study also included clinical parameters (anterior rhinoscopy) [129, 130].

In summary, in this study, bronchial asthma was more frequently associated with NAR than with AR. However, the two kinds of rhinitis could not be differentiated in terms of severity of clinical symptoms. Nasal polyposis was more frequently associated with bronchial asthma, in allergic patients.

The major allergens in the area were grass pollen, cereal pollen, mites and olive tree. Monosensitisation was uncommon and it mainly included mites. Interestingly enough, the magnitude of sensitisation was different among the major allergens, with Graminea pollen as both one of the major allergens and the one with the highest levels of sensitisation.

Urban and rural-based AR patients had no different sensitisation profiles, and could not be differentiated in terms of severity of their disease.

Acknowledgements

The authors would like to thank all the volunteers without whom this study would have been impossible, the administrative staff from the Hospital Centre and A. Raposo for invaluable help with the data base.

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3.3. Functional Characterisation of CD8+CD28+ and CD28− T Cells in Atopic Individuals sensitised to *Dermatophagoides pteronyssinus*

Published in Allergol. et Immunopathol. 2006; 34 (6): 234-41

3.3.1. Abstract

Background

For more than thirty years several investigators have suggested that CD8+ T suppressor cells played an important role in immunoregulation. Recent studies, mainly in transplantation and autoimmunity models, characterised this suppressor population by the lack of the co-stimulatory molecule CD28. These CD8+CD28− T cells are phenotypically and functionally different from CD8+CD28+ T cells and are expanded in rejection-free transplanted patients. Less is known about the former cells in atopy.

The aim of the present study was to analyse the phenotype and functional properties of human CD8+CD28− T cells upon allergen-specific PBMC proliferation in atopic and non-atopic individuals.

Methods

Peripheral blood mononuclear cells were obtained from atopic and non-atopic volunteers, after density gradient centrifugation. CD8+CD28− and CD8+CD28+ T cells were isolated using immunomagnetic beads, and the relative percentages and phenotypic markers were analysed by flow cytometry. Phenotypic studies included NK cell markers, chemokine receptors, activation markers as well as α/β and γ/δ TCR chains. Proliferation studies were performed in the isolated populations and also in co-cultures with PBMC using *Dermatophagoides pteronyssinus* as a stimulus. Proliferation was assessed by thymidine incorporation. Cytokine synthesis was evaluated in culture supernatants by Cytometric Bead Array.

Results

The relative percentages of CD8+CD28− T cells and their phenotypic expression in atopic and non-atopic volunteers were not significantly different. However, CD8+CD28− T cells proliferated more than CD8+CD28+ T cells when stimulated with *Der p*, although the cytokine synthesis was similar. In terms of allergen-induced proliferation in the co-cultures, CD8+CD28− co-cultures with PBMC proliferated more than CD8+CD28+ T cells co-cultures, but the cytokine synthesis patterns were alike.

Conclusions

Our data confirms phenotypic and functional differences between CD28+ and CD28− T cells, irrespectively of the atopic status. Purified CD8+CD28− T cells freshly isolated from the peripheral blood do not have suppressor properties neither over PBMC allergen-specific proliferation nor on cytokine synthesis.

3.3.2. Introduction

T cells that use non-cytolytic mechanisms to down-regulate the immune response (suppressor T cells or Ts) are thought to play an essential role in controlling reactivity to foreign antigens and inducing tolerance to self antigens [161]. Nevertheless, more than three decades since their discovery, an understanding of the mechanisms whereby suppressor cells exert their activity is still incomplete.

Phenotypically, CD8+ Ts are characterised by the lack of the CD28 co-stimulatory molecule [162]. CD8+CD28− T cells are thought to arise from CD8+CD28+ T cells that have proliferated several times since they have shorter telomeres [41] and share oligoclonal expansions [163]. *In vitro*, CD8+CD28− T cells arise from CD8+CD28+ T cells repeatedly stimulated in the presence of interleukin (IL)-2 [59]. In contrast, IL-4 can block this differentiation [43].

Recent evidence has been accumulating to show that CD8+CD28− T cells can inhibit T helper cell activation and proliferation in mitogen and antigen-driven responses [50, 70, 86]. *In vitro*, antigen-specific CD8+CD28− T suppressor cells have been generated by multiple rounds of stimulation of human CD8+CD28+ T cells with APC either from an allogeneic or a xenogeneic donor [70, 164]. The generated cells expressed FOXP3 [56], a gene related with regulatory function, and could inhibit proliferation of CD4+ T cells interacting directly with the APC used for priming [69, 76].

Non-antigen specific CD8+CD28− T cells with suppressor activity have also been generated from CD8+CD28− T cells in the presence of IL-2 and IL-10 [72]. These suppressor cells inhibited both antigen-specific CD4+ T cell proliferation and cellular cytotoxicity by secreting cytokines such as IFN-γ, IL-6 and IL-10 [82, 83]. Defects in this antigen-nonspecific suppression have been described in multiple sclerosis [83] and in patients with systemic lupus erythematosus [81], and are primarily seen in chronic progressive situations.

Atopic allergic diseases are immune disorders caused by aberrant Th2 cell-dominated responses to otherwise innocuous substances, such as proteins from house dust mite or grass or tree pollen [133]. Many authors believe that atopy and allergic diseases are the result of inadequate or impaired inhibition of allergen-specific T helper-type responses by the regulatory T cells [115], or even a consequence of decreased frequencies of those cells in atopic individuals [116]. There is a growing body of evidence which suggests that CD8+ T cells play an important part in regulating the IgE response to non-replicating antigens [91, 93]. Nonetheless, the presence of CD8+ suppressor T cells in human atopic patients is not yet confirmed.

The aim of the present study was to analyse the functional properties of human CD8+CD28− T cells upon *Dermatophagoides pteronyssinus* (*Der p*)-specific PBMC proliferation in atopic and non-atopic individuals.

3.3.3. Materials and methods

Subjects

Peripheral blood was obtained from 30 non-atopic and 32 atopic (with allergic rhinitis) adult volunteers (Caucasian, non-smokers), paired for age and gender. Atopic volunteers were recruited from the allergy clinic of the Cova da Beira Hospital and the non-atopic volunteers were recruited among the Hospital and the University staff.

Atopy was assessed by positive skin prick tests and specific IgE levels to *Der p*. Volunteers who received immunotherapy or were on systemic medication were excluded. Pregnant or breastfeeding women and all volunteers with disease affecting the immune system were also excluded.

The study was approved by the Hospital Ethics Committee. Informed written consent was signed by all the volunteers.

Monoclonal antibodies

The following monoclonal antibodies (mAbs) were used: anti-CD3 mAb conjugated with APC was purchased from Pharmingen (San Diego, CA, USA), anti-CD8 mAb conjugated with PerCP was purchased from Becton Dickinson (San José, CA, USA), and anti-CD28 mAb conjugated with FITC was purchased from Pharmingen.

Blood preparation and antibody staining

Freshly collected peripheral blood mononuclear cells (PBMC) were stained from whole blood after lysis of the erythrocytes (10mM Tris, 0.15M NH_4Cl, pH=7.4). Staining was performed at 4°C for 30 min in staining solution (PBS, 0.2% bovine serum albumin (BSA), 0.1% NaN_3) in round-bottomed microtiter plates (Greiner, Nürtingen, Germany) with ≈ $0.5x10^6$ cells/well. After staining, the cells were washed, resuspended in 500µl PBS and acquired in a FACSCalibur Flow cytometer (Becton Dickinson).

Flow cytometry analysis

Data were collected on 20.000 cells/sample using FACSCalibur flow cytometer equipped with an argon ion laser and a red diode laser, for quantification of CD28+ and CD28− T cells. Anti-CD8, −CD3, and −CD28 monoclonal mAb were used to define the CD3+CD8+CD28+ and CD28− T cell populations. T lymphocytes were gated for analysis based on light scattering properties and on CD3 staining. Positively and negatively stained populations were calculated by quadrant dot plot analysis determined by isotype controls.
For phenotypic analysis of the defined subpopulations, 30.000 events were collected per sample. Fluorescence dot plots and histograms were analysed using cytological software (Cell Quest Pro, Becton Dickinson). Phenotypic analysis of the TCRαβ and TCRγδ was also performed on the isolated fractions of CD3+CD8+CD28+ and CD28− T cells.

Cell isolation

Peripheral blood mononuclear cells (PBMC) from the volunteers were separated from peripheral blood (120ml) by centrifugation over Lymphoprep. Lymphocytes were further purified by allowing the cells to adhere to plastic for 1h at 37°C, 5%CO_2. CD8+ T cells were isolated from the supernatant by negative selection using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). To separate CD28+ and CD28− T cells from CD8+ cell suspensions, cells were incubated with CD28-FITC mAb (Pharmingen) and then with anti-FITC microbeads (Miltenyi Biotec). Purity of the fractions was greater than

85% as evaluated by flow cytometry analysis. The adherent fraction was collected as well and used as APC in cell cultures after mitomycin treatment.

Cell Cultures

Responder cells were cultured at a concentration of $0.5x10^6$ cells/ml for 6 days in RMPI 1640 complete medium [RPMI 1640, 2mM L-glutamine, 1% antibiotic/antimicotic (Sigma-Aldrich, St Louis, MO, USA), and 10% foetal calf serum (Biochrom, Berlin, Germany)] in 96-well flatt-bottomed plates (Nunc, Roskilde, Denmark) with or without APC and 10μg/ml purified *Der p* extract (a kind gift from Dr. Joost Van Neerven, The Netherlands). For positive control, cells were incubated with 0.5μg/ml OKT3 (eBiosciences,San Diego USA) or with 5μg PHA (Sigma-Aldrich) for 3 days in the same conditions as for *Der p*. Fourteen hours before the end of the culture [^{3}H]-thymidine was added to the wells (1μCi/well). [^{3}H]-thymidine (Amersham Biosciences, Uppsala, Sweden) incorporation was determined by scintillation counts in a TopCount (PerkinElmer, MA, USA) and results were expressed as counts per minute (cpm). Mean cpm of the triplicate cultures and standard error of the mean were calculated.
Culture conditions were as follows: CD8+CD28+ cells alone, CD8+CD28− cells alone, CD8+CD28+ cells + APC + *Der p*, and CD8+CD28− cells + APC + *Der p*.

Cell co-cultures

PBMC ($0.5x10^6$ cells/ml) were incubated in different proportions (1:1, 2:1, 4:1) with freshly isolated CD8+CD28+ and CD8+CD28− T cells for 6 days in 96-well flatt-bottomed plates with and without 10μg/ml *Der p* extract. Tritiated thymidine (1μCi/well) was added 14 hours prior the end of the culture. Cells were harvested on fibber filters, and incorporated thymidine was determined by scintillation counting.

Cytokine production

For soluble cytokine detection in the culture supernatants, the human Th1/Th2 cytokine bead array kit was used, according to the manufacturer's instructions (Becton Dickinson).

Statistical analysis

Statistical analysis of the results was performed using Minitab 14 statistical Software. Mann Whitney U test was used to assess differences between atopic and non-atopic volunteers. Wilcoxon signed rank test was used to assess differences within the same population. A p value of less than 0.05 was considered significant.

3.3.4. Results

In order to assess the proliferative response to allergen of CD8+CD28+ and CD28− T cells, we set up cultures of isolated fractions with and without APC and *Der p*. As shown in Figure 11, contrary to what occurred with unstimulated cells, CD8+CD28− T cells proliferated when stimulated with *Der p*, displaying a significantly higher level of proliferation as compared with CD8+CD28+ T cells in atopic and non-atopic individuals (p= 0.001). However, the proliferation was not significantly different between atopic and non-atopic volunteers (p>0.05).

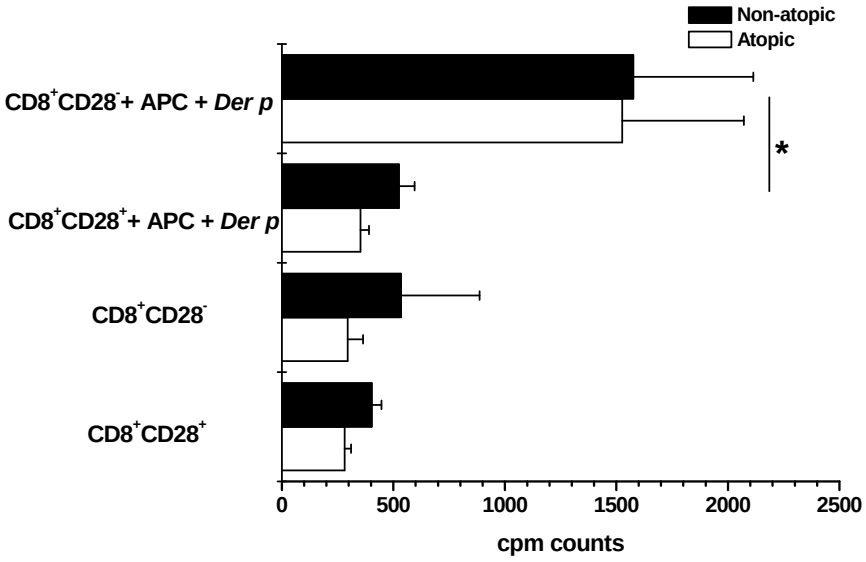

Figure 11 – CD8+CD28− T cells proliferate more than CD8+CD28+ T cells when stimulated with *Der p*.
Isolated CD8+CD28− and CD8+CD28+ T cells from 8 atopic and 10 non-atopic volunteers were incubated for 6 days in 96-well flat-bottomed plates with and without 10µg/ml *Der p* extract. Tritiated thymidine (1µCi/well) was added 14h prior to the end of the culture. Cells were harvested on fibber filters, and incorporated thymidine was determined by scintillation counting. Results show thymidine incorporation (cpm, mean ± sem). Wilcoxon signed ranked test was used for comparison between conditions

We also stimulated isolated fractions with OKT3 and, in this case, CD8+CD28− T cells proliferated less significantly as compared with CD8+CD28+ T cells (p= 0.003) (Figure 12).

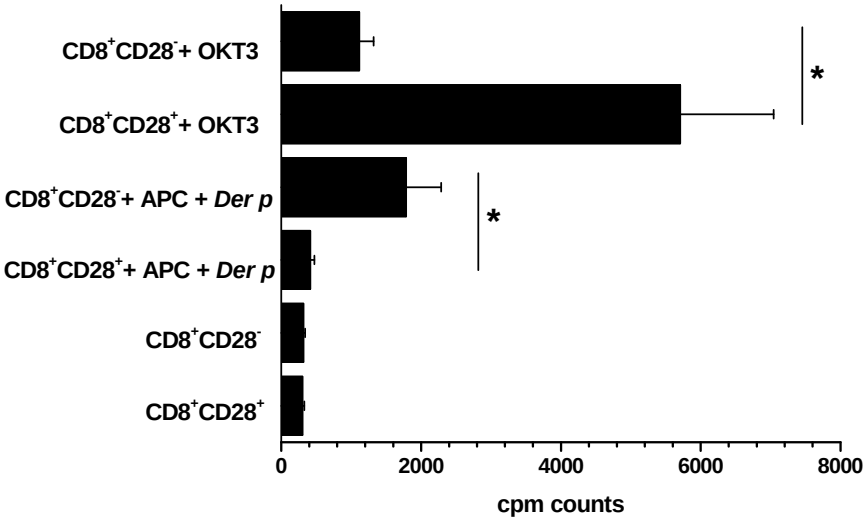

Figure 12 – CD8+CD28− T cells proliferate more than CD8+CD28+ T cells when stimulated with *Der p* but less with a polyclonal stimulus.
Isolated CD8+CD28+ and CD8+CD28− T cells from non-atopic volunteers were incubated for 6 days in 96-well flat-bottomed plates with and without 10μg/ml *Der p* extract and for 3 days with 0.5μg/ml OKT3. Tritiated thymidine (1μCi/well) was added 14 hours prior the end of the culture. Cells were harvested on fibber filters, and incorporated thymidine was determined by scintillation counting. Results show thymidine incorporation (cpm, mean ± sem) for 10 volunteers (5 females, aged 22-25 years). Wilcoxon signed rank test was used for comparison between conditions.

Cytokine synthesis was studied using the cytometric bead array (CBA) in culture supernatants collected on day 3. None of the cultures synthesised IL-5, IL-4 or IL-2, but all the *Der p*-stimulated cultures synthesised IFN-γ, TNF-α and IL-10. However, we did not observe statistically significant differences either between the cultures with CD8+CD28+ and CD28− T cells or between atopic and non-atopic individuals (Figure 13).

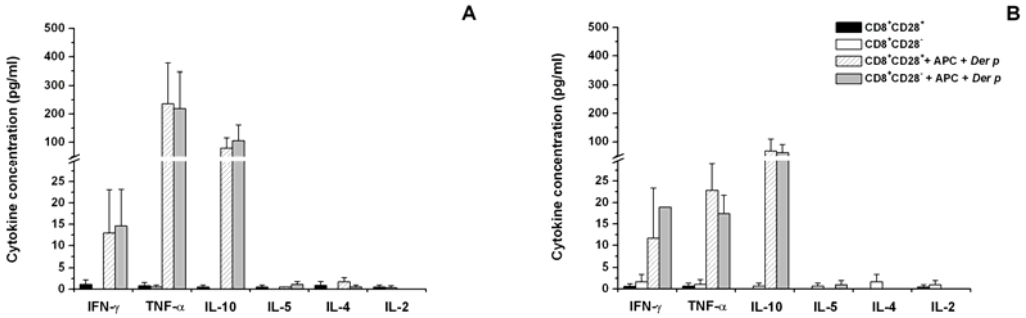

Figure 13 – Cytokine synthesis is similar for both isolated populations.
Isolated CD8+CD28− and CD8+CD28+ T cells from 4 atopic (Panel A) and 3 non-atopic (Panel B) volunteers were incubated in 96-well flat-bottomed plates with and without 10μg/ml *Der p* extract. On day 3, culture supernatants were collected and cytokine synthesis (IFN-γ, TNF-α, IL-10, IL-5, IL-4, and IL-2) was evaluated by cytometric bead array, following the manufacturer's protocol. Values shown are mean ± sem. Mann-Whiney U test was used for comparison between atopic and non-atopic and Wilcoxon signed rank test was used for comparison between conditions.

In order to study the potential suppressor properties of the isolated subpopulations, we performed co-cultures with PBMC at different ratios. None of the isolated subpopulations was able to suppress the allergen-specific PBMC proliferation, since we observed an increase in cpm counts (Figure 14).

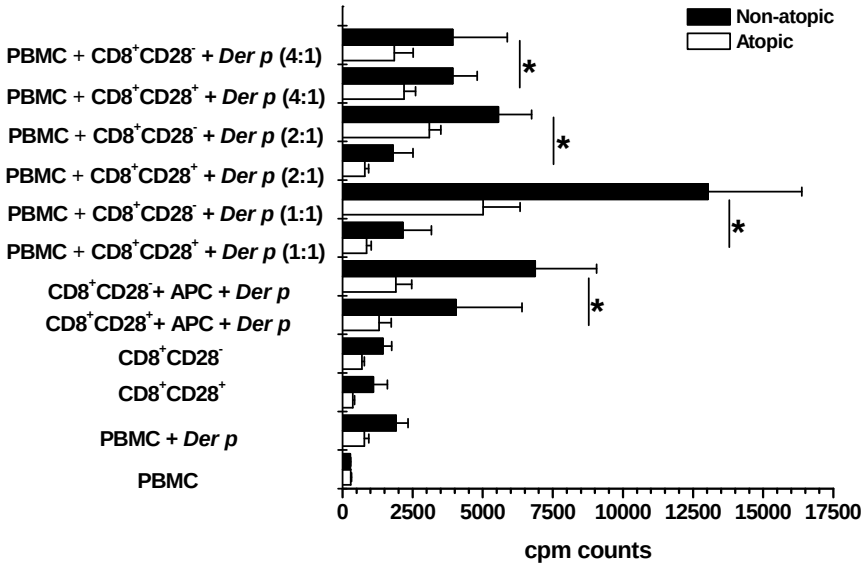

Figure 14 – CD8+CD28− co-cultures with PBMC proliferate more than CD8+CD28+ co-cultures. Isolated CD8+CD28− and CD8+CD28+ T cells from 8 atopic and 6 non-atopic volunteers were incubated in different proportions with PBMC for 6 days in 96-well flat-bottomed plates with and without 10µg/ml *Der p* extract. Tritiated thymidine (1µCi/well) was added 14h prior to the end of the culture. Cells were harvested on fiber filters, and incorporated thymidine was determined by scintillation counting. Results show thymidine incorporation (cpm, mean ± sem). Wilcoxon signed ranked test was used for comparison between conditions.

Cytokine synthesis was also evaluated by CBA, in the co-culture supernatants on day 3 and on day 5. Synthesis of IL-5, IL-4 and IL-2 was very low in all the cultures. When cytokine synthesis was compared between day 3 and day 5, only IFN-γ synthesis increased in the co-cultures. There were no significant differences in cytokine synthesis either between cultures from atopic and non-atopic individuals or between co-cultures with CD8+CD28− or CD8+CD28+ T cells (Figure 15).

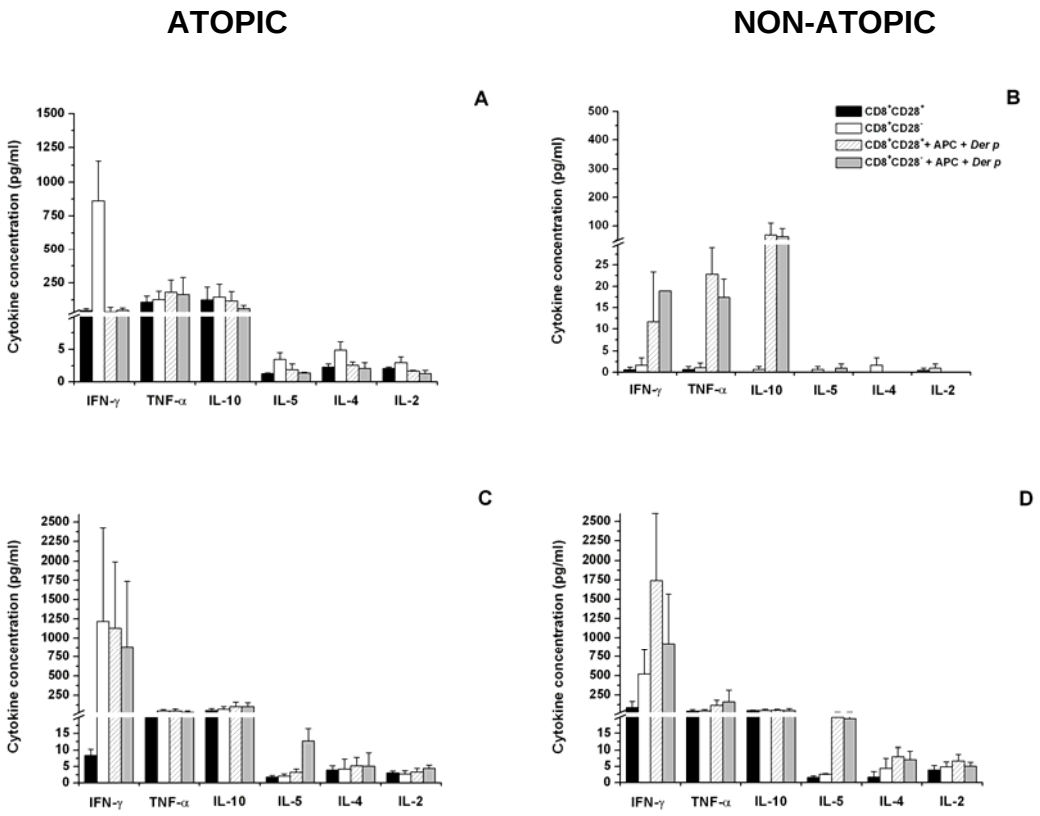

Figure 15 – Cytokine synthesis is similar in all the co-cultures stimulated with *Der p* extract. Isolated CD8+CD28− and CD8+CD28+ T cells from 4 atopic and 3 non-atopic volunteers were incubated in a proportion of 1:1 with PBMC in 96-well flat-bottomed plates with and without 10μg/ml *Der p* extract. On day 3 and day 5, culture supernatants were collected and cytokine synthesis (IFN-γ, TNF-α, IL-10, IL-5, IL-4, and IL-2) was evaluated by cytometric bead array, following the manufacturer's protocol. Values shown are mean ± sem. Mann-Whiney U test was used for comparison between atopic and non-atopic volunteers and Wilcoxon signed rank test was used for comparison between conditions.

3.3.5. Discussion

This is the first study on suppressor function of CD8+CD28− T cells in allergy. Previous studies on the regulation of atopic allergy mainly included natural T regulatory cells (CD4+CD25+) and showed inconclusive results [165, 166]. We believe that this study can enhance the knowledge and shed some light on the functional properties of the CD8+CD28− T cells and their implication in the development of atopy.

In previous studies, we observed that CD8+CD28− T cells are not phenotypically different between atopic and non-atopic individuals. This may imply that atopy is not associated with a specific phenotype of CD8+ T cells. However, it is important to mention that our data on phenotypic differences between CD28+ and CD28− subpopulations is in line with other reports in health and disease [33, 58]. Moreover, the increased expression of the NK cell

related receptors in CD8+CD28− T cells, in a context where co-stimulation is not present, may be important towards limiting T cell cytolytic responses, and act as a form of "regulation".

Classical antigen-presentation studies showed that MHC class I molecules present peptides derived from proteins synthesized within the cell, whereas MHC class II molecules present exogenous proteins captured from the environment. Emerging evidence indicates, however, that dendritic cells have a specialised capacity to process exogenous antigens into the MHC class I pathway [167]. Indeed we observed antigen-specific proliferation and cytokine synthesis by purified CD8+CD28− T cells when cultured with *Der p* which suggests that exogenous proteins can in fact be presented through the MHC class I pathway.

In accordance with our expectations, unstimulated cells did not proliferate. Interestingly enough, CD8+CD28− but not CD8+CD28+ T cells proliferated in response to *Der p*, in the presence of APC. This fact suggests that CD28− T cell proliferation is not impaired in spite of the absence of CD28 co-stimulation [168] and that CD8+CD28− T cells respond to common aeroallergens. These results should be considered with caution as the two subsets of CD8+ T cells studied here are oligoclonal [163] with respect to their TCR, and the use of this allergen might stimulate an insignificant minority of clones and lead to inconclusive results. For this reason, we performed cell cultures for 3 days with OKT3 to confirm whether multi-clonal cellular responses were different in both subpopulations. Results showed a proliferation impairment in CD8+CD28− T cells which corroborates previous results [33, 47]. This implies that our results must be confirmed by performing the same studies with another allergen.

When cytokine production was analysed, no detectable IL-5, IL-4 and IL-2 production was induced by the allergen in CD28+ or CD28− T cells. On the other hand, IFN-γ, TNF-α and IL-10 were synthesised at similar levels by both isolated populations. Other investigators mention that a subpopulation of suppressor CD8+CD28− T cells synthesises IL-10, thus mediating the suppressive effect [72]. Moreover, Seneviratne *et al.* linked low levels of IL-10 with severe atopic disease [16]. Since both of our subsets produced IL-10, we performed the co-culture studies in order to evaluate the "regulatory" capacity of these cells. The production of cytokines varied between donors, but followed similar patterns. The failure to detect the other cytokines (IL-5, IL-4, and IL-2) in the supernatants does not necessarily imply that they are not synthesised but may also suggest that they are immediately used as autocrine factors.

Finally, we performed co-culture studies and observed that freshly isolated CD8+CD28+ or CD8+CD28− T cells did not show suppressive properties, and were not able to inhibit allergen-driven PBMC proliferation or cytokine synthesis. These results are in line with previous reports namely by Suciu-Foca and Filaci groups [169], where suppressor CD8+CD28− T cells were only generated after multiple rounds of stimulation of PBMCs with allogeneic [69], xenogeneic [70] or antigen-pulsed [71] autologous APC.

However, the presence of CD8+CD28− suppressor T cells *in vivo* was observed in transplanted patients without rejection [87]. These facts may imply a need for an elevated and sustained contact with the antigen (hence the need for multiple rounds of stimulation) in order to generate antigen-specific suppressor cells. It would be interesting to further study suppressive properties of CD8+CD28− T cells, by using allergen-specific T cell lines developed from atopic and non-atopic volunteers.

In summary, in the present study we have shown that atopy is not related to an altered relative percentage or specific phenotypes in CD8+CD28+ or CD28− human T cells. Freshly immunomagnetically isolated CD8+CD28+ or CD28− human T cells have distinct phenotypes and, although sharing similar cytokine production patterns, they proliferate at different levels to common stimuli. Both subpopulations show similar proliferation capacity in atopic and non-atopic individuals and do not have any suppressor capacity.

Acknowledgments

The authors would like to thank all the volunteers without whom this study would have been impossible, and Dr. Joost Van Neerven for kindly providing the *Der p* extract.

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3.4. T Cells in Sputum of Asthmatic Patients are Activated Independently of Disease Severity or Control

Submitted to Respiration

3.4.1. Abstract

Background

T cells play an important role in bronchial asthma. Although airway CD4+ T cells have been extensively studied previously, there are hardly any studies relating CD8+ T cell activation and disease symptoms.

The aims of this study were to analyse the association between T cell activation in induced sputum T cells and asthma severity and control; and to evaluate T cell subpopulations in the same subgroups.

Methods

Fifty allergic asthmatic patients were recruited and lung function testing was performed. Airway cells were obtained by sputum induction via inhalation of hypertonic saline solution. CD3, CD4, CD8, CD28, CD25 and CD69 were studied by flow cytometry in whole induced sputum and peripheral blood T cells.

Results

Total induced sputum T cells and CD8+ T cells had a higher relative percentage of the activation markers CD25 and CD69 in comparison with peripheral blood. In sputum, the relative percentage of CD25 was higher in CD4+ T cells when compared to CD8+ T cells and the reverse was true regarding CD69. However, neither disease severity nor control correlated with the relative percentage of CD25 or CD69 expression on T cells in sputum.

Conclusions

Both CD4+ and CD8+ T cells are activated in the lungs and peripheral blood of asthmatic patients. However, there is no correlation between T cell activation phenotype in the target organ and disease severity or control.

3.4.2. Introduction

Asthma is characterised by variable airflow obstruction, airway hyperresponsiveness, and chronic airway inflammation, involving activated eosinophils, mast cells, and T lymphocytes [170]. Assessment of airway inflammation is important in asthma since it directly reflects the disease, whereas lung function testing and airway responsiveness are indirect measurements of the condition. Typically, airway inflammation has been assessed by bronchial biopsies or bronchoalveolar lavage (BAL) fluid. Examination of spontaneous or induced sputum has advantages over these invasive methods, as it is inexpensive, easy to perform, well-tolerated, non-invasive, safe and can be performed repetitively [171]. The cellular composition of sputum correlates well with that of BAL, but to a lesser extent with that of bronchial biopsies [172, 173]. In addition to cellular sputum composition, there is increasing interest in the analysis of cellular subtypes and activation, which can be performed using flow cytometry [174].

Traditionally, type 2 CD4+ T cells (Th2), have been considered to be the most important T cell subset in the pathophysiology of asthma. In fact, cytokines synthesised by CD4+ T cells are able to activate eosinophils and promote B cell synthesis of IgE. CD8+ T cells have been regarded as much less important or even as negative regulators of allergic inflammation. It was suggested that CD8+ T cells can contribute to the pathology of asthma death due to an enhancement of pre-existing airway inflammation in response to viral infection [112, 113]. Furthermore, human airway CD8+ T cells spontaneously produce increased type 1 and type 2 cytokines in subjects with asthma when compared to healthy controls [11].

Airway inflammation in asthma, involving activated eosinophils, mast cells and T lymphocytes can have varying degrees, and clinically, correlates with disease severity [175]. In fact, asthma guidelines recommend stepwise increments in anti-inflammatory medication to control increased disease activity [120]. However, few studies have analysed the relationship between disease severity and parameters of T cell activation.

The aims of this study were to compare T cell subpopulations and activation status between peripheral blood and induced sputum in asthma, and to correlate relative percentages in induced sputum with severity and control of the disease.

3.4.3. Materials and Methods

Volunteers

Fifty adult allergic asthmatic patients were recruited from the Allergy Clinic of the Cova da Beira Hospital and lung function testing was performed. Asthma was defined according to the American Thoracic Society criteria [176]. Allergy was confirmed by clinical history, skin prick testing, and determination of total and specific IgE. Asthma severity was assessed at the first appointment, according to the *Global Initiative for Asthma* (GINA) guidelines [120, 177]. Asthma control was assessed by the *Asthma Control Test* (ACT) [178], just before the induction of sputum. None of the patients had changes in the medication or respiratory infections in the month prior to the induction, and none was on oral steroids.

The study was approved by the Hospital Ethics Committee and all subjects gave their written informed consent.

Sputum induction

All patients were given detailed information and clear instructions prior to the procedure. Before sputum induction, all patients inhaled 12µg formoterol via a metered dose inhaler.

Sputum was induced and processed according to the method described by Pizzichini *et al.* [172] with minor modifications.

Baseline peak expiratory flow (PEF) was measured prior to induction, and the measurement was repeated following formoterol, and after each 5 minute inhalation of nebulised hypertonic saline (5%). The procedure was stopped if PEF fell by >20% at any time, or if bothersome symptoms occurred. After each period of inhalation, volunteers were asked to rinse their mouth with water and blow their nose, and attempt to cough sputum into a sterile container. The cumulative duration of nebulisation was 15-20 minutes.

Sputum processing

Unselected sputum was weighed and 0.1% dithiothreitol (DTT, Sigma-Aldrich, Saint Louis, MO, USA) in phosphate buffered saline (PBS) was added at a ratio of 4 ml to 1g sputum. The sputum was incubated with DTT at room temperature for 15 minutes on a rolling mixer. The same volume of PBS (4ml to 1g sputum) was added to the mixture and incubated for another 10 minutes. The suspension was then filtered through a 40µm cell strainer (Falcon). The filtrate was centrifuged at 500g for 10 minutes at room temperature to pellet cells. After two washing steps, cells were resuspendend in PBS and viability was determined by tryptan blue (Sigma-Aldrich) exclusion staining in a Neubauer haemocytometer (Merck Eurolabs, Lutterworth, UK).

PBMC preparation

Peripheral blood mononuclear cells (PBMC) were separated from 20ml heparinised peripheral blood using Lymphoprep (Nycomed, Oslo, Norway), washed twice in RPMI 1640 (Sigma Aldrich) without additives and counted.

Flow cytometry

Sputum cells and PBMC were washed in PBS containing 0.1% sodium azide (Sigma-Aldrich) and 0.4% bovine serum albumin (Sigma-Aldrich) (staining medium) and centrifuged at 300g. For each test, approximately 500.000 lymphocytes were incubated with fluorochrome stained antibodies for 30 minutes at 4ºC in the dark. Cells were then washed, resuspended in PBS and acquired using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Up to 20 000 CD3 positive events were collected per sample. All samples were stained with an APC-conjugated anti-human CD3 monoclonal antibody (mAb), FITC conjugated anti-human CD4 mAb, and PerCP conjugated anti-human CD8 mAb. To assess surface marker expression, samples were incubated with anti-CD25, -CD69, or -CD28 PE-conjugated mAbs (Becton Dickinson). Isotype-matched antibodies were used as controls. The number of positive cells for each surface marker was expressed as a percentage of CD4+ or CD8+ cells.

DTT tests in peripheral blood

As sputum has to be homogenized before flow cytometry, a possible effect of DTT treatment on surface markers has to be considered. However, in contrast to peripheral blood leucocytes, induced sputum leucocytes can only be obtained with DTT treatment.

In order to evaluate the influence of DTT on surface markers under study, we performed the same procedure of sputum processing using peripheral blood. Half the sample was treated with DTT and the other half with PBS alone in the same proportion as that of sputum (1g peripheral blood: 4ml DTT or 4ml PBS). Afterwards PBMC were isolated and stained as described.

Data analysis

Flow cytometry data were acquired and analysed using CellQuest Software (Becton Dickinson). Data are expressed as median and range. Wilcoxon signed rank test was used for comparison within the same group, and Mann-Whitney U test was used for comparison between two groups. A value of $p<0.05$ was considered significant, at a 95% confidence level.

Statistical analyses were performed using Minitab 14.

3.4.4. Results

Characteristics of patients

Fifty allergic asthmatic patients were recruited for this study.

Success rate in sputum induction was 90%; however, flow cytometry could only be performed in 41 of the samples due to insufficient number of T cells (clinical characteristics of subjects are given in Table 3).

Patients with different asthma severities were paired regarding to age and gender. Twenty one patients had intermittent asthma and were not under inhaled corticosteroids. They only resorted to rescue medication with short acting β2-agonists. Twenty patients had moderate persistent asthma; all were receiving beclomethasone dipropionate or equivalent (budesonide or fluticasone) daily (median dose 500μg per day), 11 of them were also receiving long-acting β2-agonists, and 6 were on anti-leukotrienes.

Table 3 – Clinical characteristics of study subjects.

	Intermitent Asthma (n=21)	Moderate Persistent Asthma (n=20)
Age (years)	39 ± 15	49 ± 13
Sex (F/M)	14:5	11:9
FEV1 (% predicted)	106% ± 12	92% ± 23
FEV1/FVC	82% ± 7	70% ± 10
FEF_{25-75} (% predicted)	85% ± 23	56% ± 26
Current smokers	3	4
Blood eosinophils (%)	2.4% ± 2	3.8% ± 3
Serum IgE (IU/ml)	186 ± 152	500 ± 612

Results are expressed as mean (± SE)
FEV1=forced expiratory volume in 1 second; FVC=forced vital capacity; FEF= forced expiratory flow

Sputum processing

Since DTT may affect results with sputum T cells, we also performed DTT incubations with peripheral blood. No differences were seen in staining of any of the cell surface markers (CD28, CD25 or CD69) on either CD3+, CD4+ or CD8+ cells between peripheral blood samples pre-treated with DTT plus buffer (PBS), and those treated with buffer alone (Table 4).

Table 4 – The effect of dithiothreitol (DTT) on the percentage of sputum lymphocytes expressing CD28, CD25 and CD69 (n=6)

	CD3+			CD4+			CD8+		
	DTT+PBS	PBS	p	DTT+PBS	PBS	p	DTT+PBS	PBS	p
CD28	80.7 [73.7-90.0]	83.0 [72.1-89.4]	0.402 0	97.5 [82.5-99.8]	97.1 [85.3-99.6]	0.529 0	68.5 [45.1-76.8]	71.2 [47.6-76.6]	0.402 0
CD25	28.4 [16.8-34.3]	25.2 [18.1-39.9]	0.834 0	43.2 [26.1-50.1]	42.7 [27.8-52.5]	1,000	4.6 [1.5-9.0]	3.8 [2.3-9.4]	1,000
CD69	1.3 [0.7-5.5]	1.8 [0.7-5.7]	0.138 0	0.6 [0.1-5.4]	1.0 [0.2-5.5]	0.208 0	1.8 [1.6-5.1]	2.7 [1.4-4.8]	0.402 0

Comparison between blood and sputum in all asthmatic patients

There were significant differences in the CD4/CD8 ratio between sputum and peripheral blood (3.6 vs. 2.0 respectively, p=0.005). This difference was due to a decrease in the percentage of CD8+ T cells in induced sputum (32.0% vs. 19.5%, p=0.001), with no significant change in CD4+ T cell percentages (63.0 vs. 73.5, p=0.056).

CD8+ T cells can be subdivided into functional and phenotypically different populations, on account of CD28 expression. CD8+CD28+ T cells are predominant in young healthy individuals and CD8+CD28− cells increase with aging and during inflammation and autoimmune disease. In the present study, there were no significant differences in relative percentages of CD8+CD28+ and CD8+CD28− T cells between peripheral blood and sputum (Figure 16I). Likewise there were no differences in the relative percentages of CD4+CD28+ and CD4+CD28- T cells (Figure 16F).

The relative percentage of total T cells expressing CD25 and CD69 was higher in sputum than in peripheral blood (26.9% vs. 21.3% and 57.7% vs. 3.4% for CD25 and CD69, respectively; Figs 16A and 16B). Similar results were obtained for CD8+ T cells (11.5% vs. 5.3% and 74.4% vs. 4.8%, for CD25 and CD69, respectively; Figs. 16G and 16H). In contrast, for CD4+ T cells only CD69 expression was higher in sputum when compared to peripheral blood (49.0% vs. 2.2, Figure 1E).

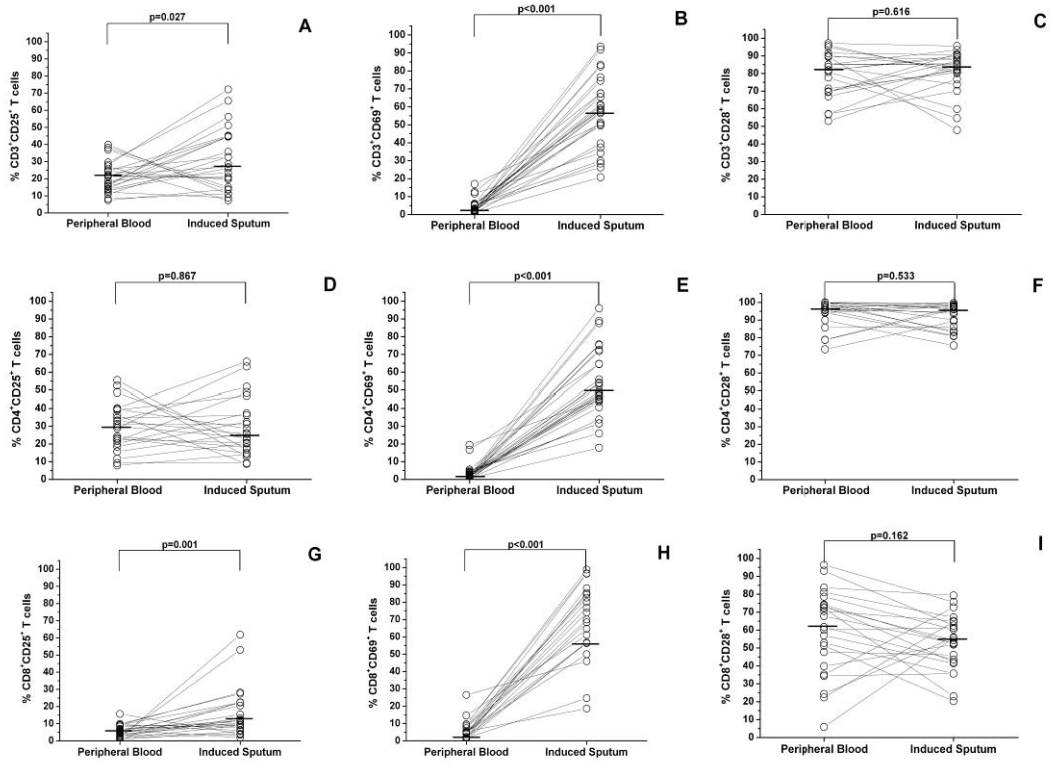

Figure 1 – CD25 (panels A, D and G, n=25), CD69 (panels B, E and H, n=28), and CD28 (panels C, F and I, n=23) surface expression in peripheral blood and induced sputum T cells from patients with intermittent and moderate persistent asthma.
The median value in each dataset is represented by a horizontal bar. Statistical analysis was performed with the Wilcoxon signed rank test.

CD8+ T cells had a lower expression of CD25 and CD28 than CD4+ T cells, both in induced sputum (10.4% vs. 25.6% and 55.6% vs. 94.6% for CD25 and CD28 respectively) and in peripheral blood (5.1% vs. 30.0% and 61.7% vs. 95.9% for CD25 and CD28 respectively; Figs 17A and 17C). In contrast, CD8+ T cells had a higher expression of CD69 than CD4+ T cells both in sputum and peripheral blood (74.4% vs. 50.8% and 4.8% vs. 2.2% respectively; Fig 17B).

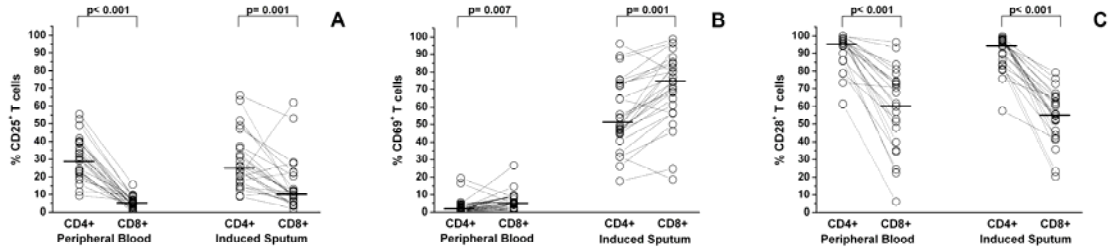

Figure 2 – Comparison of surface expression of CD25 (panel A, n=25), CD69 (panel B, n=28)) and CD28 (panel C, n=23) between CD4+ and CD8+ T cells from peripheral blood and induced sputum of patients with intermittent and moderate persistent asthma.
The median value in each dataset is represented by a horizontal bar. Statistical analysis was performed with the Mann-Whitney U test.

Comparison between blood and sputum in patients with intermittent asthma

To circumvent the possible effect of inhaled corticosteroids, we analysed the data referring only to intermittent asthmatic patients without medication, both at the time of sputum induction, and in the previous two weeks. We found similar results in these intermittent patients as those reported in the whole study population for the expression of activation markers in blood and induced sputum (Data not shown).

Relationship between severity and phenotype in sputum

Asthma severity was classified according to GINA guidelines and only intermittent or moderate persistent asthmatic patients were recruited for this study. To circumvent the possible effect of control, we show here results for controlled intermittent and controlled moderate persistent asthmatic patients alone.

There were no significant differences in the percentages of any of the sputum T cell subpopulations between patients with intermittent asthma and patients with moderate asthma. Likewise, no correlation was found between relative expression in sputum lymphocytes of CD25 or CD69 and the severity of asthma (Table 5).

Table 5 – Expression of CD25, CD69 and CD28 in sputum total lymphocytes, CD4+ and CD8+ T cells from patients with intermittent (n=11) and moderate persistent (n=6) controlled asthma.

	CD3+			CD4+			CD8+		
	Intermittent	Moderate persistent	p	Intermittent	Moderate persistent	p	Intermittent	Moderate persistent	p
CD28	83.9 [54.9-93.3]	81.9 [81.5-85.4]	0.756	96.2 [81.1-98.9]	96.0 [90.0-99.7]	0.640	52.4 [35.7-75.5]	81.9 [82.5-85.4]	0.794
CD25	44.6 [20.4-65.5]	38.65 [13.9-77.8]	0.860	37.3 [20.6-65.9]	26.1 [14.1-47.1]	0.860	11.3 [3.5-61.9]	12.6 [6.5-28.2]	0.871
CD69	57.1 [26.3-61.2]	57.4 [26.3-61.2]	0.428	53.6 [40.6-75.3]	45.6 [31.6-52.3]	0.090	74.1 [61.1-88.1]	82.4 [71.3-89.4]	0.213

Results are represented as median percentage and range, and comparisons were performed by the Mann-Witney U test.

Nevertheless, a more frequent expression of CD25 on CD8+ T cells from peripheral blood was associated with greater disease severity, with moderate persistent asthmatic patients having a higher percentage of CD25 positive cells (8.7% vs. 2.1% for intermittent and moderate persistent asthmatic patients, p=0.006, Fig 18) than the intermittent asthmatic patients.

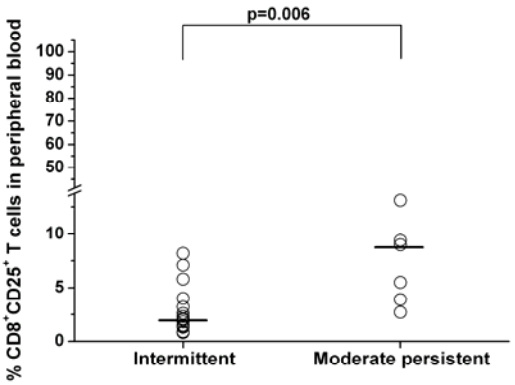

Figure 18 – Expression of CD25 on CD8+ T cells from peripheral blood of patients with intermittent (n=18) and moderate persistent (n=8) asthma.
The median value in each dataset is represented by a horizontal bar.
Statistical analysis was performed with the Mann-Whitney U test.

Relationship between control and phenotype in sputum

Asthma control was assessed prior to sputum induction by the ACT test. Asthmatic patients were divided into controlled (ACT above or equal to 20 points) or uncontrolled (ACT below 20 points) according to test results.

No correlation was found between relative expression in sputum lymphocytes of CD28, CD25 or CD69, and the control of asthma (Table 6).

Table 6 – Expression of CD25, CD69 and CD28 in sputum total lymphocytes, CD4+ and CD8+ T cells from patients with controlled (n=6) and uncontrolled (n=6) moderate persistent asthma.

	CD3+			CD4+			CD8+		
	Controlled	**Uncontrolled**	**p**	**Controlled**	**Uncontrolled**	**p**	**Controlled**	**Uncontrolled**	**p**
CD28	81.9 [81.5-85.4]	80.2 [59.8-91.1]	0.819	96.0 [90.0-99.7]	83.8 [80.7-97.5]	0.255	81.9 [82.5-85.4]	83.9 [54.9-93.3]	0.756
CD25	38.65 [13.9-77.8]	17.4 [11.1-28.6]	0.066	26.1 [14.1-47.1]	21.4 [12.9-32.4]	0.411	12.6 [6.5-28.2]	7.0 [4.1-27.5]	0.093
CD69	57.4 [26.3-61.2]	58.3 [20.8-65.5]	0.871	45.6 [31.6-52.3]	43.7 [17.8-64.5]	1.000	82.4 [71.3-89.4]	62.5 [50.0-80.2]	0.055

Results are represented as median percentage and range, and comparisons were performed by the Mann-Witney U test.

3.4.5. Discussion

This study clearly shows that, in subjects with allergic asthma, sputum CD4+ and CD8+ T cells have a phenotype of activated cells. This might be related to increased production of cytokines in the lung and to a hypothetical regulatory or inflammatory function.

In the present study, we observed higher expression of activation markers in sputum lymphocytes than in peripheral blood, consistent with an inflammatory process going on in the lung. Interestingly enough, this heightened expression was significant predominantly in CD8+ T cells which had higher expression of both early and late activation markers.

CD8+ T cells are considered to be of lesser importance in bronchial asthma. In contrast, in chronic obstructive pulmonary disease (COPD), also an inflammatory lung disease, CD8+ T cells contribute to the abnormal inflammatory process, and play an important role in the pathogenesis of disease. Increased numbers of activated CD8+ T cells in the airways may cause acute tissue damage via the release of lytic substances, such as perforin and granzymes.

CD8+ T cells are important immunoregulatory cells in some animal models of allergic disease [179], but their role in human allergic immune responses has not been well defined. A

tendency towards a high percentage of CD8+ T cells in the BAL of asthmatic patients as compared with healthy controls has been shown, resulting in a decreased CD4/CD8 ratio [114]. In fact, activated CD4+ T cells increase and produce type 2 cytokines in the peripheral blood, but CD8+ T cells are more sequestred than CD4+ T cells in the airway during an acute asthma attack [114].

Furthermore, the number of CD8+ cells in bronchial biopsies in patients with asthma has been associated with disease outcomes, as determined by loss of lung function [96]. Indeed, activated CD8+ T cell infiltration in peribronchial tissue has been associated with asthma death [112].

In our study, we found a higher CD4/CD8 ratio in induced sputum when compared to peripheral blood. Similar results have previously been reported by other investigators [172], even in healthy volunteers [174]. The imbalance obtained was due primarily to a reduction in the percentage of CD8+ T cells. This suggests that this difference may rather be due to the sources of the biological fluids, than to the presence or absence of asthma.

In the present study, we did not find any significant differences between the percentages of either CD28-expressing CD4+ or CD8+ lymphocytes in sputum and peripheral blood. CD28 is a co-stimulatory receptor, constitutively expressed on approximately 95% of CD4+ T cells and 50% of CD8+ T cells in humans, and is crucial for activation and proliferation of naïve T cells [47]. Effector T cells are less dependent on CD28 and their survival relies on extrinsic factors, such as IL-2, that are produced during activation [27]. This may suggest that T cells found in the lung in the context of asthma are not different from those in peripheral blood, in terms of co-stimulatory requirements.

We show here that in both total CD3+ cells and CD8+ T cells, CD25 and CD69 are expressed in a higher relative percentage in sputum than in peripheral blood. CD25, the interleukin-2 receptor α-chain, is a marker of both T cell activation and proliferation. CD69, which can be induced in different leucocytes, such as T cells, eosinophils, and neutrophils, is an early marker of T cell activation.

Leckie *et al.* reported similar results to ours: both in asthmatic patients and healthy controls CD8+ T cells from sputum had higher percentage of CD69 expression than CD4+ cells [174]. There were also higher percentages of both CD4+ and CD8+ cells expressing CD69 in sputum than in peripheral blood [174]. However, these authors failed to stain T cells with

anti-CD3 mAb. Instead, they used anti-human CD45 mAb to exclude other non-leucocyte events. This might lead to the inclusion of CD8+ NK cells in the analysis. Moreover, the Leckie study only included eight "very mild" asthmatic patients, while in the present study, 41 asthmatic patients with different degrees of severity were studied. In another report, Ortega *et al.* assessed the presence of activated bronchial lymphocytes in induced sputum of asthmatic patients and healthy controls. Lymphocytes from asthmatic patients showed an increased surface expression of activation markers (CD25 in T cells, and CD23 in B cells) when compared to lymphocytes of healthy non-atopic subjects [180]. However, this study only evaluated total T cells, while in our study we analysed both CD4+ and CD8+ subpopulations.

Our previous results could have been influenced by sputum processing using DTT. However, we believe that this is not the case because adding the same concentration of DTT, as used in sputum processing protocol, to whole blood from six allergic asthmatic patients did not influence the expression of the markers under study. Furthermore, other studies have also shown that DTT has no effect on the expression of CD69 and other surface markers [174, 181].

The GINA guidelines classify asthma severity in four degrees, from mild intermittent to severe persistent [177]. In the present study, we recruited two groups of asthmatic patients: one group with intermittent asthma and another group with moderate persistent asthma. Intermittent asthmatic patients were not on regular medication (only rescue medication), and all moderate persistent asthmatic patients were on inhaled corticosteroids.

We could not observe any relationship between asthma severity and the percentage of total, CD4+ or CD8+ T cells expressing the surface markers under analysis in induced sputum. In contrast, CD25 expression was increased on CD8+ T cells from peripheral blood of moderate persistent as compared to intermittent asthmatic patients.

Our results in induced sputum are not in agreement with those from a previous report. Hamzaoui *et al.* studied, for the first time, the expression of CD28 on CD8+ T cells in induced sputum. CD8+CD28- cells were found to be more expanded and expressed lower levels of IFN-γ in severe asthmatic patients than in mild asthmatic patients and age-matched healthy controls [182]. However, the authors did not include CD3 in the flow cytometric evaluation, including as CD8+ T cells probably other non-T cells (namely NK cells) which may also express CD8.

Reasons that may account for our failure to observe a relationship between asthma severity and parameters analysed in sputum in our study may include the fact that patients with moderate asthma were on inhaled corticosteroids. In fact, treatment with inhaled corticosteroids could have had a confounding effect on analysis of airway inflammation, as it has been demonstrated to reduce expression of some activation markers [108, 183]. Alternatively, disease severity may rather be related to changes in function than in the percentage or phenotype of CD8+ T cells in the bronchi of patients with asthma.

Independently of disease severity it is important to monitor control of disease. Monitorisation of disease control can be performed using both clinical scores and lung function testing. However, more patient-dependent tests of control, such as the ACT are increasingly being used. In this regard it should be stressed that clinical control is, to a certain extent, related to control of bronchial inflammation. The most frequently used marker of airway inflammation is sputum eosinophil percentage, since eosinophils are a good marker of impending loss of asthma control, and their increase is significantly associated with symptoms [173, 184]. However, some asthma exacerbations in optimally treated patients are mild and non-eosinophilic, so they cannot be monitored by eosinophilic cell counts [185].

Instead of using eosinophils, in the present study, we evaluated the possible correlation between T cell subpopulations and their activation status, and asthma control, as assessed by the ACT. For clarity of results we show here only the results for controlled and uncontrolled moderate persistent asthmatic patients.

No association was found between relative expression in sputum lymphocytes of CD28, CD25 or CD69, and the control of asthma. As previously discussed for disease severity, it is possible that this lack of association was due to the fact that all moderate persistent asthmatic patients were taking inhaled corticosteroids. Studies with a different design, allowing suspension of medication may help to clarify this issue.

In summary, in this study, we show that T cells are activated in the lung, which may suggest that both subpopulations are participants in the inflammation process at the target organ. However, we have not been able to demonstrate a relationship between activation of T cell subsets and severity of asthma or asthma control. Further studies are needed in order to characterise the function of T cells in the lung, mainly in the CD8+ subpopulation.

Acknowledgements

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4. General Discussion

4.1. Demographic aspects

The prevalence of allergic diseases is increasing in most countries of the world, particularly in areas with previously low or medium levels of prevalence [186]. A large number of papers have been published over the past years extending our knowledge on the epidemiology, diagnosis, management and co-morbidities of allergic diseases.

Data regarding prevalence and sensitisation profiles in bronchial asthma and rhinitis are still scarce in Portugal. Nevertheless, the prevalence of allergic diseases in this country increased between ISAAC phases I and III (7 years apart), both in allergic rhinoconjunctivitis and bronchial asthma in the two age groups under study (6-7 years and 13-14 years) [186]. Data in adults is restricted to the ECRHS study in volunteers between 20-44 years old, which showed an estimated prevalence of 5% for bronchial asthma and of 17% for rhinitis [124].

Several regional [118, 129, 131, 187] and one Iberian study [130] evaluated the sensitisation profiles, prevalence and disease severity in asthma and rhinitis in Portugal. However, they were either only questionnaire based [118, 187], included data of a private clinic practice [129, 131], or were not specifically carried out in the Cova da Beira region [130].

In order to increase our present knowledge of the demographic aspects of the main allergic diseases in Portugal, we collected data from patients attending the Allergy Clinic of the Cova da Beira Hospital Centre. It is important to stress that in the first Portuguese pollen map, carried out in the country between 1998 and 1999, the Cova da Beira region had the highest pollen counts in Portugal (Mapa Polínico em Portugal (1998-1999), SPAIC/Schering-Plough). Surprisingly, the region is not included in the "*Rede Portuguesa de Aerobiologia"* (www.rpaerobiologia.com), and no station/monitoring centre exists nearby. This should be a goal for the near future.

All patients with diagnosed asthma and/or rhinitis were included for further study. The asthma study (Paper I) included patients admitted to the Allergy Clinic between 2003 and 2006 (1078 patients); whereas the rhinitis study (Paper II) comprised patients admitted to the Allergy Clinic between 2003 and 2007 (1092 patients). Both referral patients and newly diagnosed ones were enrolled.

Exclusion criteria included patients under 18 years of age, patients not currently living in the area, and patients with discordant results in specific allergy tests.
We diagnosed asthma and rhinitis by positive history and examination, backed up by lung function testing and specific allergy tests (skin prick test and serum-specific IgE). According to IgE involvement, two broad categories were considered: allergic and non-allergic. We applied current guidelines for the classification of this type of pathology regarding severity and control aspects, including both the severity of symptoms and quality of life outcomes. Another important aspect was that we considered co-morbidities of bronchial asthma and rhinitis, and applied the concept of "one airway one disease". More specifically, patients with rhinitis were evaluated for asthma by history, chest examination and assessment of airway obstruction. On the other hand, patients with asthma were evaluated for rhinitis by history and physical examination, including anterior rhinoscopy.

Thirty percent of our asthmatic patients and twenty eight percent of our rhinitis patients were non-allergic. Non-allergic asthmatic patients were older, predominantly female, and had a higher prevalence of drug allergy/drug intolerance. This is in agreement with previous reports in other countries [125, 139].

We used anterior rhinoscopy to detect and characterise nasal polyposis. This is of especial interest, since nasal polyps can significantly decrease the quality of life of patients with rhinitis and worsen concomitant bronchial asthma. In fact, in our study, there was a trend for AR patients with nasal polyposis to have bronchial asthma. In contrast, in rhinitis patients, who had moderate/severe disease, the prevalence of polyposis was low. This was probably due to the fact that the diagnosis of polyposis was based on anterior rhinoscopy and not all patients underwent nasal endoscopy, which might have underestimated the true prevalence of nasal polyps.

Interactions between the lower and the upper airways are well known and have been extensively studied since the 1990s. This eventually led to the development of the ARIA project and to the elaboration of specific guidelines on the subject [136, 188]. It is reported that over 80% of asthmatic patients have rhinitis and 10-40% of patients with rhinitis have asthma [136].
In terms of this association, in our population (Paper I), rhinitis was more frequently associated with allergic than with non-allergic asthma. In contrast, bronchial asthma was

more frequently associated with NAR than with AR, although, overall, there were more AR patients with asthma (Paper II). Reasons that account for this may include the fact that allergic rhinitis may precede the development of asthma in a typical allergic march in adults; furthermore, immunotherapy in allergic rhinitis patients may delay or even prevent the development of further sensitisations and of bronchial asthma. In contrast, non-allergic rhinitis and asthma may share common triggers, even if they do not follow a specific timeline. Finally, there was only a partial overlap between our patients with mainly rhinitis (Paper I) or asthma (Paper II).

Besides analysing the differential association between rhinitis and asthma in allergic or non-allergic contexts, it is quite important to ascertain the severity of both rhinitis and asthma. In this regard, neither allergic nor non-allergic asthma (nor rhinitis) could be differentiated in terms of the severity of clinical symptoms. This is somehow unexpected, as it is commonly thought that non-allergic diseases are more severe. This may be due to the fact that there were fewer patients with the non-allergic forms of these diseases, thereby not allowing a fully balanced analysis of results. In addition, one must keep two aspects in mind: firstly, disease severity increases with age, and secondly, non-allergic patients are older. Thus, apparently, non-allergic diseases will appear as more severe if we do not pair the samples for age.

Sensitisation to aeroallergens was assessed by skin prick testing and/or serum specific IgE determination. Skin prick testing was not performed in cases of severe skin disease or if a very exuberant reaction was expected. Dermographism and inconclusive skin prick tests (such as negative reaction to Histamine) excluded skin test results from further analysis. Skin prick testing included a battery of 14 single extracts or extract mixtures from the supposedly most prevalent allergens in the area, reaching a total of 35 aeroallergens.
The major allergens in the area were grass pollen, cereal pollen, mites and olive tree pollen. Monosensitisation was uncommon and it mainly included mites. This sensitisation pattern was similar for asthmatic and allergic rhinitis patients.
Frequency of sensitisation evaluated by specific IgE was slightly different but directly correlated with that from the skin prick tests. This finding has two main causes: firstly, for some patients, data on both skin prick testing and specific IgE were unavailable; secondly even after correcting for this bias by selecting only patients with both exams, the skin prick tests proved more sensitive, as expected.

Interestingly enough, in spite of similar prevalence within the major allergens, a particular species or family seems to be preferentially associated with high levels of specific IgE. This was the case of grass pollen (Poaceae family), both one of the major allergens and the one with the highest levels of sensitisation. This is in accordance with previous studies in Portugal [129], [131] and the Iberian Peninsula [130], reporting common polysensitisation involving mites and pollens. However, these studies evaluated only severity, prevalence, and sensitisation with only minor emphasis on clinical aspects. Moreover, sensitisation was evaluated by skin prick test alone.

In our study, correlation between specific IgE levels and severity of the disease was not found possibly due to the polysensitisation profile of most of the volunteers. Nevertheless, it has also been described that specific IgE levels do not often directly correlate with clinical symptoms [189, 190, 191].

Epidemiological studies carried out in different geographical regions of the world have shown a significant and consistent association between levels of airborne pollutants (diesel exhaust particles, ozone, nitrogen dioxide, and sulphur dioxide) and increased asthma and rhinitis symptoms [132]. The prevalence of atopy and allergic rhinitis is higher in urban than in rural areas, both due to increased air pollution and protective farm effect.

In urban areas, pollen grains can become coated with fuel residues and combustion products, and this binding of pollen to diesel exhaust particles may modulate the allergenic epitopes and increase their allergenicity [132]. Moreover, nonspecific irritants such as air pollution may aggravate symptoms in symptomatic patients and induce symptoms in asymptomatic patients.

Urban- and rural-based asthmatic patients in our study had different sensitisation profiles, but could not be differentiated in terms of severity of their disease. In contrast, urban and rural-based AR patients had similar sensitisation profiles.

Our findings may be related to the relative size of the sensitiser particles, and to how deep they can travel through the airways. In fact, intact pollen grains (10 to 100μm on average) do not typically enter the lower regions of the respiratory tract, and main symptoms are located in the eyes, nose and nasopharynx [155]. In our specific population, most of the patients, both asthmatic and AR, were sensitised to Poaceae pollen, which have pollens varying from 20-80μm, with cultivated ones (cereals) having larger pollens. Provocation tests with allergen could be a way to clarify this issue, especially because there is concomitantly asthma and rhinitis in a very high percentage of the volunteers.

One limitation of our studies is that the sample of adults we analysed was drawn from a specialised allergy practice. In this sense, it may differ significantly from the general population of patients with rhinitis and asthma, since patients treated by specialists appear to have more severe symptoms than those seen by general practitioners. In fact, many rhinitis patients dismiss the disease. In addition, severity of asthma and rhinitis patients in our studies was classified at the first appointment, even though many of them were not treatment-naïve at that time.

Moreover, as patients were selected by presenting rhinitis to the rhinitis study and by presenting asthma to the asthma study, there is a certain degree of overlap among the groups. However, it is important to notice that the majority of the population enrolled for the rhinitis study had moderate/severe disease (82% in the allergic rhinitis group and 80% in the non-allergic rhinitis group), and was most likely referred to the Allergy Clinic by its rhinitis symptoms. In contrast, a similar trend was not seen in the asthmatic patients, as the majority of the population under study presented intermittent (52% in the allergic rhinitis group and 45% in the non-allergic rhinitis group) or mild persistent disease (29% in the allergic rhinitis group and 34% in the non-allergic rhinitis group).

Nevertheless, from our point of view, this is a very interesting study as it included clinical parameters, in contrast to previous studies that were mainly questionnaire-based. As the clinical definition of the diseases is difficult to use in the epidemiological settings of large populations, the questionnaires use simpler "working definitions", that are less specific. Our study could have been improved had we had access to auxiliary diagnostic testing, namely endoscopy and rhinomanometry for rhinitis evaluation and eNO for asthma assessment. The first methods are usually reserved for ENT (ear, nose and throat) outpatient clinics; the latter one is not currently available at the Hospital, in spite of a try-out period with one such system in the Lung Function Testing Unit.

As a result of the demographic studies, we developed a thorough patient database, useful for studying the cellular aspects of allergic pathologies. We believe that the sensitisation profiles will increase the specificity of the coming evaluations, and will serve to direct avoidance strategies and even landscape planning.

In addition, these studies reinforced the need to treat the upper and lower airway diseases with a combined strategy in terms of efficacy and safety.

4.2. CD8+ T cells in allergy

In both allergic asthma and rhinitis, there is chronic inflammation of the respiratory tract, which is mediated by the increased expression of multiple inflammatory proteins, including cytokines, chemokines, adhesion molecules, and inflammatory enzymes. In both diseases, there are acute episodes or exacerbations, when the intensity of this inflammation increases. Several studies have suggested a role for T cells in asthma, as T cell infiltration is a feature of the late-phase response to allergen in the lung. By specific immunostaining of bronchial mucosal biopsies, obtained via the fiberoptic bronchoscope, T cells could be identified, as well as the mRNA for the cytokines they may secrete.

In asthmatic patients, there is an increase in the number of CD4+ T cells in the airways, which are predominantly Th2 cells [192]. Th2 cells have a central role in allergic inflammation by secreting the cytokines IL-4, IL-5, IL-9 and IL-13. IL-4 and IL-13 drive IgE production by B cells, IL-5 is responsible for eosinophil differentiation in the bone marrow and enhances their effector capacity; and IL-9 attracts and drives the differentiation of mast cells.

On the other hand, CD8+ T cells are considered to be much less important, although a number of studies describe a role for these cells in the development of AHR and airway inflammation [95, 97, 100]. In asthma patients, airway infiltrating CD8+ T cells have the capacity to produce Th2 cytokines and modulate the disease [11], increased numbers of CD8+ T cells have been found in the lungs of patients after a fatal asthma attack [112], and the number of bronchial wall CD8+ T cells, but not the number of eosinophils or thickness of the basement membrane, was found to correlate significantly with decline in lung function [96].

The precise role of CD8+ T cells in allergic inflammation is still elusive, and it might be that different CD8+ T cell populations exert different functions. Moreover, CD8+ T cell function may be different between the sensitisation phase and the actual disease.

Atopy can be assessed by studying specific IgE levels in serum; it is likely that allergic disease encompasses other alterations at the T cell level in peripheral blood, not only restricted to the target-organs. With our project, we aimed at studying CD8+ T cells in the peripheral blood of atopic volunteers with allergic diseases (Paper III). As a subpopulation of CD8+ T cells, CD8+CD28− T cells showed distinct functional properties in other

disease settings *in vivo* and *in vitro,* we studied CD28 positive and negative CD8+ T cell populations independently.

Atopy was not related to an altered relative percentage or specific phenotypes in CD8+CD28+ or CD28− human T cells. However, freshly immunomagnetically isolated CD8+CD28+ or CD28− human T cells had distinct phenotypes and, although sharing similar cytokine production patterns, they proliferated at different levels to common stimuli. Proliferation to the allergen extract *Der p* was also differential, as only CD8+CD28- proliferated in response to *Der p* in the presence of APC.

It would be important to repeat this study with different allergens, especially so because house dust mite allergen has proteolytic activity. This activity is responsible for augmenting the permeability of pulmonary epithelium, thereby contributing to the uptake of the allergens and increasing the likelihood of developing an allergic response [193]. Thus, in this case, the biological activity of the allergen enhances its allergenicity *in vivo*. In *in vitro* systems, the mitogenic potential may also be influenced by the enzymatic activity, although no studies on this aspect have been published.

Both CD8+ T cell subpopulations showed similar proliferation capacity in atopic and non-atopic individuals, and did not show suppressor capacity. One must bear in mind that this assessment roughly mimics the real stimuli *in vivo*, as in a setting of allergic disease the stimuli are not punctual, but more or less continuous over long periods of time. It can also be argued that peripheral blood T cells do not share the same cellular environment as their counterparts at the target organs. Furthermore, allergen contact is much more limited.

In fact, previous studies with unprimed CD8+CD28− T cells from the peripheral blood showed that these cells do not have regulatory properties, and do not express markers related to suppression [56].

To clarify the potential suppressive function of the CD8+ T cells, we aimed at developing allergen-specific T cell lines. With that approach, we were expecting to develop both allergen-specific Th2 and CD8+ cell lines, studying the suppressive potential through a co-culture system. This still remains an ongoing project, as until today, we were unable to obtain a cell line with the allergen extracts we have used (Appendix).

T cells in peripheral blood may not be a mirror image of their counterparts at the target organs, unless migration from the target-organs back to peripheral blood exists. It is

possible that once at the target-organ, CD8+ T cells do not return to the peripheral blood, and allergen-induced specific features cannot be "seen" there.
In fact, some researchers believe that even in non-atopic asthmatic patients a IgE-mediated response develops only at the target-organ, as both phenotypes (allergic and non-allergic) cannot be distinguished in terms of clinical and pathophysiological features [125, 194].
Thus, a regulatory potential of CD8+ T cells in allergy can only be denied after carefully studying organ-based CD8+ T cells. So far, suppressor functions of CD8+ T cells in humans have been found in disease setting encompassing massive antigen stimulation, such is the case of transplanted patients [195].

We, therefore, went on to study CD8+ T cells at the target-organ, verifying not only their presence, but also the activation-status in asthmatic patients. CD8+ T cells from the lung could have been obtained from bronchoalveolar lavage fluid, but we chose induced sputum instead, as this is a far safer method and also non-invasive. Sputum cells are derived mainly from the central, more proximal airways; while cells from BAL and bronchial washing (BW) sample more peripheral airways and the alveolar compartment. Nevertheless, cellular sputum composition correlates well with that found both in BAL and BW.

In our sputum study, (Paper IV), we show that CD8+ T cells are activated, which may suggest that they are participants in the inflammation process in the target organ. As inflammation is related to disease severity, we further evaluated activation markers in allergic asthmatic patients with different degrees of disease severity. We were unable to demonstrate a relationship between activation of T cell subsets and severity of asthma or asthma control.
The main problem in the study design was the ethical impossibility to withdraw inhaled corticosteroids from more severe patients. Ideally, all study volunteers should be without corticosteroid medication for at least two weeks, controlled only by inhaled β-agonists.
As corticosteroids influence the expression of activation markers, they could mask expected differences [108, 183]. Hence, it would be important to repeat the study without interfering medications in a closely monitored setting.
This same problem also influenced our evaluation on control. A thorough analysis should be made by evaluating control and specific subpopulations and activation markers in a population of asthmatic patients with the same disease severity. Furthermore, if corticosteroid withdrawal is impossible, patients should at least also be under similar

dosages of corticosteroids, apart from being paired regarding age, gender and disease severity. We performed such an analysis in a small sample of intermittent asthmatic patients that were only on beta-blockers. However, no significant differences were found, either in terms of subpopulations, or in terms of activation markers.

We have studied CD8+ T cells only in asthmatic patients, since we could not obtain any suitable sputum sample from a wide sample of normal controls. Therefore, we cannot exclude the possibility that CD8+ T cells may also be activated in the sputum of healthy controls, although it is unlikely, as suggested by Ortega et. al, who assessed the presence of activated bronchial lymphocytes in induced sputum from asthmatic patients and healthy controls [180]. However, Leckie *et al.* failed to demonstrate a significant difference between the relative expression of CD69 in CD8+ T cells from the induced sputum of mild asthmatic patients and healthy controls [174].

A thorough study of several other phenotypic markers and a functional study were impossible to perform, as CD8+ T cells existed in a reduced percentage in the induced sputum samples. The percentage of CD8+ T cells in the peripheral blood is approximately 30% of all T cells in the peripheral blood, decreases in induced sputum and the CD4/CD8 ratio increases even more in asthmatic patients, as the disease is mostly Th2 mediated.

In summary, in this thesis, we have clarified the demographic characteristics of the asthmatic and rhinitis patients of the Allergy Clinic of the Cova da Beira Hospital. Furthermore, we have characterised T cells, especially the subgroup of CD8+ T cells, both phenotypically and functionally in allergic patients. Finally, we have attempted to devise novel strategies to isolate peripheral blood T cells and to derive allergen-induced T cell lines. Regarding our hypotheses, firstly, freshly isolated peripheral blood CD8+ T cells were unable to inhibit proliferation and cytokine synthesis of CD4+ effector T cells induced by the exposure to *Der p* extract. Secondly, induced sputum CD8+ T cells from asthmatic patients were activated, expressing higher relative percentages of the activation markers CD25 and CD69 when compared to CD8+ T cells from the peripheral blood. Thirdly, although activated, CD8+ T cells activation in induced sputum from asthmatic patients did not correlate either with disease severity or with disease control.

5. Appendix

5.1. Isolation of Leucocytes from Depletion Filters

Introduction

Peripheral blood $CD8^+CD28^-$ T cells exist in a variable percentage (up to 40% of all CD8+ T cells). The great majority of studies performed to date used buffy-coats or freshly drawn peripheral blood as a source of lymphocytes. In order to obtain functional leucocytes for performing the cell culture studies and implement the new methods, we tried a method using leucocyte depletion filters. These blood product preparation units are normally employed by blood banks to prepare a leucocyte-poor blood product and are commonly discarded after use.

This procedure is the object of two United States Patents that we used as reference: Kuroda *et al.* United States Patent number 4416777 [196] and Rashidbaigi *et al.* United States Patent number 5989441 [197]. The first patent describes a multi-step process to facilitate removal of red blood cells from leucocytes using a variety of elution solutions: phosphate buffered saline (PBS) in combination with polyvinyl-pyrrolidone, sodium casein, polyvinyl-alcohol or gelatine [196]. The second patent involves isolating leucocytes from filters which have been used to purify red blood cells or platelets back-flushing the used filters with a haemolysis solution, such as cold ammonium chloride, and collecting the functional human leucocytes [197]. Using this method, the recovery from Leukotrap™ filters was between 6 and $8x10^8$ leucocytes per filter unit, i.e. a 30-40% recovery of total leucocytes. The viability was more than 95%. Leucocytes recovered by the claimed method were functional, i.e., the cells were capable of being activated by virus or mitogen to produce cytokines, such as interferon, or other immunomodulators. Both methods require processing the filters within 24 hours after blood collection; otherwise the viability of the leucocytes is greatly impaired.

Several authors published alterations to these patents in scientific journals [198]. Based upon all these references we delineated a method which was applied to several filters.

Materials and Methods

Filters were maintained at 4°C until used and processing was always within 24 hours after blood collection. Each filter was back-flushed eight times with red cell lysing solution (ammonium chloride 0.83%) using a 60ml syringe. The collected suspensions were divided into two fractions, the first fraction containing the first four and the second fraction the last four suspensions. In order to remove red blood cells, the eluted leucocyte preparations were further purified in a density gradient using Lymphoprep™ (Axis-Shield). The nebulous phase was collected, washed twice in fresh PBS and incubated overnight in Petri dishes in RPMI complete medium. The non-adherent fraction was collected, washed and counted in a haemocytometer by the Trypan blue exclusion method.

In order to evaluate purity (as well as size and granularity) obtained leucocytes were acquired in a FACSCalibur.

Results and Discussion

The process of isolating leucocytes from depletion filters was developed in order to isolate a great amount of leucocytes from a usually discarded material. Prior methods have been reported in which leucocytes which remain trapped behind in the filter units have been recovered. For example, one method involves back-flushing five times with 50 mL PBS at 4° C using a 60 mL syringe (R. Longley et al, J. Immuno. Methods, 121:33-38 (1989)). By applying our modified method, we expected to retrieve functional leucocytes free from erythrocytes and platelets from the recycled filters. However, our results were disappointing.

Obtained fractions were always contaminated with leucocytes other than lymphocytes and monocytes, even after separation over Lymphoprep™ (Figure 19) and overnight adherence to plastic (Figure20). Moreover, lymphocytes had different characteristics regarding forward and side scatter when compared to lymphocytes isolated from freshly drawn peripheral blood. Leucocytes obtained were very "sticky" to plastic and glass surfaces and between them. Besides all this we had low recoveries of leucocytes.

Although we used fluorochrome-conjugated anti-CD3, anti-CD4, and anti-CD8 monoclonal antibodies for confirmation purposes, we did not evaluate viability. This could have been done using a PI/Annexin V method for flow cytometry. As such, we can argue

that the leukocytes obtained may be necrotic or apoptotic cells. This might explain their different forward and side scatter characteristics.

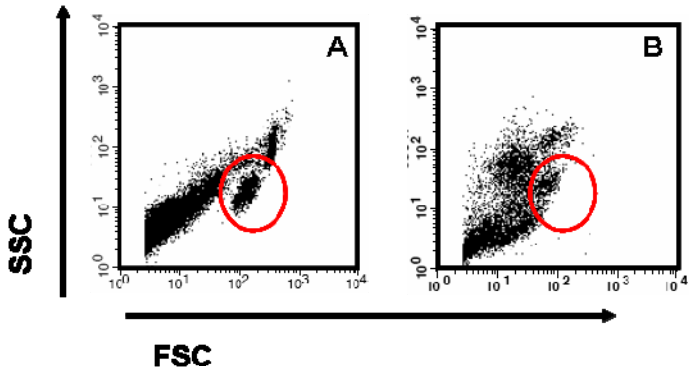

Figure 19 – Flow cytometry dot plots of filter mononuclear cells.
Obtained fractions were contaminated with granulocytes even after density gradient centrifugation and overnight adherence to plastic.
Panel A depicts a dot-plot from whole peripheral blood; Panel B depicts the first fraction of the recovered leucocytes at the end of the procedure.

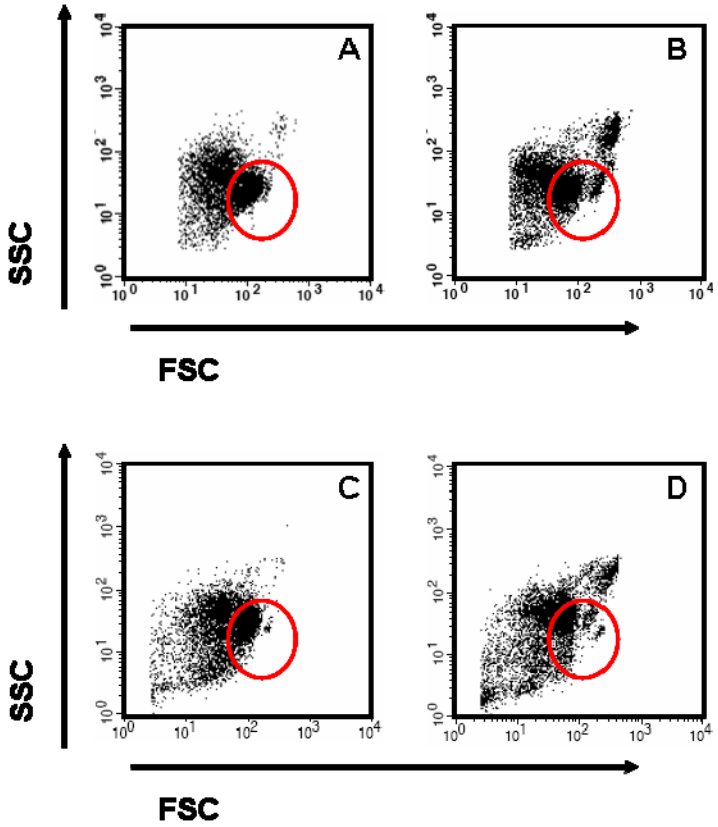

Figure 20 – Overnight adherence to plastic increases purity of leucocyte depletion filters' fractions.
After density gradient centrifugation, cells were allowed to adhere to a Petri plastic dish overnight. The non-adherent fraction was collected and after one washing step cells were passed through a flow cytometer. Panels A and B depict the first and second fractions respectively after density gradient centrifugation; panels C and D depict the same fractions after overnight adherence to plastic.

After careful analysis we abandoned the process in favour of freshly drawn peripheral venous blood. The latter circumvents our problem of scarce and contaminated fractions of mononuclear cells, since it allows simpler, quicker and purer isolations. Furthermore, it only implies a small discomfort to the volunteers. The drawback was that we needed more volunteers to establish/implement the methods instead of using an available source of leucocytes.

Notes:

Meyer *et al.* published an alternate method for leucocytes elution in the *Journal of Immunological Methods* in late 2005 [199]. The method was published when we were already using freshly drawn peripheral blood and we did not try it. The main difference is the use of a supplemented filter elution medium (PBS without Mg2+ or Ca2+ containing 5mM of EDTA-Na2 and 2.5% sucrose) leading to higher cell yields. However, the total leucocyte recovery with this method is low compared to whole blood, and also lower than in standard buffy coats. Relative recoveries for lymphocytes and monocytes are higher than for total leucocytes, which makes the method interesting as yet another source of mononuclear cells.

The method led to a European Patent (EP1484390) "Methods of preparing peripheral stem cells from leucocyte reduction filters" application. The last event published (06/02/2008) was that the application deemed to be withdrawn because the reply to the examination report was not received in time.

5.2. Generation of Allergen-specific T cell lines

Introduction

Allergic diseases are associated with an influx of inflammatory cells such as eosinophils and T cells into target-organs. Allergen-specific T cells seem to orchestrate the whole allergic process through the production of various mediators (such as IL-4, IL-5, IL-9 and IL-13), as well as cell-to-cell contact. The relative importance of the various subpopulations of T cells has not been fully addressed in allergic disease.

Allergen-specific T cells exist in very small numbers in the peripheral blood and allergen-specific proliferative responses are usually weak, making it hard to manipulate the specific response and measure the actual regulatory or suppressor functions. As we have previously shown, CD8+ T cells are able to proliferate, albeit weakly, in response to *Der p* extract.

In order to better clarify the role of the CD4+ and CD8+ T cell populations in allergic atopic diseases, it is important to develop allergen-specific T cell lines that will allow the study of the finer functional details of these cells. Our aim was to generate allergen-specific human T cell lines, specific for the major aeroallergens in the Cova da Beira region, namely Graminea pollens.

Materials and Methods

Subjects

Atopy was assessed by positive skin prick tests and specific IgE levels to common aeroallergens. Volunteers who received immunotherapy or were on systemic medication were excluded. Pregnant or breastfeeding women and all volunteers with disease affecting the immune system were also excluded. Informed written consent was obtained from all the volunteers. The project was approved by the Ethics Committee of the Cova da Beira Hospital.

Allergen Extracts

Leti provided us with the following *Phleum pratense* extracts:

L extract	**LP extract**	**B extract**
S-319/L	65-06/LP	S-319/L
16/09/2002	01/06/2006	16/09/2002
Cad. Sep 2007	Cad. 31/05/2011	Cad. Sep 2007
130,8 mg	137,7 mg	130.8 mg
S319/02 4834/16		P 185.4 HEP/mg

Peripheral blood was collected from 8 adult atopic volunteers all sensitised to *Phleum pratense*.

Density gradient isolated PBMC were cultured at a concentration of $1x10^6$ cells/ml in triplicates in RMPI 1640 complete medium [RPMI 1640, 2mM L-glutamine, 1% antibiotic/antimicotic (Sigma-Aldrich, St Louis, MO, USA), and 5% foetal calf serum (Biochrom, Berlin, Germany)] in 96-well flatt-bottomed plates (Nunc, Roskilde, Denmark) with or without several different concentrations of L or LP extract (3, 10, 30, 50, 100, and 300μg/ml of extract) for 4, 5, 6, or 7 days. For positive control, cells were incubated with 5μg PHA (Sigma-Aldrich) in the same conditions as for the extracts. Fourteen hours before the end of the culture [^{3}H]-thymidine was added to the wells (1μCi/well). [^{3}H]-thymidine (Amersham Biosciences, Uppsala, Sweden) incorporation was determined by scintillation counts in a TopCount (PerkinElmer, MA, USA) after harvest and results were expressed as counts per minute (cpm). Mean cpm of the triplicate cultures and standard error of the mean were calculated.

Results

After performing the cell cultures with the PBMC obtained from the atopic volunteers sensitised to *Phleum pratense* cells were harvested and proliferation was determined by [^{3}H]-thymidine incorporation. We did not observe proliferation for the cultures stimulated with any of the *Phleum* extracts for the days and concentrations tested (Figure 21), although

the positive culture control originated significantly high proliferation. The B extract seems to be toxic to the PBMC as no proliferation was observed.

Other allergen extracts were also provided to us with similar results.

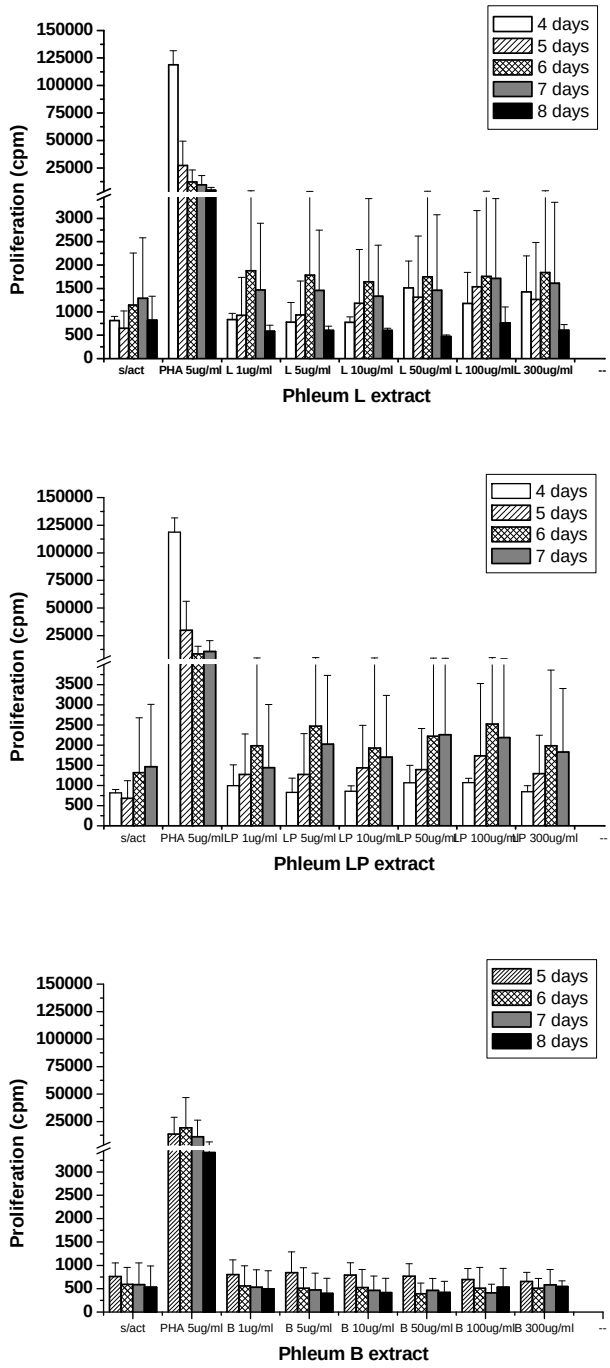

Figure 21 – Proliferation of PBMC to *Phleum pratense* extracts.
Isolated PBMC from 6 atopic volunteers were incubated for 4, 5, 6, 7, or 8 days in 96-well flat-bottomed plates with and without different concentrations of Phleum pratense L, LP, and B extracts. Phytohemaglutinin was used as a positive control (5µg/ml). Tritiated thymidine (1µCi/well) was added 14 hours prior to the end of the culture. Cells were harvested onto fibre filters, and incorporated thymidine was determined by scintillation counting. Results show thymidine incorporation (cpm, mean ± standard deviation).

Discussion and Conclusions

With this study we intended to use the allergen-specific T cell lines for allergen-induced proliferation and suppression studies. We also aimed at evaluating the suppressor properties of CD8+ T cells on allergen-induced cytokine synthesis.

Allergen-specific T cell lines established from allergic patients provide the opportunity to investigate the function and characteristics of T cells responding to allergens [200], [201] and determine the influence of substances which may modulate T cell functions [202].

Developing allergen-specific T cell lines is a very complicated process, since the frequency of allergen-specific T cells in PBMC tends to be low. Thus, allergen responses are usually quite weak and the concentration of allergen required to get proliferation is often high, especially when we compare it with other antigens. Bearing this in mind we carefully selected the volunteers and the allergens, including only dialysed allergens.

One limiting factor could have been the use of fetal calf serum instead of human AB serum, as it was referred in previous published studies, since it can lead to diminished proliferations.

All volunteers under study were highly sensitive to *Phleum pratense*, as assessed by skin prick test and serum-specific IgE. However, the extracts under test did not induce proliferation on PBMC isolated from the peripheral blood of those patients. All the volunteers tested had high total IgE (above 400 UI/ml), were at least class 3 for specific IgE (>3.5 KU_A/l) to *P. pratense*, and had a clinical history compatible with *P. pratense* related allergic disease.

One must bear in mind that, as in skin prick tests, the presence or absence of specific IgE in the serum is not directly associated with the presence of allergen-induced symptoms, and, in fact, many symptom-free subjects have detectable allergen-specific IgE levels in the serum. Furthermore, in symptomatic subjects the titre of allergen-specific IgE in the serum is usually unrelated to the severity of symptoms, as severity depends not only on IgE antibodies, but also on the releasability of mediators, on the response of the target organ to mediators and on non-specific hypersensitivity [188].

It could be expected that this lack of correlation might also exist between specific IgE serum levels and allergen-specific T cell proliferation *in vitro*. It may also be that a good regulatory response exists and depleting the PBMC of regulatory/suppressor T cells may give a better chance on obtaining the lines.

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