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**Fine characterization of immunological mechanisms
mediated by the major allergens of *Parietaria judaica* and
hypoallergenic hybrid, rPjEDcys.**

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Index

Abstract	1
Introduction	2
1. Immune system	2
1.1. Innate immunity	3
1.2. Adaptive immunity	5
1.2.1. B Lymphocytes (B cells)	6
1.2.2. T Lymphocytes (T cells)	6
1.2.2.1. Cytotoxic T Lymphocytes (CTLs)	7
1.2.2.2. T helper cells (Th cells)	7
1.2.2.3. T regulatory cells (Tregs)	10
2. Allergy	15
2.1. Cytokines and allergy	18
2.2. Natural Killer (NK) cells and their role in allergy	19
2.3. T regulatory cells and their role in allergy	20
3. Allergen-Specific ImmunoTherapy (SIT)	21
3.1. Immuno deviation during SIT	25
4. <i>Parietaria judaica</i> (Pj)	27
4.1 <i>Parietaria judaica</i> allergens	27
Objective	30
Material and Methods	31
1. Production of recombinant proteins	31
1.1. Affinity chromatography	31
1.2. Dialysis	31
1.3. Second affinity chromatography	32
1.4. Gel filtration chromatography	32
1.5. Detoxi-Gel Endotoxin Removing Gel	32
1.6. Protein quantification assay	33
1.7. Limulus Amebocyte Lysate test (LAL test)	33
2 Patient selection	34
3. Human Peripheral Blood Mononuclear Cells (PBMC) isolation	34
4. Flow cytometry	35
5. 5(6)-Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA-SE) staining	36
6. Cytokine Secretion Assay: IL-5, IL-13, IFN- γ and IL-10	36
7. Immunophenotyping of CD4 ⁺ CD25 ⁺ cells	38
8. Immunophenotyping of CD4 ⁺ CD25 ⁺ CD127 ⁺ T regulatory cells	38
9. Immunophenotyping of CD4 ⁺ CD25 ⁺ GARP ⁺ and CD4 ⁺ CD25 ⁺ LAP ⁺ T regulatory cells	38
10. Multiparametric analysis of CD4 ⁺ CD25 ⁺ GARP ⁺ LAP ⁺ T regulatory cells	39
11. Immunophenotyping of CD4 ⁺ CD39 ⁺ GARP ⁺ and CD4 ⁺ CD39 ⁺ LAP ⁺ T regulatory cells	39
12. Immunophenotyping of CD4 ⁺ CD25 ⁺ PDI ⁺ cells	39
13. CD4 ⁺ CD25 ⁺ CD127 ⁺ Tregs isolation from human PBMC	39
14. TRIzol RNA extraction	41
15. cDNA synthesis	42
16. Real Time-PCR	43
17. Statistical analysis	44

Results	45
1. Production of recombinant proteins: rPar j1, rPar j 2 and rPjEDcys	45
2. Human PBMC proliferation assay: innate and adaptive immune response to Parietaria major allergens and the hypoallergenic derivate.....	47
2.1 Cytofluorimetric analysis on CD4 ⁺ CFSE ⁺ cells.....	48
2.2 Cytofluorimetric analysis on CD8 ⁺ CFSE ⁺ cells.....	49
2.3 Cytofluorimetric analysis on CD16 ⁺ CFSE ⁺ cells.....	51
2.4 Cytofluorimetric analysis on CD56 ⁺ CFSE ⁺ cells.....	52
3. IL-5, IL-13, IL-10 and IFN- γ cytokine secretion assay after PBMC stimulation with wild type allergens and rPjEDcys	54
4. Modulation of CD25 expression on CD4 ⁺ cells in response to Parietaria major allergens and rPjEDcys..	62
5. Study of allergen-specific CD4 ⁺ CD25 ⁺⁺ CD127 ⁻ Tregs after PBMC stimulation with Parietaria major allergens and hypoallergenic hybrid.....	65
6. Magnetic separation of CD4 ⁺ CD25 ⁺⁺ CD127 ⁻ Tregs after stimulation with Parietaria major allergens and hypoallergenic hybrid	69
7. Molecular characterization of allergen-specific CD4 ⁺ CD25 ⁺⁺ CD127 ⁻ T Regulatory Cell	76
8. Analysis of GARP and LAP expression on CD4 ⁺ CD25 ⁺⁺ cells in response to Parietaria major allergens and rPjEDcys	79
9. Study of GARP and LAP co-expression on CD4 ⁺ CD25 ⁺⁺ cells after PBMC stimulation with the mixture of wild type allergens and the hypoallergenic derivate	87
10. Analysis of CD39 expression on CD4 ⁺ cells in response to Parietaria major allergens and rPjEDcys.....	89
11. Evaluation of putative cross-talk between two suppressive pathways of human Treg cells	93
12. Analysis of PD1 expression on CD4 ⁺ CD25 ⁺⁺ cells in response to Parietaria major allergens and rPjEDcys	103
Discussion	107
Conclusion	113
References	113
ACKNOWLEDGEMENTS	123

Abstract

Allergy is a hypersensitivity disease IgE-mediated, affecting more than 30% of the population living in the industrialized countries. The symptoms of allergic reactions can be transiently ameliorated pharmacologically, but the only curative treatment of allergies is Allergen-Specific Immunotherapy (SIT). Recombinant hypoallergenic derivatives with reduced allergenic activity have been engineered to reduce side effects during SIT. *Parietaria judaica* (Pj) pollen contains two major allergens belonging to the family of non specific-Lipid Transfer Proteins (ns-LTP): Par j 1 and Par j 2. The allergens Par j 1 and Par j 2 are recognized in approximately 95% of allergic patients. By means of DNA recombinant technology, a hybrid hypoallergenic (PjEDcys), expressing disulphide bond variants of Par j 1 and Par j 2, has been generated. The aim of this research project is to study the immunological mechanisms activated by the major allergens of *Parietaria judaica*, rPar j 1 and rPar j 2, and the hypoallergenic hybrid rPjEDcys. Moreover, the project I am involved in is trying to address the question whether this engineered hypoallergenic derivative can be a potential product for safer Allergen Specific Immunotherapy. *In vitro* analysis suggested that rPjEDcys has a reduced allergenicity and maintains T cells reactivity. In particular we showed that PBMC of Pj allergic patients stimulated *in vitro* with the hybrid and the *wild-type* recombinant allergens scored a percentage of proliferating CD4⁺ cells higher than unstimulated samples. We also demonstrate that components of innate immune system (CD56⁺ cells) proliferate in response to *wild-type* allergens and rPjEDcys. Furthermore, cytokine secretion assays on CD4⁺ cells demonstrated that rPjEDcys induces a lower the secretion of two Th2 cytokines that are critical in the development of allergy such as IL-5 and IL-13 compared to *wild-type* allergens. Furthermore we observed the induction of a Treg cell subset (defined as CD4⁺ CD25⁺⁺ CD127⁻) in response to rPjEDcys and the *wild-type* allergens. However, the number of these Treg cells and the intensity of CD25 expression is higher in response to hypoallergen hybrid than *Parietaria* major allergens. We also characterized these cells at molecular level by REAL-TIME PCR. Moreover, we addressed the kinetic of functional surface marker expression, such as GARP (Glycoprotein A Repeats Predominant), LAP (Latency-Associated Peptide) CD39 and PD1 on CD4⁺ cells. Our analyses demonstrated that rPjEDcys induces a number of GARP-LAP-CD39 co-expressing cells and CD4⁺CD25⁺⁺PD1⁺ higher than *wild-type* recombinant allergens. These results suggest that rPjEDcys represents a useful approach for immunotherapy of allergic disease.

Introduction

1. Immune system

The immune system is a complex interactive network of cells, tissues and organs that work together to defend the body against attacks by “foreign” insults while keeping a state of tolerance to self and innocuous non-self antigens. Structurally, the immune system is a collection of cells, molecules, tissue, organs and circulatory systems. Immune system cells are produced and mature in specialised areas of the body called primary lymphoid organs such as the thymus or bone marrow. They are transported via the cardiovascular and lymphatic circulatory systems to peripheral tissues or specialised secondary lymphoid organs such as the lymph nodes or spleen.

Although scientists have learned much about the immune system, it is highly complex and continuous studies have to be performed to understand how the human immune system targets invading microbes, infected cells and tumors while ignoring healthy tissues. Our environment contains a large amount of organisms and substances that damage the host through a very broad selection of pathogenic mechanisms. Therefore human immune system uses a complex array of protective mechanisms to locate and eliminate these organisms and environment substances from the body and maintain the body in a healthy state for many years.

A general feature of the human immune system is the ability to detect structural features of the pathogen, toxin, tumor cells or allergenic structure (non self) that mark it as distinct from body’s own cells (self). The body’s immune defenses normally coexist peacefully with cells that carry distinctive “self” marker molecules. However, when host immune cells encounter cells or organisms carrying foreign markers they attack [1-2].

Once immune cells recognize the enemies, they become activated and begin to produce and secrete cytokines, molecules that mediate and regulate their own growth and cell behaviour, and chemokines, molecules that stimulate cell movement and recruit other immune cells into the infection site. These cells can produce and release a large amount of chemical mediators and activate other cells involved in immune response in order to attack organisms and substances that invade body systems and cause disease [1]. These events allow us to maintain the homeostasis of the body.

This view of the immune system describe it as a multilevel dynamic system of cells, molecules, tissue, organs and circulatory systems that protects organisms from infection with a different

defense stage of increasing specificity (Fig. 1) [1-2].

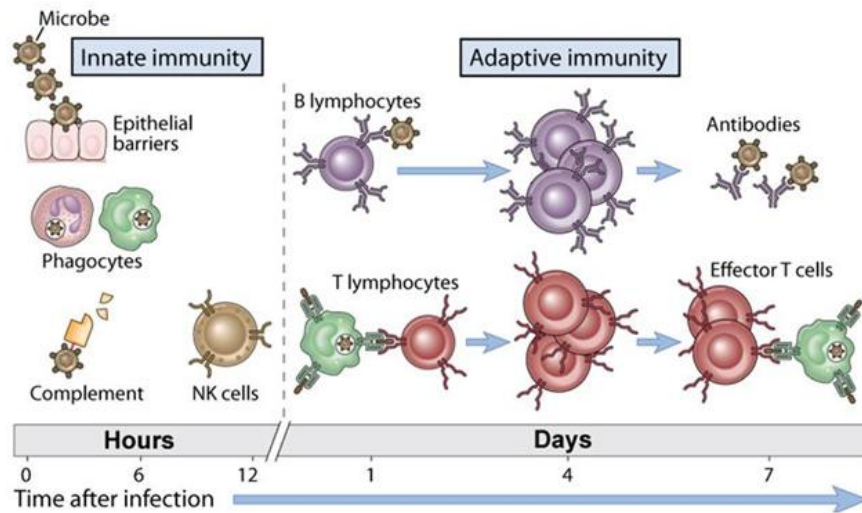


Fig 1. Innate and Adaptive Immunity: the mechanisms of innate immunity provide the initial defense against infections. Adaptive immune responses develop later and consist of activation of lymphocytes. The kinetics of the innate and adaptive immune responses are approximations and may vary in different infections [1].

1.1. Innate immunity

Innate immunity is an evolutionarily ancient part of the host defense mechanisms and provides the first line of defense against invading bacteria [1].

The innate immune system includes physical, chemical and cellular barriers. The main physical barriers are skin and mucous membranes. Chemical barriers include specialized soluble molecules that have antimicrobial activity, these represent one of the most ancient forms of defense against infection; the most noteworthy antibacterial substance is the enzyme lysozyme. Innate responses frequently involve complement, acute-phase proteins, and cytokines. The molecules collectively referred to as acute-phase proteins enhance resistance to infection and promote the repair of damaged tissue. Cytokines act as messengers both within the immune system and between the immune system and other systems of the body, generating an integrated network that is involved in the regulation of immune responses.

Innate immune cells include populations of white blood cells such as circulating Dendritic Cells (DCs), neutrophils, Natural Killer (NK) cells, Natural Killer T (NKT) cells, monocytes, eosinophils and basophils, along with tissue-resident mast cells and macrophages [1-3].

The cellular response to a microbial agent of infection that overcomes the initial barriers of skin

and mucous membranes is rapid, typically initiating within minutes of invasion. Innate immune cells accomplish general pattern recognition by using a variety of Pattern Recognition Receptors (PRRs) that can be expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids and recognized microb-specific molecule called Pathogen-Associated Molecular Patterns (PAMPs) [4-6]. The innate immune system consists of all the immune defenses that lack immunologic memory, their response does not change, regardless of the type of pathogen involved. Thus, a characteristic of innate responses is that they remain unchanged however often the antigen is encountered.

NK cells are a type of cytotoxic lymphocyte critical to the innate immune system. NK cells are known to differentiate from the common lymphoid progenitor-generating B and T lymphocytes and mature in the bone marrow, lymph nodes, spleen, tonsils, and thymus, where they then enter into the circulation [1-2]. These cells can be distinguished from other lymphocytes with the lack of the T cell receptor and surface immunoglobulin, but these cells express many NK cell-specific surface molecules. Human NK cells express structurally and functionally two distinct families of Major Histocompatibility Complex (MHC) class I receptors: Killer cell Immunoglobulin (Ig)-like Receptors (KIR) and lectin-like receptors. These cells have the ability to both lyse target cells and also provide an early source of immunoregulatory cytokine [7-8]. Human NK cells comprise 15% of all lymphocytes and are defined phenotypically by their expression of CD56, an isoform of the human neural-cell adhesion molecule with unknown function on human NK cells and lack of expression of CD3, T cell co-receptor and CD16, the low-affinity Fc γ receptor III (Fc γ RIII). However, two distinct populations of human NK cells could be identified, based on their cell-surface density of CD56 and CD16. The majority (90%) of human NK cells have low-density expression of CD56 and express high levels of CD16 (NK cells CD56^{dim}CD16^{bright}); whereas 10% of NK cells are CD56^{bright}CD16^{dim/-}. Functional studies of these subsets by Lanier and colleagues revealed that resting CD56^{dim}CD16^{bright} cells are naturally more cytotoxic than CD56^{bright} CD16^{dim/-} NK cells. Consistent with these cytotoxic differences, CD56^{dim} CD16^{bright} NK cells have a major number of cytoplasmic granules containing proteins such as perforin and proteases (known as granzymes) and higher level expression of KIRs than CD56^{bright} CD16^{dim/-} cells.

By contrast CD56^{bright}CD16^{dim/-} NK cells have low level expression of KIRs but have the capacity to produce higher levels of immunoregulatory cytokines, such as interferon (IFN)- γ , Tumour Necrosis Factor (TNF)- α , Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and IL-5 than CD56^{dim} CD16^{bright} NK cells (Fig.2) [7-8].

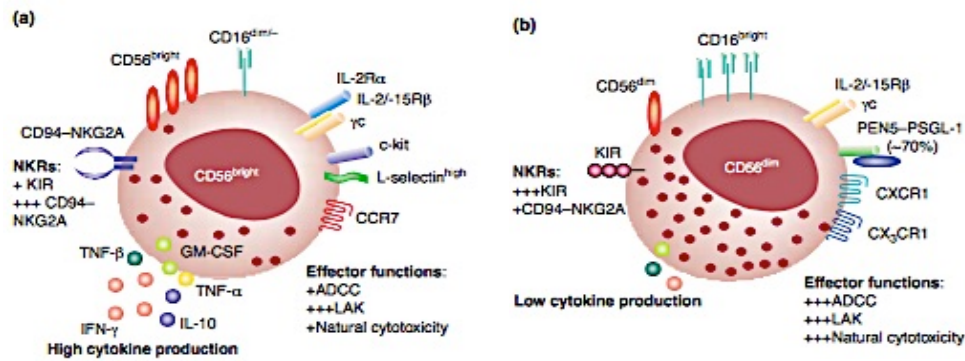


Fig. 2. Schema of human natural killer (NK)-cell subsets: (a) CD56^{bright} CD16^{dim/-} NK cells produce high levels of cytokines following stimulation. This subset has low-density expression of CD16 and low natural cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC). Therefore, this NK-cell subset expresses a number of cytokine and chemokine receptors constitutively. (b) CD56^{dim} NK cells produce low levels of NK-derived cytokines but are potent mediators of ADCC, LAK activity and natural cytotoxicity, and have a more granular morphology than CD56^{bright} CD16^{dim/-} NK cells. The CD56^{dim} CD16^{bright} NK-cell subset has high-level expression of KIRs [7].

1.2. Adaptive immunity

Innate immunity, alone, may not be sufficient to protect a host against an invading pathogen or to prevent disease from occurring. However, if innate immunity fails, the pathogen may yet be detected and attacked by the mechanisms of adaptive immunity. The innate and adaptive immune responses synergistically act as protection against invading organisms, but they differ in a number of ways. The adaptive immune system shows delayed responses to pathogens.

The two main cell populations of the adaptive immune system are T and B lymphocytes (T and B cells), which recognize a high diversity of antigens. The adaptive immune is also called specific immune response and consists of antibody responses and cell-mediated responses, which are carried out by B and T cells, respectively. The B and T cell receptors are random products of somatic gene rearrangement during maturation. The genes encoding these receptors are assembled in random product by the activity of enzymes called RAG-1 and RAG-2 (Recombination Activation Gene). Defence against extracellular microbes and microbial toxin is mediated by B cells, instead intracellular microorganisms are detected by T lymphocytes via cell-mediated immune responses [1-2].

1.2.1. B Lymphocytes (B cells)

B cells are a subset of lymphocytes involved in the production of antibodies that are released in blood plasma and lymph, where they bind specifically to foreign antigens.

B cells arise from hemopoietic stem cells in the bone marrow, where B cells pass through several distinctive developmental stages, during which they acquire their antigen specificity. Upon the first exposure to a microbe or an antigen and the recognition of foreign antigens through the B Cell Receptor (BCR), the *naïve* B-lymphocytes are activated and differentiated into antibody-producing plasma cells and memory cells. When the same antigen reenters the body, the circulating antibodies provide immediate protection against infection.

The antibodies secreted by plasma cells can be grouped into different antibody classes or isotypes. There are five different antibody isotypes known as IgA, IgD, IgE, IgG and IgM that perform different roles. [1-2]

1.2.2. T Lymphocytes (T cells)

T cells, or T lymphocytes, are a subset of lymphocytes defined by their development in the thymus, where they develop to recognize cell surface molecules presenting foreign antigens (antigen-MHC). MHC molecules are encoded by the Major Histocompatibility Complex (MHC) genes and determine histocompatibility. All T cells express CD3 antigen and a unique T Cell Receptor (TCR), but they differ for CD4 and CD8 expression, which act as coreceptor. The initial step of differentiation of the *naïve* cells is the antigenic stimulation as a result of interaction of TCR and CD4⁺ or CD8⁺ with antigen-MHC class II or MHC class I complex respectively, presented on professional antigen presenting cells (APCs).

Naïve T cells express CD28, the receptor for the peripheral membrane proteins (type B7) found on APCs that provide co-stimulatory signals (in addition to the TCR) required for T cell activation and survival. Blockade of CD28 is effective in stopping T cell activation, a mechanism that the immune system uses to down-regulate T cell activation. Association of the TCR of a *naïve* T cell with MHC:antigen complex without CD28:B7 interaction results in a T cell that is anergic.

T cells contribute to immune defenses in two major ways. Some T cells (CD4⁺ T cells) direct and regulate immune responses, whereas others T (CD8⁺ T cells) cells attack directly infected or cancer cells [1-2].

1.2.2.1. Cytotoxic T Lymphocytes (CTLs)

The CD8⁺ T cells are Cytotoxic T Lymphocytes (CTLs) which attack directly or kill infected cells expressing foreign antigen fragments in the context of their class I molecules. Moreover, CTLs are useful for attacking tumor cells. CD8⁺ T cells use multiple mechanisms to kill tumor or infected cells; they release two preformed cytotoxic proteins, granzymes and perforin, and secrete cytokines such as interferon- γ (IFN- γ) and tumor necrosis factor (TNF). Both perforin and granzymes are required for effective cell killing. The granzymes are cellular proteases, they are formed in the CD8⁺ T cell only after antigen-specific activation of the cell and trigger apoptosis in the target cell, activating an enzyme cascade that induces the DNA degradation. Instead, the perforins make pores on cell surface through which the granzymes can move into the target cell. The cytokines expressed by CD8⁺ T cells (IFN- γ and TNF- α) are also important because they mediate many antitumor effects. IFN- γ is well known for its antiangiogenic properties. Experimental evidences suggest that IFN- γ and TNF- α released from CTL are key effector molecules for the eradication of tumor by the destruction of tumor stroma [9].

1.2.2.2. T helper cells (Th cells)

The CD4⁺ T cells are called T helper cells; these play an important role in the regulation of the immune system, coordinating the activity of the immune response. As suggested by their name, Th cells modulate the function of B cells by inducing antibody secretion and isotype switching. Th cells are not able to directly kill infected cells, but they play an essential role also in the regulation of CD8⁺ cytotoxic T cells.

The helper T cells recognize foreign antigen in the context of their MHC class II molecules. When an antigen-presenting cell (APC) activates a naïve helper T cell in a peripheral lymphoid tissue, this T cell can differentiate into effector T cell subset. Mosmann and Coffman originally classified CD4⁺ T lymphocytes into Th1 and Th2 subsets [9-10].

They showed that T *naïve* cells (T helper type 0, Th0) could differentiate into Th1 or Th2 effector cells; many factors could influence Th1/Th2 differentiation. Among these ones there are the dose and the nature of antigens and the interaction with different APCs but the polarization process is driven mainly by cytokines produced by APCs. IL-12 and IL-4 are defined as the critical cytokines initiating the downstream signaling cascade to induce *naïve* T CD4⁺ cells to differentiate into Th1 or Th2 cells [11-12]. IL-12 appears to induce IFN- γ , a key factor in Th1 polarization from Th0.

IFN- γ acts to up-regulate the master regulator for Th1 differentiation, T-box transcription factor (T-bet) showing a positive feedback loop for Th1 differentiation. On the contrary, IL-4 and IL-2 are critical for Th2 differentiation and up-regulate the expression of the master regulator GATA-binding protein (GATA3). GATA3 is indispensable for Th2 differentiation and Th2 cytokine production. It is also essential for inhibition of Th1 differentiation and IFN- γ production [13-14].

These two types of effector helper T cells (Th1 and Th2) are functionally distinct, can be characterized by the cytokines they secrete and play different immune responses.

If Th0 cells differentiate into a Th1 cells, they will secrete IFN- γ and TNF- α . Under physiological conditions, Th1 pathway primarily acts in the defense against intracellular pathogens. IFN- γ is the most characteristic cytokine produced by activated Th1. It shows a multitude of functions; for example it promotes phagocytosis and elimination of intracellular pathogens by upregulation of the expression of high affinity IgG Fc region receptor (Fc γ RI). Activation of the Fc γ R via antigen containing immune complexes lead to the pathogen phagocytosis and can lead to the generation of reactive oxygen species which in turn actively participate in phagocytosis.

Th1 cells may also stimulate B cells to secrete specific subclasses of IgG antibodies that can coat extracellular microbes and activate the complement system. Th1 immune response is essential in the clearance of intracellular pathogens but if they are over activated under pathological conditions they could promote autoimmune diseases. In fact, exceeding Th1 responses have been found to be associated with autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis and type 1 diabetes.

Otherwise, Th2 cells mediate host defense against extracellular parasites. Moreover, Th2 cells are well known for their involvement in allergies, these cells are in fact the main causes of allergic inflammatory diseases and asthma [11-12, 15].

Th2 responses are associated with a high production of a large range of cytokines, including IL-4, IL-5, IL-9 and IL-13. Th2 cytokines exert their effects on many cell types in the organism. In fact, Th2 cells are able to stimulate and recruit specialized subsets of immune cells, such as eosinophiles, basophiles, neutrophiles and mast cells, towards the site of infection or in response to allergens. Th2 cytokines also induce Ig ϵ gene transcription in B cells, which results in subsequently class switching towards the IgE isotype, a critical mediator of allergy.

Although, initially, immunologists believed that there were fundamentally 2 types of CD4⁺ T cells, Th1 and Th2, the repertoire of effector CD4⁺ T cell subsets has recently expanded to include additional effector T cell subsets like Th17 and T regulatory cells (**Tregs**) (Fig. 3).

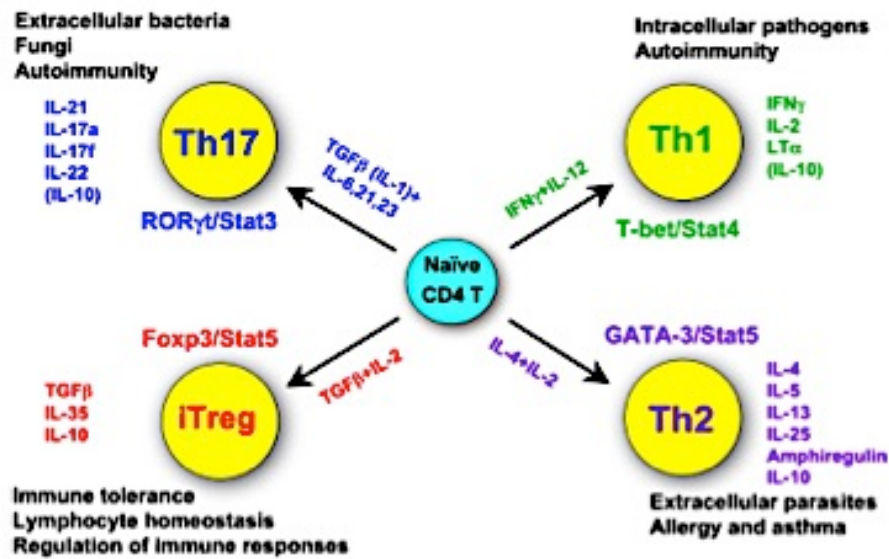


Fig. 3 The major subclasses of effector helper T cells: these cells are functionally distinct and can be distinguished by the cytokines they secrete [11].

The differentiation of the different lineages depends on the complex network of specific cytokine signaling and transcription factors followed by epigenetic modifications. Th17 cells secrete a characteristic profile of cytokines including IL-17A, IL-17F and IL-22. The natural role of Th17 cells in the immune system is to protect the organism from extracellular bacteria and fungal infections but an uncontrolled or inappropriate Th17 activation has been linked to several autoimmune pathologies such as arthritis, multiple sclerosis, psoriasis and lupus [16-18].

The second important effector T CD4⁺ cells named T regulatory cells have been identified in 1970 by Gershon and Kondo. These cells showed a distinct suppressive activity and for this reason they were initially named suppressive cells. Our immune system consists of a complex network of regulatory response that maintain the immune homeostasis. Tregs are the main cells involved in the regulation of both innate and adaptive immune response. This feature makes these cells important in maintaining peripheral immune tolerance.

Tregs are CD4⁺CD25⁺⁺ and have suppressive effects that are essential for maintaining self-tolerance and controlling pathological immune responses, in order to control autoantigen reactions such as those which cause autoimmunity diseases. The generation of Tregs constitutes an essential mechanism in the establishment and maintenance of peripheral tolerance [19-21].

1.2.2.3. T regulatory cells (Tregs)

As mentioned before, Tregs are a heterogeneous group of CD4⁺ T cells, therefore, during the Third International Conference on Regulatory T Cells and Th Subsets and Clinical Application in Human Diseases held in Shanghai in 2012, several recommendations were developed to simplify the nomenclature of Tregs.

Tregs can be divided based on their origin into: naturally occurring Treg cells, which develop in the thymus (**tTregs** or **nTregs**); and induced Treg cells that develop in the periphery (**pTregs** or **iTregs**) [22].

In addition to their origin, Tregs could be divided based on their surface markers and their mechanism of action.

Naturally occurring regulatory T cells are the best-characterized Tregs and constitute a distinct cell lineage in the periphery, being 5%–10% of the total peripheral CD4⁺ T cells. Although there is no cell surface marker that uniquely identifies tTregs, there are cell surface proteins that are preferentially expressed on tTregs. The cells constitutively express a high level of **CD25** [high affinity interleukin-2 receptor, alpha chain (IL-2R α)], however this receptor is not only expressed by Treg cells but by all activated T cells. In contrast, **CD127** [interleukin-7 receptor, alpha chain (IL-7R α)] is absent in Treg and although it is down regulated in activated effector T cells, this marker allows to distinguish Tregs from effector T cells. Besides the expression of CD25 and the absence of CD127, these cells constitutively express other several activation markers including the glucocorticoid-induced tumor-necrosis factor (TNF) receptor-related protein (GITR), L-selectin [CD62 ligand (CD62L)], cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152) and OX40 (CD134) [23-26].

Even so, it has been observed that none of these markers exclusively identifies Tregs since they can also be expressed in various degrees on activated effector T cells. tTregs are characterized by the expression of the transcription factor Fork head Box P3 (**FoxP3** or scurfin) is key transcription factor indispensable for Treg development in the thymus [26].

In mice, CD4⁺ Tregs are a homogenous population, in which all CD4⁺ and CD25⁺ cells are Tregs. In humans, the Tregs are a heterogeneous population, in which not all CD25⁺ cells are Tregs. Several studies have identified the transcription factor FoxP3 as the “master regulator” of tTreg, in which it is constitutively expressed at high levels, but now it is well evident that the tTreg activity is not only regulated by FoxP3 expression [26-27]. Previous studies have revealed the specific role of FoxP3 in the development and function of natural Tregs. First findings were obtained in a mice

model of FoxP3 deficient recombinant mice and the natural mutant *scurfy* mice, which showed the crucial role of FoxP3 in the development of tTreg. Scurfy mice have a frameshift mutation in the FoxP3 gene that results in a truncated protein lacking the fork head domain. FoxP3 is sufficient for the development and the function of Tregs in mice. In fact, the destruction or spontaneous mutation of FoxP3 gene causes the absence of Treg cells in mice and induce the lymphoproliferative autoimmune syndrome characterized by multiorgan lymphocytic infiltration and uncontrolled cytokine secretion [27-28].

The human FoxP3 gene maps on X chromosome and it has been observed that FoxP3 mutation leads to autoimmune disease with clinical effect similar to those observed in *scurfy* mice. This syndrome is known as immune dysfunction polyendocrinopathy enteropathy X-linked (IPEX) and is a rare recessive disorder showing an X-linked hereditary pattern: only males are affected, whereas the carrier mothers are healthy. Patients with IPEX have shown multiorgan lymphocytic infiltration and typically develop autoimmune diseases that are related to Tregs deficit [28]. Novel findings obtained in mice model revealed that FoxP3 is able to upregulate or down regulate more than 700 genes; FoxP3 directly regulates 10% of these [29].

Not all human Treg cells constitutively express FoxP3. The other type of regulatory T cells, named peripherally generated T regulatory (pTreg), can differentiate in the periphery from non-Tregs and could acquire FoxP3 expression in response to foreign antigens and show suppressive functions similar to tTregs. These cells can be generated under more various conditions, for example they can be generated in lamina propria of the intestine in response to microbiota and food allergens, in every chronically inflamed tissue or grafts. pTregs development always needs TCR stimulation and the cytokines TGF- β and IL-2 [30]. Surely, the existence of different subsets of Treg and lack of a define cell surface signature makes it difficult to understand the role of each regulatory cells in the regulation of our immune system.

Different Treg subsets have been identified based on the phenotypic expression of functional markers and their suppressive activity. Treg uses many mechanisms to limit the activation of other immune cells and ensure an immune tolerance. These include the contact-dependent and independent mechanisms that comprise the secretion of immunomodulatory cytokines, the metabolic disruption modulation of DCs, and the cytolysis of target cells [31-32].

The main mechanism by which Treg cells control immune responses is through the secretion of high levels of the immunosuppressive cytokines IL-10 and TGF- β . In vivo studies have shown that IL-10 and TGF- β might be required for Treg control of immune response in allergy.

IL-10, has potent immunomodulatory effect that suppresses effector T cell response in two different ways; directly by suppressing IL-2 and INF- γ secretion and T cell proliferation or indirectly by inhibiting inflammatory factors and activating tolerogenic pathways [26]. However, Treg cells are not the only T cell subset that secretes IL-10; other T cell subsets can express IL-10, depending on stimulation and environmental conditions.

Treg cells show a typical kinetics of IL-10 secretion, they produce high concentrations of IL-10 early after antigen specific stimulation while IL-10 production by other Th cell clones or peripheral blood cells occurs late after stimulation and it is less concentrated [33].

TGF- β is another important pleiotropic cytokine with immunoregulatory properties, which plays an essential role in the maintenance of immunological tolerance. This cytokine has multiple suppressive effects on T cells, B cells, macrophages and other immune cells [34].

Moreover, TGF- β could be found in the latent form associated with Latency-Associated Peptide (**LAP**) on Treg cell surface, but their suppressive mechanism is mediated by TGF- β secretion rather than through cell surface-bound TGF- β [35]. Activation of TGF- β is a highly regulated process at the post-translational level; TGF- β is synthesized in cells as a pro-TGF- β precursor. Following a homodimerization step, pro-TGF- β is cleaved by furin convertase, which forms a complex that contains mature TGF- β associated to LAP. The C-terminal homodimer corresponds to mature TGF- β , while the N-terminal homodimer is LAP peptide. TGF- β is active only after the dissociation from LAP. More recently, an orphan toll-like receptor called Glycoprotein A Repetitions Predominant (**GARP**, or LRRC32) has been identified as a novel marker for Tregs involved in regulatory function, in particular associated with TGF- β suppressive mechanism [35]. The GARP protein was identified as a cell surface protein of Treg cells. Mutational study by Unutmaz et al revealed that deletion of the cytoplasmic portion of GARP did not affect Treg phenotype or function; on the contrary the transmembrane region and the extracellular region were important in its function infact the depletion of either region abrogated Treg suppressor function. Flow cytometry analysis on the cell surface expression of GARP showed the absence of this protein on the cell surface of any resting Treg or T conventional cell [36]. Thanks to the discovery of GARP more light was given to TGF- β on Treg cell biology.

Some published data showed that GARP is selectively up-regulated on Tregs after TCR activation, this surface molecule has been described as unique activation marker of human Tregs [37].

Instead, FoxP3 and CD25 are constitutively expressed on Tregs and cannot be used to evaluate activation status [38].

GARP mRNA was found expressed at low basal level in other cell types, but GARP protein was not expressed, these observation highlight that there is a post-transcriptional regulation of GARP [35-36]. GARP expression is selectively restricted to the FoxP3⁺ cells and this has led to the hypothesis that GARP is an effector molecule regulated by FoxP3 expression. Silencing FoxP3 by shRNA expression in Tregs showed a reduction of GARP up-regulation. On the contrary reduced GARP expression with shRNA in Tregs induced a moderate reduction of suppressive activity but did not change FoxP3 expression. These findings highlighted that GARP could have a potential contribution into Treg-mediate suppression [37-39].

Tran et. al. showed that GARP function is to associate with latent transforming growth factor- β (proTGF- β) on the surface of FoxP3⁺ Tregs and regulate its activation [40] (Fig 4).

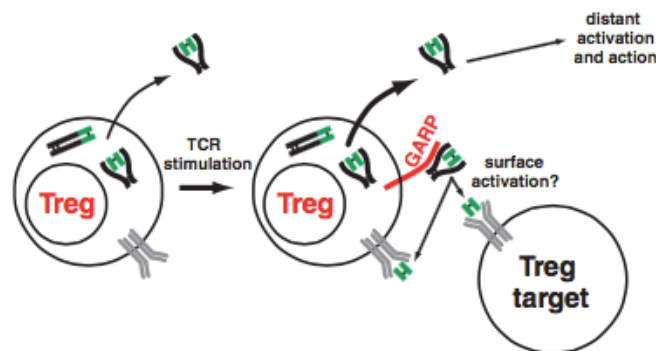


Fig 4. Model of TGF- β production by Human Treg cells. Functional association between GARP and LAP proteins [45].

Metabolic disruption is another strategy used by Tregs to suppress other cells. A primary mechanism is the consumption of local IL-2, which they need for survival and proliferation.

Recent studies on murine model indicated that ATP is implicated in the pathogenesis of allergy by triggering the migration of eosinophils and dendritic cells (DCs) and promoting the maturation and activation of DCs [41]. Some reports have shown that T regulatory cells express ectoenzyme **CD39** [Ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1)] and **CD73** [ecto-5'-nucleotidase] and use these enzyme to mediate immunosuppression through the production of adenosine. Human Treg cells have been reported to express CD39 on their surface to hydrolyze

extracellular adenosine triphosphate (ATP) and adenosine diphosphate (ADP) into adenosine monophosphate (AMP). Thereafter, AMP is processed into immunosuppressive adenosine by the CD73 ectoenzyme. This mechanism is important in the immune regulation, in fact the purinergic mediators, like ATP and adenosine, are released into the extracellular space and act as positive or negative signals modulating the immune responses. ATP induces pro-inflammatory responses, such as the release of inflammatory cytokines. On the contrary, adenosine mediates anti-inflammatory effects by inhibiting activated immune cells in a negative feedback loop.

The inhibitory effect of adenosine on T cells is mediated by the ability to block T-cell receptor signaling due to the binding of adenosine to A_{2A} receptors (the main adenosine receptor) and the accumulation of intracellular cAMP (Fig. 5) [42-45].

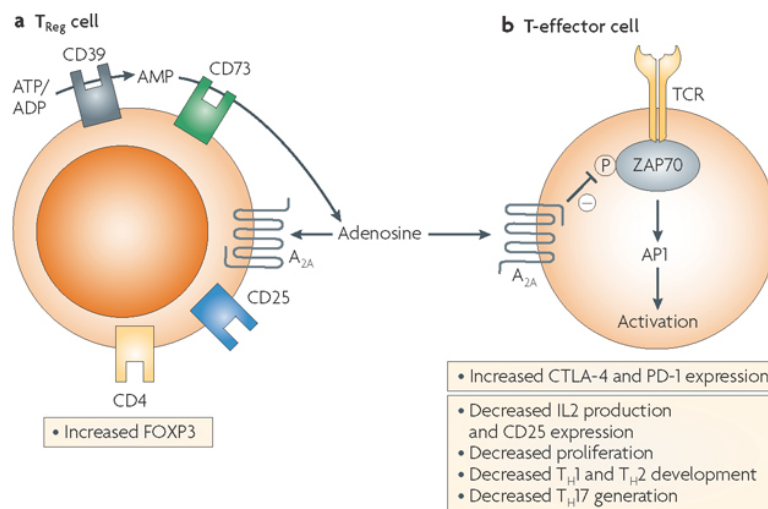


Fig. 5. Metabolic suppression pathway: **a.** Regulatory T (T_{Reg}) cells produce adenosine following sequential degradation of ATP/ADP via CD39 (ENTPD1; ectonucleoside triphosphate diphosphohydrolase 1) and CD73 (ecto-5'-nucleotidase) **b.** Adenosine activates A_{2A} receptors on T-effector cells to inhibit T-cell receptor (TCR)-mediated signalling by preventing ZAP70 phosphorylation and activation of the transcription factor activator protein 1 (AP1). This decreases interleukin 2 (IL2) production and CD25 expression resulting in decreased T effector cell proliferation. In addition, it decreases the development of both T helper 1 (T_{H1}) and T_{H2} cells, as well as the generation of T_{H17} lymphocytes [76].

Tregs can also inhibit T cell responses by cell contact dependent mechanisms. These mechanisms of immuno-immunosuppression are mediated by inhibitory receptor, as CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4 or CD152) and **PD-1** (Programmed Death 1 receptor or CD279). PD1 is an immunoinhibitory receptor that belongs to the CD28 family with a is a critical role in the regulation of T cell activation and function during immunity and tolerance.

It is upregulated on T cells upon their activation and highly expressed on Tregs.

Its ligand, PD-L1, is expressed on antigen presenting cells (APCs) such as dendritic cells, macrophages and B cells, T cells, and a variety of nonhematopoietic cells including vascular endothelium, and at sites of immune privilege including the placenta and the eye. PD-L2 expression in contrast was reported to be elevated on DCs from the lung and from draining pulmonary lymph nodes after antigen exposure. Interactions between PD-1 and its ligands, help to regulate the balance between stimulatory and inhibitory signals needed for regulate the balance between T- cell activation, tolerance and immunopathology (Fig. 6) [33, 64].

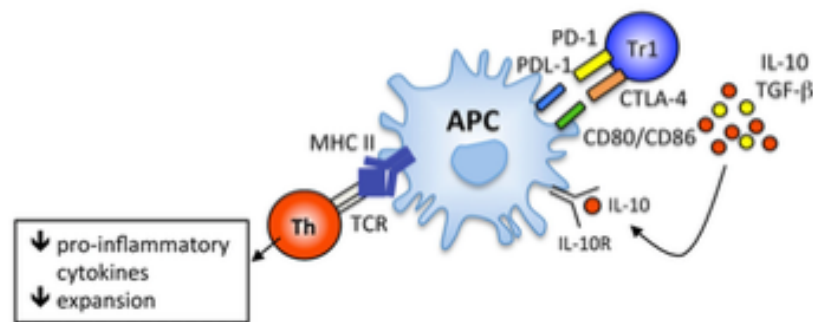


Fig. 6. Cell- contact dependent mechanisms: PD-1 on Treg cells down-regulate or prevent the up-regulation of MHC class II and co-stimulatory molecules on APC upon interaction with their ligand, which limits effector T cell activation [64].

This receptor was first observed in T cells undergoing cell death, but several studies, involving PD1, have demonstrated an important role in tumor immunity (47) and autoimmune disease (48). Instead, the contribution of PD1 pathway to the development of allergic airway responses in bronchial asthma has recently been recognized (49-50).

2. Allergy

Allergy is one of the most widespread diseases of the modern world. The term allergy was originally defined by Clemens Von Pirquet to describe “an altered capacity of the body to react to a foreign substance”.

Although the immune system protects the organism against disease, the normally protective immune mechanisms can sometimes cause injurious reactions in the host, as a consequence of deregulated immune responses; these reactions are known as hypersensitivity reactions [1, 2].

“Allergy is a hypersensitivity reaction initiated by immunological mechanism”, as defined by the EAACI nomenclature task force, and it is one of the most widespread diseases of the modern world [51].

Allergic disorders are associated with the production of allergen-specific IgE and the expansion of allergen-specific T-cell populations (Th2 cells). These disorders are due to an aberrant immune response, against various harmless environmental proteins (known as allergens), mediated through key effector cells, the Th2 cells and an associated cytokine pattern including IL-4, IL-5, and IL-13 [52].

The allergic inflammation is often classified into three temporal phases. Early-phase reactions are induced within seconds to minutes of allergen exposure. During this phase, differentiated Th2 cells are able to produce large amounts of IL-4 and IL-13, which are essential in the induction of class switching recombination of the immunoglobulin heavy chain in B cells and the subsequent production of allergen-specific IgE antibodies. Once formed and released into the circulation, IgE binds to the high affinity receptor FcεRI, on the surface of mast cells and basophiles as well as to Antigen Presenting Cells (APCs), which in turn allows for an increased uptake of allergens. Upon re-exposure, allergens activate inflammatory cell recruitment and induce the release of inflammatory mediators, which are responsible for Early (EARs) and Late Allergic Responses (LARs). The early allergic response occurs within minute of contact with allergens, the binding of the allergen to IgE initiates a process of intracellular signaling, which leads to degranulation of cells, with the release of inflammatory mediators, such as histamine and leukotrienes, these substances cause immediate allergic inflammation and promote vascular permeability, smooth-muscle contraction and mucus production.

The second phase, called late-phase reactions (LARs) occurs within several hours after the first allergic symptoms and can last for days or even weeks. During the late-phase reaction, mast cells responding to allergen release a wide range of newly synthesized cytokines, chemokines and growth factors that cause an influx of eosinophiles and Th2 cells. Eosinophiles are the major cell type involved in allergic inflammation, and upon activation they release inflammatory mediators and cytotoxic proteins (including major basic protein, eosinophil peroxidase and eosinophil-derived neurotoxin). Finally, when allergen exposure is continuous or repetitive, a chronic allergic inflammation develops; this persistent inflammation occurs at sites of repeated allergen exposure (Fig. 7) [52-54].

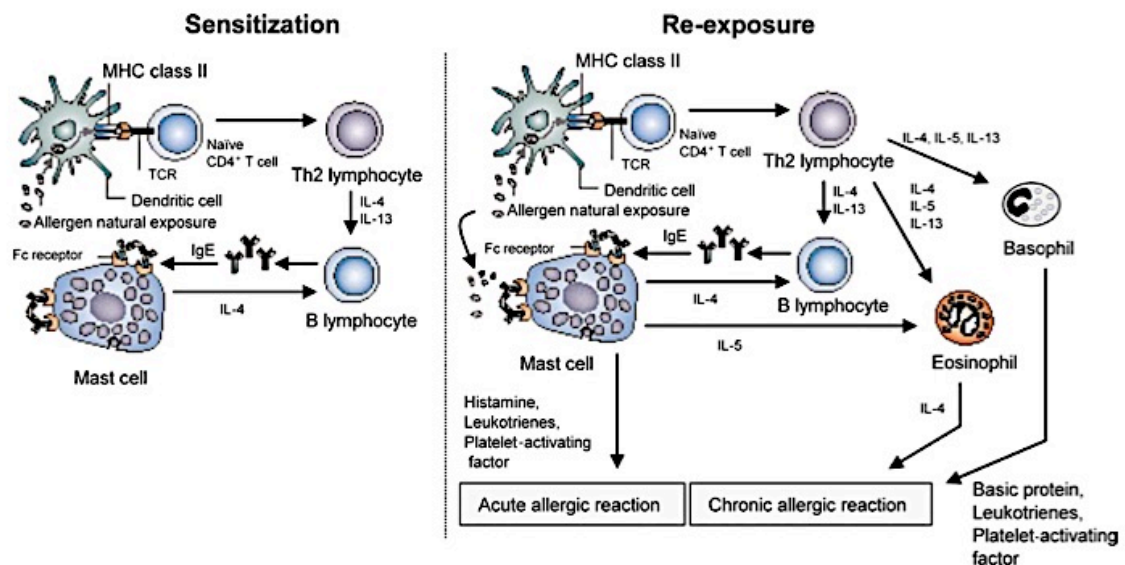


Fig 7. Schematic representation of allergy pathophysiology. In atopic individuals, first exposure to an allergen leads to activation of Th2 lymphocytes and stimulation of IgE synthesis. Upon re-exposure, binding of the allergen to IgE causes mast cell degranulation and further activation of Th2 cells with resulting eosinophil inflammation [54].

The immunological processes leading to the development of the pathogenesis of allergy are relatively defined, but it remains to understand why some individuals respond to allergen exposure with atopic immune responses and others do not. It is well known that both genetic and environmental factors are involved. Interestingly, the increase in atopic disorders observed has been partly attributed to an increase in the industrialization and development of countries. Moreover, epidemiologists have studied that the increased prevalence of allergic diseases is associated with a reduced microbial exposure [55-56]. The hygiene hypothesis, proposed by Strachan 1989, asserts that a reduced microbial stimulation of Toll-like receptors (TLRs, a type of pattern recognition receptor) on cells of the innate immunity, i.e. dendritic cells (DCs) and natural killer (NK) cells in early life is associated with an increasing propensity for allergy sensitization. In accordance to this theory the exposure to microbial antigens during childhood prevents the development of an allergen-specific Th2 response towards an allergen-specific Th1 response [56].

2.1. Cytokines and allergy

Allergic diseases are complex inflammatory disorders that involve many cytokine pathways. Many studies show, that allergic disease represents a specialized form of T cell-mediated immunity, where T CD4⁺ cells, particularly Th2 cells, and their products play a central role in the development and the persistence of chronic inflammation. It has been thought that the Th2 cell-cytokines have been involved in triggering hypersensitivity disorders such as allergy.

In allergic patients, Th2-type cytokines lead to the development of allergic symptoms following exposure to allergens. Th2 cells orchestrate the allergic response through the secretion of a series of cytokines, such as IL-4, IL-5 and IL-13, which act to enhance the allergic inflammation that may lead to chronic inflammation in tissues often exposed to allergens. **IL-4** is crucially involved in the development of allergic responses. This cytokine induces T *naïve* cell polarization bias the Th2 cells, this cytokine promotes the ϵ isotype switch and secretion of IgE by B lymphocytes, recruits basophiles, eosinophiles and monocytes. Besides, IL-4 enhances IgE-mediated immune responses through the upregulation of IgE receptors on the cell surface: both the low-affinity IgE receptor (Fc ϵ R2 or CD23) on B cells and mononuclear phagocytic cells and the high-affinity IgE receptor (Fc ϵ R1) on mast cells and basophiles [57-58].

Other cytokines produced by activated allergen-specific Th2 cells that also contribute to the pathogenesis of allergic asthma are IL-5 and IL-13. **IL-5** has been associated with the cause of several allergic diseases, such as nasal rhinitis, asthma, and atopic dermatitis.

It has been recognized as the major maturation and differentiation factor for eosinophiles during allergic inflammation. In fact, it is selective for eosinophiles and basophiles and it is well known that these cells are the two main effector cell types involved in allergic inflammation. The secretion of this cytokine causes the massive influx of eosinophiles into the lung during the course of an allergic response [59]. IL-5 binding to its receptor modulates various functions of eosinophiles, including expansion and differentiation of eosinophile precursors, upregulation of expression of its own specific α receptor chain during human eosinophile development, cellular adhesion, chemotaxis, degranulation, cytotoxicity, prolonged survival and activation. Eosinophiles contain numerous basic and cytotoxic granule proteins (including major basic protein, eosinophile peroxidase and eosinophile-derived neurotoxin) that are released upon activation [59-60]. These proteins could result in being toxic for the respiratory epithelium and may cause bronchoconstriction. Since, the eosinophiles are the major cell type involved in allergic

inflammation, IL-5 has been proposed as an excellent therapeutic target in allergic diseases. The current therapies include two monoclonal anti-IL-5 antibodies (mepolizumab, reslizumab) and a monoclonal antibody directed at the IL-5 receptor (benralizumab). Therapeutic strategies aimed at the inhibition of IL-5 and or the reduction of serum IL-5 levels may be a promise in the treatment of eosinophilic diseases as well as allergies [61]. **IL-13** is another Th2-derived cytokine, which shows similar effects to those of IL-4. IL-13 induces Ige gene transcription in B cells, which subsequently results in class switching towards the IgE isotype as well as IL-4. Unlike IL-4, it is not able to regulate T cell differentiation of Th0 into Th2 cells. IL-13 is able to regulate the Th2-mediated response via its ability to stimulate gene expression of a variety of gene such as those coding for adhesion molecules, chemokines, and metalloproteinases in order to promote the recruitment, homing, activation and survival of a wide range of inflammatory cells [62-63]. IL-13 is able to induce a multitude of effects on airway epithelial cells resulting in an increment of mucus production by submucosal glands and epithelial cells [64].

This cytokine induces the production of chemokines that can synergize with IL-5 to selectively recruit eosinophiles to the lung. IL-13 is suspected to be the central mediator in the development of allergic response and it has been proposed to be mainly involved in the chronicity of allergic inflammation, and to play a critical role in the development of asthma [65-66]. Studies in mouse model of allergic disease have shown that inhibition of IL-13, by genetic knockout or IL-13 binding proteins or even the anti IL-13 receptor, could be therapeutically beneficial [66-68].

2.2. Natural Killer (NK) cells and their role in allergy

The role of T cells and cytokines associated pattern in allergy have been well characterized, but little is known about the role of innate immunity cells. It is known that the imbalance of Th1/Th2 T-cell polarization and the bias towards Th2 cytokine production plays an important role in both the initiation and maintenance of these events. It is well known that allergic diseases involve not only Th2 cells, but much rather involve more complex immune disorders [39]. Recent studies have suggested a role for NK cells in allergy, showing defects in CD56^{bright} CD16^{dim} NK cells in allergic patients compared to healthy individuals [69-70]. These observations have suggested that these cells might play a role in bias towards Th2 response in poly-allergic patients and healthy individuals. NK cells are considered versatile immunomodulatory cells, it has been shown that human NK cells are able to polarize *in vitro* into two functionally different subsets NK1 or NK2,

producing cytokine subsets similar to Th1 and Th2 cells. NK cells grown in the presence of IL-12 (NK1) produce predominantly IFN- γ , whereas NK cells grown in the presence of IL-4 (NK2) produce IL-5 and IL-13. NK cells support DCs to the induction of appropriate T CD4⁺ responses [68-70]. Studies in healthy patients reported that, during a tolerogenic immune response, components of the innate immune system (CD56^{bright} CD16^{dim} cells) are activated by allergens to produce IFN- γ and IL-10 cytokines. Moreover, in contrast to the diminished levels of IFN- γ ⁺CD56⁺ NK1 cells, high levels of IL-4⁺CD56⁺ NK2 cells in PBMC of asthmatic patients have been shown. The cytotoxic capacity of NK cells in patients with allergic rhinitis was also higher compared with that seen in healthy subjects. There are good evidences that NK cells also take part in allergic diseases through their contribution to allergen-specific immune suppression and IgE production [71-75].

2.3. T regulatory cells and their role in allergy

The initial immune model of the Th1/Th2 imbalance associated with the ‘hygiene hypothesis’ has recently been questioned by further evidences that peripheral T-cell regulation plays a crucial role in the control of harmful T-cell responses [76].

It has been proposed that an impairment in the induction of regulatory responses is the underlying mechanism involved in the differentiation of pathogenic T cells driving to inflammatory responses including aberrant Th2 response that drives to allergic diseases. Our immune system consists of a complex network of regulatory response that maintain the immune homeostasis.

Moreover, the description of a new subset of CD4⁺ T cells named Tregs revisited the “immune regulation” concept.

Tregs are the main cells involved in the regulation of both innate and adaptive immune response. This feature makes these cells important in maintaining peripheral immune tolerance and more generally in the control of immune response against tumors, viral infections and transplants. Defects in their function can induce inflammation and autoimmune diseases [77-79].

In recent years Tregs have been described as key mediators during the sensitization phase of allergic response. Studies on immune response to allergens in healthy non-atopic individuals showed that Tregs play a crucial role in preventing Th2 response to allergens and maintaining the peripheral tolerance. On the contrary, the T regulatory function results impaired in allergic individuals. Therefore, the activation of Th2 response to allergens is a consequence of an impaired

mechanism of tolerance that is normally characterized by T cell anergy and Treg activity mediated by secretion of suppressive cytokines such as IL-10 and TGF- β [80-81]. Studies on allergic murine models showed that the depletion of Tregs during the sensitization phase led to the development of allergic inflammation, with increased recruitments of inflammatory cells, secretion of Th2 cytokines and high levels of serum IgE pointing out the pivotal role of Treg cells in the regulation of immune response to allergens. However, the allergic inflammation can be suppressed and the immune response restored by the adoptive transfer of Treg expressing IL-10 and TGF- β . Therefore, the possibility to control Treg function could have many therapeutic potentials for the treatment of allergy. Many studies have been carried out to characterize Treg phenotypes and biological activity.

3. Allergen-Specific ImmunoTherapy (SIT)

During the last decades, the prevalence of allergic diseases has increased dramatically in the industrialized high-income countries.

The World Health Organization (WHO) reports Non-Communicable Diseases (NCDs) to be the major cause of adult mortality and morbidity in the world. Chronic respiratory diseases are the third main cause of death after cardiovascular disease and cancer. The allergic diseases are the most common chronic respiratory diseases worldwide.

The prevalence of allergic diseases has dramatically increased over the past decades, affecting up to 30% of the population in industrialized countries. The understanding of the mechanisms underlying allergic diseases, as well as those operating in non-allergic healthy responses, plays an important role in the development of new therapeutic strategies for allergic disease.

Several potential therapies have been proposed for the treatment of allergic diseases. It is possible to impair the activation of allergen-specific Th2 cells, either directly or indirectly, inhibiting antigen-presenting cells: for example by treatment with anti-inflammatory drugs, such as glucocorticoids.

Another therapeutic option for the treatment of allergic diseases is to inhibit effector molecules that cause the allergy clinical symptoms: such as the treatment with antihistamines, leukotriene antagonists as well as neutralizing antibodies specific for Th2 cytokines or antibodies specific for IgE. Therefore, various drugs can transiently ameliorate the symptoms of IgE-mediated allergy reactions, but although these treatments are highly effective for controlling allergic diseases they

are not able to modify the natural history of the allergic diseases [82-84].

The only valid treatment able to modify the underlying pathological mechanisms of immune response and to have a long-lasting effect is allergen-Specific ImmunoTherapy (SIT) which was first introduced by Leonard Noon in 1911 [85].

He originally hypothesized that patients suffering from hay fever were sensitive to a “toxin” contained in grass pollen that caused allergic symptoms [85-87]. He suggested that the inoculation of small doses of pollen extracts would induce antitoxins and be of benefit for the patient.

This procedure consists in giving increasing amounts of allergen extracts into the patient with the aim to reduce the symptoms on a re-exposure to those particular allergens.

This principle is still true today, more than 100 years later, and it is the basis for current allergen immunotherapy. The repeated administration of allergens to allergic individuals has the aim of modifying the allergen-specific immune response and of activating immunomodulatory mechanisms in treated patients, in order to induce tolerance towards higher doses of allergen and improve the quality of life during natural allergen exposure (Fig. 8).

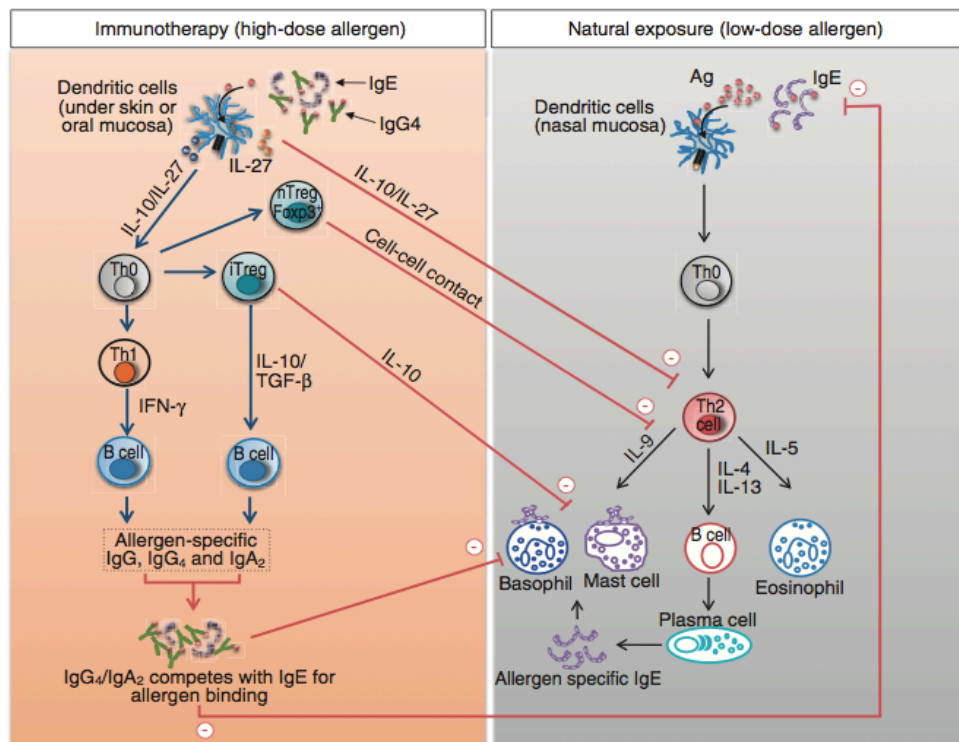


Fig. 8. Mechanism of immunological tolerance induced by repeated administration of high doses of allergens. Low-dose and repeated allergen exposure at mucosal surfaces in atopic individuals drives IgE-facilitated antigen presentation and Th2 mediated allergic inflammation. High-dose allergen administered by sublingual or subcutaneous immunotherapy results in immune deviation from a Th2 to a Th1-driven response [86].

Several studies have demonstrated the efficacy of allergen-specific immunotherapy, but although it is a clinical practice used for almost one century, the molecular mechanisms involved in successful immunotherapy are not fully understood and several factors seem to influence the immune response such as the concentration of the allergen, the type of antigen-presenting cells and the type of adjuvants used for the formulation of vaccine.

So far, allergen-specific immunotherapies have been carried out with allergen extracts from natural sources that have often shown great variations of allergen contents without taking care of the individual sensitization profile of patients. Several studies have reported that the administration of natural allergen extracts could induce new IgE specificities against allergens, which were in the natural extracts and not recognized by the patient before treatment. The main disadvantages that limits the use of allergen extracts from natural source in SIT are related to their heterogeneity and poor quality of these extracts which may lack important allergens and also show non standardized composition [88-91].

Many strategies have been developed in order to reduce systemic anaphylactic reactions during SIT and overcome the IgE-mediated side effects observed with allergen extracts.

The use of recombinant purified allergens can overcome many, if not all, of the problems associated with the use of natural allergen extracts, such as poor quality, allergenic activity and poor immunogenicity [85, 92-94]. Thanks to recombinant DNA technology, cDNAs of most important allergens have been isolated and expressed as recombinant proteins. The number of cloned and purified allergens has increased substantially over the past decade. This allows the production of pure allergen molecules in order to produce defined and safe allergy vaccines.

The use of recombinant 'native' allergens, which retain the sequence, conformation and biological activity of the natural molecule, has several advantages over the use of natural allergen extracts.

Recombinant 'native' allergens are effectively proteins that can be produced under defined conditions and purified by using procedures such as affinity chromatography [88]. Some reports about allergen-specific immunotherapy performed with recombinant allergens have been published, showing the efficacy of these vaccines for the treatment of allergic disorders [93-94]. During an allergen immunotherapy trial, in 2006, birch pollen allergic patients received recombinant Bet v 1a allergen; the results of this study showed that the treatment with recombinant allergen Bet v 1a was as effective as the treatment with the natural extract [95-96].

Moreover, several studies have shown that immunotherapy with high doses of allergens was more

effective than that with low doses for symptom reduction. However, the administration of increasing doses of recombinant ‘native’ allergens to patients may induce severe and life-threatening IgE-mediated anaphylactic side effects. Therefore, effective dosages are limited by potential systemic reactions [97].

To overcome this problem, the development of recombinant DNA technology has allowed to modify the major allergens *in vitro* and to create hypoallergenic variants with reduced allergenicity (reactivity of allergen-specific IgE) but maintained immunogenicity (Fig. 9).

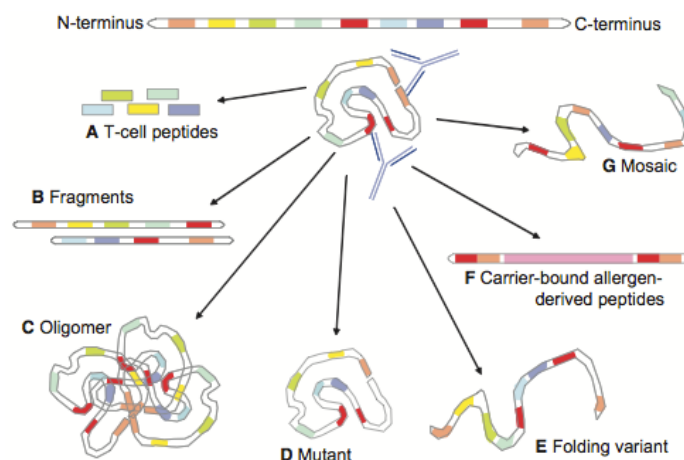


Fig. 9. Approaches for the generation of hypoallergenic allergy vaccines [86].

Different approaches can be used to produce hypoallergenic variants with reduced IgE binding activity but they have to preserve the sequence and structural motifs necessary for T-cell recognition, termed T-cell epitopes, and for induction of IgG antibodies reactive with the natural allergen (blocking antibodies) [98].

Studying the relationship between the structure of the allergen and its function is a pre-requisite for the manipulation of a gene. The allergen gene modification usually requires knowledge of B and T cell epitopes. The reduction of IgE binding activity can be obtained either by mutation and/or deletion of crucial amino acid residues involved in IgE recognition or by the disruption of the three-dimensional structure of the allergens. This, can also be obtained by fragmentation, or by oligomerization, or by fusion of allergen variants [99-101].

A novel approach to negatively regulate immune response to allergen is the generation of recombinant hybrid allergen proteins. Hybrid allergenic molecules are proteins consisting

of multiple allergens (either omo- or etero-) fused in an engineered single molecule. Hybrid allergenic molecules can contain two or more recombinant native allergens or alternatively contain the most important epitopes or polypeptides of one or more allergens. Some of these molecules can also retain T-cell epitopes and lack the IgE reactivity of the native molecules. These hybrid allergen proteins have been created for the use as combination vaccines; an applicable strategy whenever the induction of simultaneous immune responses against unrelated antigens with different immunogenicity is desired. The advantages of this strategy have been shown by a few reports where oligomerization induced immunological changes like reduced allergenicity and increased immunogenicity [102].

3.1. Immuno deviation during SIT

A healthy immune system is dynamic and balanced between Th1 and Th2 cell responses and it is able to change between the two cell-mediate responses as needed. Th1 and Th2 regulate immune system function in a delicately balanced relationship. However, both genetic and environmental factors could cause a Th1 or Th2 dominant-response and induce an immune disorder. Thus, when Th1 cells are overactive they can suppress the Th2 mediated immune response and *vice versa*. Allergen specific Immunotherapy has been shown to modify T cell response in several ways. [103-104]. T cells are considered to be the main immune cells involved in the modification of allergic immune response during allergen specific immunotherapy. The shift from Th2 cells, that characterize the atopic immune response, to the protective Th1 response, is induced by the administration of repeated high doses of allergen, as occurs by specific immunotherapy. Allergen-specific immunotherapy alters the balance of cytokines produced by T helper lymphocytes, by modulating or down regulating the production of cytokines such as IL-4, IL-5 and IL-13, and by inducing the upregulation of IFN- γ associated with the induction of an ameliorating Th1 response. Clinical evidence suggests allergen immunotherapy induces a Th1-redirection of immune response by modifying peripheral and mucosal predominant Th2 response into a protective Th1-polarization, as evidenced by a reduced Th2 cytokine level and an increased production of IFN- γ and IL-10 cytokines [105].

IFN- γ is a pleotropic cytokine that plays an essential role in both the innate and adaptive phases of an immune response. An important role of IFN- γ is to activate antigen-presenting cells (APC), such as dendritic cells and macrophages, resulting in increased phagocytosis, increased MHC class

I and II expression. It induces the production of IL-12 and reactive oxygen species, which are important in the elimination of intracellular pathogens. IFN- γ acts not only as a potent activator of the Th1 phenotype, but also as a suppressor of Th2 development, exercising inhibitory effects on Th2 cytokine production [106].

In line with this evidence, IFN- γ and IL-12 may suppress Th2 responses of allergic diseases. Epidemiological studies have clearly shown that a failed Th1 response, characterized by the cell-mediated pro-inflammatory response through the production of IFN- γ , predisposes towards the development of allergic diseases. Moreover, patients with severe asthma present a reduced level of IFN- γ in response to allergen if compared to healthy patients [106]. Another inhibitory role of IFN- γ is its ability to regulate the switching of heavy chain class into antibody-secreting B cells, inhibiting immunoglobulin class switching to IgE. Otherwise, IFN- γ induces IgG₄ production, which acts as a competitive response to that of specific IgE, by blocking IgE-facilitated antigen presentation. IgG₄ are also able to neutralize inhaled allergens, through the interactions with Fc γ receptors (Fc γ R), promoting Fc γ R-mediated endocytosis of allergen-IgG complexes [106]. Th1 and Th2 are implicated in inflammatory reactions, so as to minimize the damages of these reactions; the allergen immunotherapy triggers an anti-inflammatory immune response.

IL-10 plays a key regulatory role in peripheral tolerance during allergen specific immunotherapy and natural exposure to allergen. This cytokine acts as a general inhibitor of proliferative and cytokine response of both Th1 and Th2 [107].

IL-10 is produced by a large number of immune cells (B lymphocytes, macrophages and DCs) in addition to the regulatory CD4⁺ T cells. The repeated administration of allergen induces a state of peripheral anergy in specific T cells, which is characterized by blocked CD28 co-stimulatory signaling and suppressed proliferative and T-cell cytokine responses.

IL-10 is a potent anti-inflammatory and immunosuppressive cytokine that mediates its major immunosuppressive function by inhibiting APC functions and cytokine production of macrophages and dendritic cells and inhibiting Th1 cell-mediated immunity [107-108]. IL-10 can also suppress the allergic response reducing pro-inflammatory cytokine production by Th2 cells and inducing Th2 cell anergy by acting directly on these cells and through other immune cells [108].

The IL-10 produced by allergen specific Tregs suppresses allergic inflammation also through direct action on mast cells, basophiles and eosinophiles. Another critical role of IL-10 is its ability to promote the survival, proliferation, and differentiation of human B cells. It has two different

effects on B cells; it can differently modulate Ig responses by inhibiting IgE production and enhancing the production of blocking IgG₄.

The knowledge of these molecular mechanisms is fundamental in understanding the regulation of immune response and the immunological process involved in peripheral tolerance and their possible therapeutic applications.

4. *Parietaria judaica* (Pj)

Parietaria judaica is a species of herbaceous perennial plant belongs to genus of dicotyledonous weeds of Urticaceae family. This family is considered to be the most common group of allergenic plants in the Mediterranean area and *P. judaica* and *P. officinalis* have been reported to be the most common causes of allergy in this area. The *Parietaria* pollen shows very strong allergenic properties that give rise to seasonal allergies with clinical manifestations such as rhinitis, conjunctivitis and asthma. *Parietaria judaica* has a very long period of pollination, from February to September. In the southern regions and islands, this period is often biphasic with a first phase that starts around the months of February-March and then reaches its peak in the months of April-May; while in the northern regions there is only one peak in the period of May-June. *Parietaria* pollen is one of the main sources of allergens in Mediterranean area. In fact, about 30% of all the allergic subjects in southern Italy present a skin prick test (SPT) reactivity to the *Parietaria judaica* (Pj) pollen extract [109].

4.1 *Parietaria judaica* allergens

Recombinant DNA technology allowed to isolate and characterize the *Parietaria judaica* allergens. The composition of the allergenic extract of *Parietaria judaica* pollen has been studied and its allergens have been identified and characterized by immunochemical and molecular-biological techniques [109]. Among them, Par j 1 and Par j 2 were classified as the two major allergens of *Parietaria* pollen, which belong to the family of the non-specific Lipid Transfer Proteins (ns-LTPs) [110-112]. These proteins are widely distributed in the plant kingdom and form multigene families of related proteins, characterized by their ability to transport lipid molecules through membranes *in vitro*. Recent studies have shown that the ns-LTPs have a protective function, such as antimicrobial activities and are thought to participate in plant defense

mechanisms. This hypothetical function is also supported by the induction of the expression of many ns-LTP genes in response to biotic infections or application of fungal elicitors and by the enhanced tolerance to bacterial pathogens. Due to their possible involvement in plant defense mechanisms, nsLTPs are recognized to be pathogenesis-related (PR) proteins and constitute the PR-14 family. Two minor allergens have recently been isolated from *Parietaria* pollen: the Par j 3, showing high similarity to profilins and the Par j 4 with high level of similarity to calcium-binding proteins from other allergenic sources [113-114].

Besides, two different isoforms of Par j 1 have been isolated and named Par j 1.0101 (a 14,4 kDa protein) and Par j 1.0201 (a 10,7 kDa protein). These proteins are isoallergenic forms of the major allergen Par j 1. These isoforms demonstrated a 96% amino acid sequence similarity within the first 102 amino terminal regions. Overall, the two isoforms differ for the presence of a 37 aminoacids COOH-terminal tail in the Par j 1.0101 allergen [115-116].

Bonura et al studied the correlation between the pairing of disulfide bonds and the human IgE-binding activity. In particular, Par j 1 and Par j 2 have a characteristic structure stabilized by four disulfide bonds. Hypoallergenic molecules were generated by targeting these disulfide bonds through site-directed mutagenesis of specific cysteine residues with serine. These molecules showed an altered conformation and a decreased IgE-binding activity but a maintained T cell reactivity.

In a published paper the authors described the immunological characterization of hypoallergenic mutants of Par j 1 developed by site-directed mutagenesis [117].

Four different variants of the major allergen of Par j 1 were constructed by changing the three-dimensional structure carrying serine substitutions of cysteine residues into positions 4, 29, 30, 50 and 52 [117]. The same approach was used for the Par j 2 allergen.

Recently, the Par j 1 hypoallergens were evaluated *in vivo* in a murine model of allergic sensitization. Another published paper by Bonura et. al. described a hybrid hypoallergenic molecule, named rPjEDcys, generated by head to tail fusion of the two major *Parietaria* allergens, Par j 1 and Par j 2, both mutated in cysteine residues at position 4, 29 and 30 respectively (Fig. 10) [118].

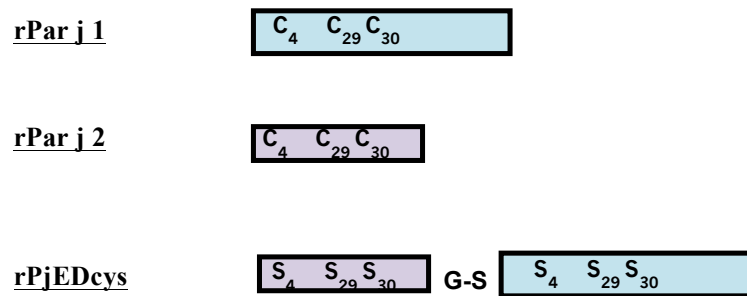


Fig. 10 Schematic representation of rPar j 1, rPar j 2 and engineered rPjEDcys.

The advantages of this strategy have been shown by few reports; oligomerization induces immunological changes like reduced allergenicity and increased immunogenicity. In accordance with these data, rPjEDcys displayed a reduced allergenicity and retained T cell reactivity.

The reduced allergenic activity of rPjEDcys combined with its retained immunogenicity was demonstrated by human *in vitro* studies that showed rPjEDcys retained the capability to stimulate CD3⁺ cell proliferation and in mouse *in vivo* studies in which BALB/c mice immunized with the rPjEDcys induced antibodies studied for their ability to bind allergens of *Parietaria* pollen.

Moreover, the immunogenicity of the hypoallergenic hybrid was studied *in vivo* looking at the pattern of antibody production in a mouse model of sensitization. BALB/c mice were immunized with the hybrid and the induced antibodies were studied for their ability to bind the major allergens of the *Parietaria* pollen. Despite of the changes introduced by mutagenesis, the rPjEDcys was able to induce a strong IgG response towards the rParj1 and rParj2 molecules. Both ELISA inhibition and western blot experiments performed with the *Parietaria* pollen extract demonstrated that the rPjEDcys induced IgG antibodies were able to recognize the natural major allergens. This suggested that an immunization protocol performed with the recombinant hybrid could induce Par j 1 and Par j 2 specific antibody responses. These responses could be capable of inhibiting IgE mediated presentation, blocking basophile histamine release and reducing seasonal IgE production and allergic symptoms during the natural course of sensitization against the *Parietaria judaica* [118].

Objective

The aim of this research project is to study the immunological mechanisms activated by the major allergens of *Parietaria judaica*, rPar j 1 and rPar j 2, and the hybrid hypoallergenic mutant rPjEDcys containing their main B and T-cell epitopes in one molecule. Despite their common evolutionary origin in fact, Par j 1 and Par j 2 allergens display some cross-reactive and independent IgE epitopes. The project I am involved in is to address the question whether this engineered hypoallergenic molecule can be used as a potential pharmaceutical product for a safer allergen-specific immunotherapy.

Therefore, the generation of a single protein comprising the two major allergens of *Parietaria* pollen as pharmaceutical product may reduce production costs, providing a therapy that could modify allergen-specific immune response towards the two major *Parietaria* allergens.

The use of rPjEDcys in the treatment of allergy may present many advantages with respect to the natural extracts used:

- 1) one pharmaceutical product to protect against Par j 1 and Par j 2 sensitivity;
- 2) a characterized and standardized product;
- 3) a safer product with reduced anaphylactic activity.

However, there is a need of a new insight into the mechanisms of inflammatory and allergic diseases.

The study of the processes at molecular level enables us to better understand the regulation of the immune responses. Intensive studies have to be performed to understand the mechanisms of action of allergen specific immunotherapy in order to decrease the side effects and increase the efficiency of the current therapies and to develop novel therapies.

Therefore, in this experimental setting, the use of the major allergens of *Parietaria judaica* and the hybrid allowed me to study the immunological mechanisms activated by the major allergens of *Parietaria judaica*, Par j 1 and Par j 2, and to investigate the differences between the wild type allergens and the hypoallergenic mutant rPjEDcys.

Material and Methods

1. Production of recombinant proteins

The recombinant allergens (Par j 1, Par j 2 and PjEDcys) were expressed as histidine-tagged proteins (pQE30 vector, Qiagen, UK) in *Escherichia coli* (strain M15, Qiagen, UK). Recombinant clones were grown over night at 37°C in 10 ml of 2YT broth (Bacto-tryptone 16g/l, yeast extract 10 g/l and NaCl 5g/l at pH 7.0) containing 100 µg/ml ampicillin. The bacteria were diluted 1:40, incubated for 2 hours at 37°C. Isopropylthio-β-galactoside (IPTG) was added to the final concentration of 1 mM and growth was continued for a further 3 hrs. Cells were harvested by centrifugation (5,000 rpm for 15 minutes at 4°C) and disrupted by using a sonicator device (Heat System Ultrasonic W-285) in a starting buffer containing 10 mM of sodium phosphate, 0.5 M NaCl (for rPar j 2 and rPjEDcys) or 1 M NaCl (for rPar j 1), 10 mM imidazole and 6 M urea at pH 7.4. The cell lysates were centrifuged at 10,000 rpm for 10 minutes to remove cell debris. Following the lysis, the lysate becomes very viscous due to the release of genomic DNA into the solution. It is very important to reduce viscosity and to avoid clogging of the column. Therefore, supernatant was filtered through a sterile 0.8µm syringe disc-type filter to disrupt genomic DNA and used for purification of the proteins.

1.1. Affinity chromatography

Proteins were purified by using HisTrap chelating Column (GE Healthcare, Hertfordshire, UK) equilibrated in starting buffer. The column was washed in wash buffer (10 mM sodium phosphate, 0.5 or 1 M sodium chloride, 50 mM imidazole and 6 M urea at pH 7.4) to reduce unspecific binding and to remove the contaminants. Histidine-tagged proteins were eluted by using a discontinuous imidazole gradient (elution buffer: 50–500 mM imidazole). The eluted proteins were diluted 1:50 in starting buffer without imidazole and reloaded onto a HisTrap chelating column for a second purification phase. Eluted fractions were analyzed by SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue.

1.2. Dialysis

Refolding of the solubilized proteins is initiated by the removal of the urea by means of dialysis, using membranes with molecular cutoff of 3.500 Da. The fractions containing the recombinant allergens were first pooled and diluted 1:20 in starting buffer and dialysed against a buffer

containing 10 mM sodium phosphate and 0.5 or 1 M sodium chloride, at pH 7.4 for 90 minutes.

1.3. Second affinity chromatography

After dialysis, a third purification step is performed using buffers that do not contain urea. The sample was recovered and reloaded onto another HisTrap chelating Column (GE Healthcare, Hertfordshire, UK) equilibrated in starting buffer without urea. The column was washed in a wash buffer and the histidine-tagged proteins have been eluted by using a discontinuous imidazole gradient (50–500 mM). Eluted fractions were analyzed by SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue.

1.4. Gel filtration chromatography

Eluted fractions containing recombinant proteins were desalted using a Sephadex G-25 Superfine column (GE Healthcare, Hertfordshire, UK). Sephadex G-25 is a well-established gel filtration medium for desalting, separations of low and high molecular weight molecules and buffer exchange. The positive fraction was loaded onto Sephadex G-25 superfine column equilibrated in 1X Phosphate Buffered Saline (PBS) solution without Ca^{2+} and Mg^{2+} (Dulbecco's phosphate-buffered saline (D-PBS)) and the recombinant proteins were then eluted using 1X PBS solution. Eluted fractions were analyzed by SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue.

1.5. Detoxi-Gel Endotoxin Removing Gel

Many recombinant proteins are expressed in *Escherichia coli* vectors. These products are always contaminated with endotoxin derived from the outer cell membrane of gram-negative bacteria.

Due to the diverse and potentially harmful biological response to these molecules, the presence of endotoxins in biologically derived products prepared for therapeutic use is a major problem.

Detoxi-Gel Endotoxin Removing Gel (Pierce, USA) is commonly used to remove endotoxins from protein solution. This technique uses immobilized polymixin B, a peptide antibiotic that has a very high binding affinity for the lipid A of most endotoxins, allowing its removal from solution. Detoxi-Gel Endotoxin Removing Gel was regenerated in 5 ml of 1% sodium deoxycholate in sterilized water and washed with 5 ml of sterilized water and 5 ml of PBS 1X before the fraction containing recombinant protein was added. The recombinant proteins (rPar j 1, rPar j 2 and rPjEDeys) were eluted using PBS 1X (w/o Ca^{2+} and Mg^{2+}) and gravity-flow fractions were collected.

1.6. Protein quantification assay

Protein concentration was determined by reference to a standard curve with known concentrations of Bovine Serum Albumin (BSA). Protein calibration curve was constructed using BSA at concentrations of 250 ng/μl, 500 ng/μl, 750 ng/μl and 1000 ng/μl. A calibration curve of fraction that has an unknown protein concentration was prepared with an increasing quantity of protein solution. The samples were analyzed by SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue and quantification of protein loaded was performed with a Bio-Rad ChemiDoc using the software Quantity One. The recombinant proteins were stored at -80°C.

1.7. *Limulus Amebocyte Lysate test (LAL test)*

Each protein was tested for endotoxin content using the Multi-test *Limulus Amebocyte Lysate* (LAL) pyrogen plus test (Bio-Whittaker, VWR International, Milan, Italy)

This is highest sensitive system of detection and quantification of the amount of bacterial endotoxin in a sample, which is derived from the circulating amebocyte of horseshoe crab *Limulus Polyphemus*. The maximum sensitivity is 0.03 EU/ml (λ) (EU = endotoxin units). LAL assay is a gel-clot assay, based on the interaction of endotoxins with the proenzyme Factor C found in circulating amebocytes. When the LAL is combined with a dilution of the sample containing endotoxin, clots upon exposure to endotoxin. LPS contamination is usually expressed in EU / micrograms. In order to assess the amount of endogenous LPS, serial dilutions of recombinant proteins were made. The first solution was prepared at a concentration of 1 μg of protein in 100 μl of water for bacterial endotoxin (LAL reagent water) (Lonza, endotoxin content: <0.005 EU/ml) test and serial dilutions of this solution were prepared (1:1, 1:2, 1:4, 1:8, 1:16) in LAL reagent water. The serial dilutions allow us to detect the endpoint dilution, as the last dilution of endotoxin that still gives a positive result. The test is not valid without a positive and a negative control. Positive control is prepared with 100 μl of standard endotoxin at a concentration of 2 λ (0.06 EU/ml). Negative control contains only 100 μl of LAL reagent water.

In each vial, a volume of 100 μl of LAL reagent was added to an equal volume of the samples and controls. Each dilution, as well as positive and negative controls, was assayed in duplicate.

The vials were incubated in the heat-stable apparatus at 37 ± 1°C for 60 ± 2 min, avoiding vibration. After the one hour incubation period, the positive and negative results were recorded. A result was recorded as positive when a gel was formed in reaction tube and remained when the vial

was inverted through 180 degrees. A negative result was indicated by the absence of a gel or by the formation of a viscous gel that did not maintain its integrity when inverted.

LAL test sensitivity allows us to detect an endogenous endotoxin content lower 0.003 ng LPS/ μ g of recombinant protein.

2. Patient selection

Patients included in the study were over 18. They were chosen, during the *Parietaria judaica* pollen season, for their allergic clinical history towards the *Parietaria judaica* pollen and for the positive skin prick test in response to commercial extracts.

None of them had previously received treatment with glucocorticosteroid or antihistamine within the last months. Patients with uncontrolled asthma or receiving previous *Parietaria judaica* specific immunotherapy were excluded from the study. Each patient signed an informed consent before the blood collection.

3. Human Peripheral Blood Mononuclear Cells (PBMC) isolation

Human Peripheral Blood Mononuclear Cells (PBMC) were isolated from heparinized peripheral blood of *Parietaria* allergic patients, by Ficoll density gradient centrifugation (LYNPHOPREP Axis-Shield-Norway). 15 ml of heparinized peripheral blood was diluted (1:1) in HBSS (Hank's balanced salt solution -EUROCLONE-) supplemented with 1X penicillin/streptomycin. The sample is centrifuged at 2,500 rpm for 20 minutes at 20°C in a swinging-bucket rotor without brake. After centrifugation, the following layers will be visible in the tube, from top to bottom: plasma and other constituents, a layer of mononuclear cells (PBMC), Ficoll-Paque, and erythrocytes & granulocytes which should be present in a pellet form at the bottom. This separation allows an easy harvest of PBMC. The mononuclear cell layer was aspirated and the recovered interface was carefully transferred into a new 15 ml conical tube. The recovered cells were washed three times in HBSS and then the cells were counted using a Thoma counting chamber. The cells were resuspended in complete RPMI (RPMI 1640 with a final concentration of: HEPES 25 mM, glutamine 2 mM, sodium pyruvate 2.5 mM, 1X penicillin/streptomycin, 10% fetal calf serum (FCS) or human AB serum) at final concentration of 1×10^6 cells/ml or 5×10^6 cells/ml.

4. Flow cytometry

Immunostaining and flow cytometry analyses were performed according to standard procedures.

All antibodies were purchased from Miltenyi Biotec or BD Biosciences (Tabel 1). Isotype controls for each antibodies were included in all experiments.

Each samples was incubated for 20 minutes in the dark in the refrigerator (4°C). A minimum of 100,000 cells per sample were then analyzed a BD FACS Calibur (BD Biosciences, San Jose, CA, USA) and CyAn ADP flow cytometer (DAKO) only for multiparametric analyses. Data interpretation was performed with WinMDI 2.9 software (Windows Multiple Document Interface for Flow Cytometry 2.9), Summit 4.3 software or Flowjo v10.1 software (Tree Star, Ashland, OR). Analysis gates were set on live lymphocytes by forward and side scatter profile.

Antibody	Fluorochrome	Provider	Antibody dilution
anti-CD4	PE (Phycoerythrin)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-CD4	PerCP (Peridinin chlorophyll protein)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-CD4	FITC (Fluorescein isothiocyanate)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti- CD8	PerCP (Peridinin chlorophyll protein)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-CD14	PerCP (Peridinin chlorophyll protein)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-CD16	PE (Phycoerythrin)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-CD25	PE (Phycoerythrin)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-CD25	FITC-VioBright (Fluorescein isothiocyanate)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-CD39	FITC-VioBright(Fluorescein isothiocyanate)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-CD39	PE (Phycoerythrin)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-CD56	PerCP-Cy5.5 (Peridinin chlorophyll protein-Cy5.5 conjugates)	BD-Bioscience	1 µl/ 1 X 10 ⁶ cells
anti-CD127	FITC (Fluorescein isothiocyanate)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-GARP	PE (Phycoerythrin)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-GARP	APC (Allophycocyanin)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-LAP	PE (Phycoerythrin)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
anti-PD1	PE (Phycoerythrin)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
REA Control (S)	PE (Phycoerythrin)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
Mouse anti-IgG2a	PE (Phycoerythrin)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells
Mouse anti-IgG2a	PerCP (Peridinin chlorophyll protein)	Miltenyi Biotec	1 µl/ 1 X 10 ⁶ cells

Mouse anti-IgG2a	FITC-VioBright (Fluorescein isothiocyanate)	Miltenyi Biotec	1 μ l/ 1 X 10 ⁶ cells
Mouse anti-IgG1	PE (Phycoerythrin)	Miltenyi Biotec	1 μ l/ 1 X 10 ⁶ cells

Table 1 – References of antibodies used for flow cytometry.

5. 5(6)-Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA-SE) staining

PBMC from ten allergic patients were resuspended in 1X PBS w/o calcium and magnesium (GIBCO) at a concentration of 1 X 10⁷ cells/ml and labeled with a solution containing 5 μ M 5(6)-Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA-SE, Molecular Probes, Nebr.,USA) and maintained for 4 minutes at room temperature in the dark. After this period, the reaction was stopped by adding ice-cold RPMI medium supplemented with 10% FCS.

The cells were washed twice in RPMI medium and were resuspended in RPMI complete medium with 10% FCS at final concentration of 1 X 10⁶ cells/ml.

CFSE-labeled cells were incubated with antigen, prepared as described before, at 1 and 10 μ g/ml for 7 days. Control samples were cells incubated with medium only and cells incubated with LPS at 1 μ g/ml (Escherichia coli 026:B6, SIGMA).

After 7 days the cells were collected by centrifugation washed and stained with anti-CD4 PE, anti-CD8 PerCP, anti-CD16 PE and anti-CD56 PerCP-Cy5.5.

6. Cytokine Secretion Assay: IL-5, IL-13, IFN- γ and IL-10

Cytokine secretion assay was carried out in order to detect T cell secreted cytokines upon antigen specific stimulation. This technique allows the multiparameteric analysis of viable cytokine-secreting cells down to frequencies of 10⁻⁵. It was performed in order to detect and isolate viable antigen-specific T cells after a specific *in vitro* stimulation. T cells secrete cytokines, only transiently, upon stimulation and therefore there are normally only very few T cells secreting cytokines in peripheral blood. However, memory/effector T CD4⁺ cells rapidly restart to secrete cytokines after an *in vitro* stimulation.

In particular, PBMC from ten allergic patients were resuspended in RPMI complete medium with 10% human AB serum and seeded at a final concentration of 5 X 10⁶ cells/ ml.

PBMC were incubated with antigen, prepared as described before, at 20 μ g/ml for 16 hrs at 37°C, 5% CO₂ and moreover, purified costimulatory anti-CD28/49d were added at a final concentration

of 1 µg/ml each sample. The control sample was treated as the antigen-stimulated sample except for antigen addition.

After 16 hrs, the cells were collected and resuspended in 4 ml of cold buffer (RPMI complete medium with 1% human AB serum) at a final concentration of 10^6 cells/ml and divided into four different tubes per sample, in order to investigate four different cytokines, as IL-5, IL-13, IFN- γ and IL-10. A total of 10^6 cells were used for each cytokine secretion assay.

The cytokine secretion assay involved the following steps:

1) PBMC were washed by adding 1 ml of cold buffer and centrifuged at 1500 rpm for 5 minutes at 4°C; after centrifugation supernatant was pipette off completely.

2) PBMC were resuspended in 90 µl of cold buffer per 10^6 total cells, adding 10 µl of a cytokine-specific catch reagent (cytokine-specific 'catch' antibody, conjugated with a CD45-specific monoclonal antibody that labels all leucocytes equally) and incubated for 5 minutes on ice. Afterwards, the cells were resuspended in 14 ml of warm medium (RPMI complete medium with 5% human AB serum) for 45 minutes at 37°C to allow cytokine secretion. The secreted cytokine binds to the cytokine-specific Catch Reagent on the secreting cells; during this step each tube was rotated every 5 minutes to resuspend settled cells. The cytokine secretion was stopped by placing each tube on ice. Cells were washed with a cold buffer and centrifuged at 1,500 rpm for 10 minutes at 4°C. After centrifugation, cell pellet was resuspended in 90 µl of cold buffer and the cytokines were subsequently labeled with 10 µl of a cytokine-specific "Detection" antibody, which is conjugated to a fluorochrome like phycoerythrin (PE) (Fig. 11).

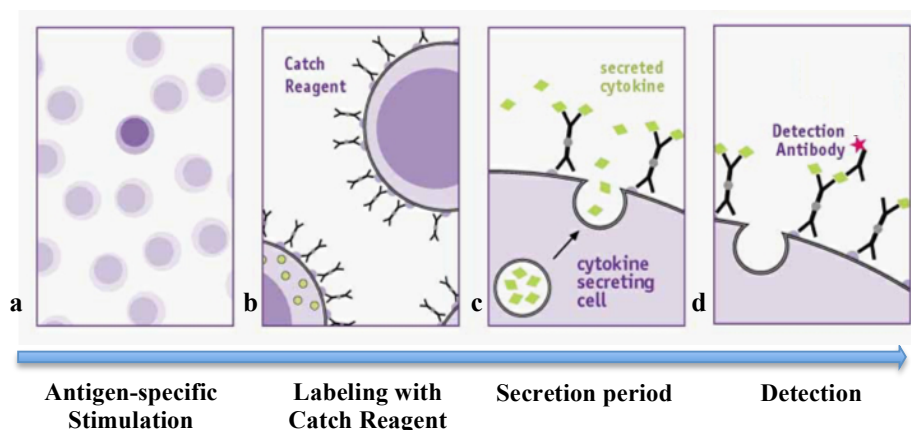


Fig 11. Schematic representation of Cytokine Secretion Assay: after a short antigen specific restimulation (a) a cytokine specific Catch Reagent is attached to the cell surface of all cells (b). The cells are then incubated for 45 minutes at 37°C to allow cytokine secretion (c). The secreted cytokine binds to the cytokine-specific Catch Reagent on the secreting cells and is subsequently labeled with a second cytokine-specific "Detection" antibody, which is usually conjugated to a fluorochrome like phycoerythrin (PE) for sensitive analysis by flow cytometry (d).

Cells stained with cytokine-specific detection antibody for IL-5, IL13, IL-10 or IFN- γ , were further stained with anti-CD4 FITC and anti-CD14 PerCP antibodies in 100 μ l of FACS buffer (1X PBS, 1% FBS, 0.1% sodium azide).

Labeled cells were incubated for 20 minutes in the dark at 4°C and analyzed by flow cytometry.

Before FACS analysis, 1 μ l of Propidium Iodide (PI) (0.5 mg/ml, Sigma) was added to each sample to distinguish between live and dead cells. The dead cells and monocytes were excluded according to PI and CD14-PerCP staining.

7. Immunophenotyping of CD4⁺ CD25⁺⁺ cells

PBMC from four allergic patients were cultured in RPMI complete medium with 10% FCS and seeded in 24 well tissue culture plate at a final concentration of 1 X 10⁶ cells/ml.

PBMC were incubated with the antigens, prepared as described before, at 10 μ g/ml for 8 days at 37°C and 5% CO₂. The control sample was treated as the antigen-stimulated sample except for antigen addition. Cells were harvested every day, washed by adding 1 ml of cold FACS buffer and centrifuged at 1500 rpm for 5 minutes at 4°C; after centrifugation supernatant was pipetted off completely.

PBMC were stained with anti-CD4 FITC and anti-CD25 PE.

8. Immunophenotyping of CD4⁺ CD25⁺⁺ CD127⁻ T regulatory cells

PBMC from five allergic patients were cultured as described in the previous paragraph. PBMC were incubated with the antigens, at 10 μ g/ml, for 8 days at 37°C and 5% CO₂. Cells were harvested at 6 days, 7 days and 8 days and washed by adding 1 ml of cold FACS buffer.

PBMC were stained with anti-CD4 PerCP and anti-CD25 PE, anti-CD127 FITC.

9. Immunophenotyping of CD4⁺ CD25⁺⁺ GARP⁺ and CD4⁺ CD25⁺⁺ LAP⁺ T regulatory cells

PBMC from four allergic patients were cultured as previously described. PBMC were incubated with the antigens, at 10 μ g/ml, for 3 days at 37°C and 5% CO₂. Cells were harvested at 2hrs, 4hrs, 6hrs, 18hrs, 1day, 2days and 3days and washed by adding 1 ml of cold FACS buffer.

PBMC were stained with anti-CD4 PerCP and anti-CD25 FITC, anti-GARP PE or anti-LAP PE.

10. Multiparametric analysis of CD4⁺ CD25⁺⁺ GARP⁺ LAP⁺ T regulatory cells

PBMC from three allergic patients were cultured as previously described and were incubated with the antigens, at 10 µg/ml, for 6hrs. Cells were harvested at 2hrs and 6hrs and washed as previously described by adding 1 ml of cold FACS buffer.

PBMC were stained with anti-CD4 PerCP and anti-CD25 FITC, anti-GARP APC and anti-LAP PE

11. Immunophenotyping of CD4⁺ CD39⁺ GARP⁺ and CD4⁺ CD39⁺ LAP⁺ T regulatory cells.

PBMC from two allergic patients were cultured and incubated as previously described for 8 days. Cells were harvested for a short time course at 2hrs, 4hrs, 6hrs for a longer time course each day until day 8. Each time, PBMC were stained by adding anti-CD4 PerCP and anti-CD39 FITC, anti-GARP PE or anti-LAP PE.

12. Immunophenotyping of CD4⁺ CD25⁺⁺ PD1⁺ cells

PBMC from two allergic patients were cultured and incubated as previously described for 8 days. Cells were harvested for a short time course at 2hrs, 4hrs, 6hrs for a longer time course each day until day 8. Each time, PBMC were stained by adding anti-CD4 PerCP and anti-CD25 FITC and anti-PD1 PE.

13. CD4⁺CD25⁺⁺ CD127⁻ Tregs isolation from human PBMC

The isolation of CD4⁺CD25⁺⁺CD127⁻ Tregs from human PBMC was performed following a two-step procedure or sequential sorting: depletion followed by a positive selection. This strategy is useful for isolation of extremely rare cell population, which express CD4, CD25 with high intensity and does not express CD127. It can be useful first to deplete non-target cells, which express the same antigen used for successive positive selection of the target cells. Positive selection can then be carried out with the pre-enriched fraction to obtain a pure cell population. Therefore, during the first magnetic labeling, all non-CD4⁺ and CD127^{high} cells were labeled with a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and CD235a, as a primary labeling reagent. Then, these cells were magnetically labeled with anti-biotin monoclonal antibodies conjugated to Micro Beads, as a secondary labeling

reagent (Fig. 12 a). The labeled cells were subsequently depleted by separation over a LD MACS Column (Miltenyi Biotec), which was placed in the magnetic field of a MACS Separator (Miltenyi Biotec). The unlabeled cells pass through the column and therefore CD4⁺ T cells were collected as a negative fraction. Hence the first step allows the depletion of non-CD4⁺ cells (Fig. 12 b). In the second step, the CD4⁺ CD25⁺⁺ CD127⁻ Tregs are directly labeled with anti-CD25 antibodies conjugated to Micro Beads (Miltenyi Biotec).

These were isolated by a positive selection from the pre-enriched CD4⁺ T cell fraction through separation over a MS MACS Column (Miltenyi Biotec), placed in a magnetic field (Fig. 12 c).

The unlabeled CD4⁺ CD25⁻ CD127^{high} T cells passed through the column and were collected as a negative fraction. After removing the column from the magnetic field, the magnetically retained CD4⁺CD25⁺⁺CD127⁻ Tregs were eluted as the positively selected cell fraction (Fig. 12 d). In particular, PBMC were isolated as described. PBMC from six were incubated with antigens, prepared as described at 10 µg/ml for 8 days. A negative control sample was treated as the antigen-stimulated sample but without the addition of antigen. After 8 days, the cells were collected and resuspended in 14 ml of HBSS supplemented with 1X penicillin/streptomycin. Cell suspension was centrifuged at 1,500 rpm for 10 minutes at 4°C. After centrifugation, the cell pellet was resuspended in 90 µl of HBSS, supplemented with 1X penicillin/streptomycin. The cells were

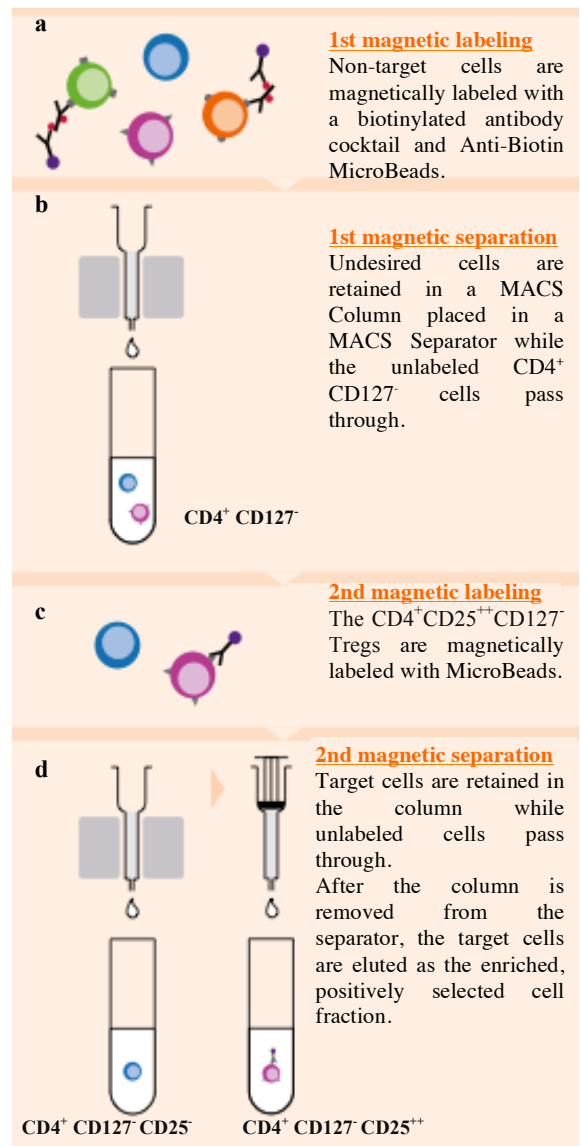


Fig 12. Schematic representation Magnetic Sequential Sorting: two-step procedure, depletion followed by positive selection.

- 1) Depletion of non-CD4⁺ T cells: After a long antigen specific restimulation PBMC are magnetically labeled (a) and labeled cells were subsequently depleted by separation over LD MACS Column (b).
- 2) Positive selection of CD4⁺CD25⁺⁺ cells: Indirect magnetic labeling of CD25⁺⁺ cells with Anti-PE MicroBeads (c) and isolation on MS MACS Column (d).

subsequently labeled with 10 μ l of CD4⁺ T Cell Biotin-Antibody Cocktail per 10⁷ total cells. Each sample was mixed and incubated for 5 minutes on ice. After that, each sample was magnetically labeled with 20 μ l of anti-biotin monoclonal antibodies conjugated to Micro Beads, mixed well and incubated for an additional 10 minutes on ice. After the magnetic labeling, 1ml of buffer were added to each cell suspension and proceeded to the magnetic separation.

First of all, LD MACS Columns were placed in the magnetic field of MACS Separator and prepared by washing with 2 ml of HBSS buffer. After this step, the magnetic labeled cells were applied onto the column. The column was washed with 2 x 1 ml of HBSS buffer and unlabeled cells that passed through it were collected. These cells represented the unlabeled pre-enriched CD4⁺ cell fraction.

In order to isolate CD4⁺CD25⁺⁺CD127⁻ Tregs from human PBMC, unlabeled pre-enriched CD4⁺ cell fraction was centrifuged at 1,500 rpm for 10 minutes at 4°C. After centrifugation, the cell pellet was resuspended in 90 μ l of HBSS, and the cells were subsequently labeled with 10 μ l of anti-CD25 Micro Beads. The magnetically labeled was processed as previously described and applied onto a MS MACS column. The column was washed with 3 x 0.5 ml of HBSS buffer and the unlabeled cells passed through the column and the flow-through contained the unlabeled CD4⁺CD25⁻CD127^{high} T cells. After that, 1 ml of HBSS buffer was applied onto the column, which was then removed from the magnetic separator, and the magnetically labeled CD4⁺CD25⁺⁺CD127⁻ cells were immediately flushed out by firmly pushing the plunger into the column. The purity of the enriched cell fractions was above 70%, as determined by flow cytometry. The two cell fractions were stained with anti-CD4 PerCP and anti-CD25 PE.

14. TRIzol RNA extraction

For further functional characterization the CD4⁺CD25⁺⁺CD127⁻ cell fraction obtained from six allergic patients were collected after magnetic separation and resuspended in 1 ml of TRIZOL Reagent.

During sample homogenization or lysis, TRIZOL Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components.

The samples were centrifuged at 11,500 rpm for 10 minutes at 4°C to remove the cell debris and the supernatants were transferred into a new tube. Afterwards the samples were incubated for 5 minutes at room temperature to leave complete dissociation of nucleoprotein complexes.

200 µl of chloroform per 1 ml of TRIZOL Reagent were added and each sample was mixed vigorously for 15 seconds and incubated at room temperature for 2 minutes. Afterwards the samples were centrifuged at 12,000 rpm for 5 minutes at 4°C to induce a phase separation where the proteins were extracted into lower red, phenol-chloroform phase, the DNA was extracted in an interphase and RNA remained in a colorless upper aqueous phase.

After transferring the aqueous phase, the RNA was recovered by precipitation with 500 µl of isopropyl alcohol. The samples were incubated at room temperature for 10 minutes and then centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant was removed completely and the pellet was washed with 1 ml of 75% ethanol at 9,000 rpm for 5 minutes at 4°C.

The RNA pellet was resuspended in 10 µl of RNase-free water and stored at -80°C. RNA integrity is critical for a successful RNA quantitation; it was evaluated using a 1% agarose gel electrophoresis (data not shown). Concentration and purity of RNA were determined by measuring the absorbance in a spectrophotometer. The RNA concentration was determined by measuring the absorbance at 260 nm; an absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml.

The ratio of the absorbance at 260 nm and 280 nm (A₂₆₀/A₂₈₀) provides an estimate of the purity of RNA with respect to contaminants.

15. cDNA synthesis

After RNA extraction from CD4⁺CD25⁺CD127⁻ cell, the cDNA synthesis was performed using the QuantiTect Reverse Transcription cDNA kit according to the manufacturer's instructions (Qiagen). This kit provides a fast procedure for efficient transcription and effective genomic DNA elimination.

Briefly, the reaction was performed with 1 µg total RNA, the purified RNA sample was incubated in 2 µl gDNA Wipeout Buffer 7X at 42°C for two minutes and then placed immediately on ice.

This step allowed the genomic DNA elimination. The reverse transcription step was performed using a Master Mix prepared from 1 µl Quantiscript Reverse Transcriptase, 4 µl Quantiscript RT buffer 5X and 1 µl RT Primer Mix. The Master Mix was added to each RNA sample and the entire reaction was incubated at 42°C for 15 minutes and was then inactivated at 95°C for 3 minutes. Afterwards, the reaction was diluted in 80 µl of RNase-free water and stored at -20°C.

16. Real Time-PCR

Real-time PCR is a form of polymerase chain reaction (PCR) where the progress of reaction can be monitored as it occurs and allows to calculate the cDNA target quantity in a sample. To improve the characterization of CD4⁺CD25⁺⁺CD127⁻ Treg cells isolated as previously described, the cDNA obtained from six allergic patients was analyzed for the expression of FOXP3, IL-10, GARP and TGF- β 1. cDNA expression for each sample was standardized using the 18S ribosomal RNA.

Quantitative real time-PCR was performed in a STEP ONE PLUS (Applied Biosystems) using a Fast SYBR Green PCR kit (Applied Biosystems) and specific primers for the different analyzed target. PCR was carried out in 20 μ l.

For each sample the following mixture was prepared:

- appropriate amount of cDNA,
- 10 μ l FAST SYBR Green Master Mix (2x),
- 0.2 μ l primers 10 μ M,
- H₂O to a final volume of 10 μ l.

Gene	Provider	Catalog number	UniGene	Refseq Accession	Band size (bp)	Reference position
<i>18SrRNA</i>	<i>Qiagen</i>	<i>PPH05666E</i>	<i>N/A</i>	<i>X03205</i>	<i>100</i>	<i>1447</i>
<i>FOXP3</i>	<i>Qiagen</i>	<i>PPH00029C</i>	<i>Hs.247700</i>	<i>NM_014009</i>	<i>89</i>	<i>2191</i>
<i>IL-10</i>	<i>Qiagen</i>	<i>PPH00572C</i>	<i>Hs.193717</i>	<i>NM_000572</i>	<i>63</i>	<i>168</i>
<i>GARP</i>	<i>Qiagen</i>	<i>PPH12836A</i>	<i>Hs.151641</i>	<i>NM_005512</i>	<i>178</i>	<i>486</i>
<i>TGF-β1</i>	<i>Qiagen</i>	<i>PPH00508A</i>	<i>Hs.645227</i>	<i>NM_000668</i>	<i>91</i>	<i>1477</i>

Table 2 – References of primers used for Real Time - PCR

The relative changes in gene expression were analyzed by $\Delta\Delta C(t)$ method. Cycling conditions were: initialization 20 sec at 95°C followed by 40 cycles of 3 sec at 95°C and 30 sec at 60°C. After the cycling process, a melting curve analysis was performed to exclude unspecific PCR products and established the purity of the amplified genes.

The melting curve analysis (65°C to 95°C, step 0.5°C) reveals the presence of non specific annealing products as primer dimers and secondary non specific amplicon which can result from primer elongation or non specific annealing events.

17. Statistical analysis

All the statistical analyses were performed using the StatView 5.0.1 software. A Wilcoxon test for comparisons between the two recruited groups was applied. Student's paired t-test was used for comparisons in allergic subjects. $p < 0.05$ was accepted as statistically significant.

Results

In order to study the immunological mechanisms activated by the major allergens of *Parietaria judaica*, Par j 1 and Par j 2, and to investigate the differences between the wild type allergens and the hypoallergenic mutant rPjEDcys, the recombinant proteins were produced and were utilized to stimulate human Peripheral Blood Mononuclear Cells (PBMC) from *Parietaria judaica* allergic patients. Flow cytometry provides a better way to characterize cells of innate and adaptive immune system, based on morphological characterization (forward and side scatter) and on the expression of cellular markers. Therefore, staining of cells with antibodies has been used to identify, count and characterize different cell subset of PBMC from allergic patients, based on the expression of specific cell surface antigens.

1. Production of recombinant proteins: rPar j1, rPar j 2 and rPjEDcys

The recombinant proteins rPar j 1, rPar j 2 and rPjEDcys used in the following experiments were expressed as His-tagged proteins in *E. coli* and isolated by following different purification steps using various chromatographic techniques as described in "Materials and Methods".

Briefly, the recombinant allergens were first purified by affinity chromatography using His-Tag Columns and then eluted in a buffer containing imidazole. The total protein content was analyzed by the colorimetric Bio-Rad protein assay and aliquots from eluted fractions were analyzed by SDS-polyacrylamide gel and stained with the Coomassie Brilliant Blue dye. Gel analysis was performed using the image analysis system Chemidoc (Bio-Rad). The fractions containing recombinant proteins were diluted and purified in a second step of affinity chromatography followed by dialysis to dilute or remove urea from the samples.

In order to completely remove the urea from the preparation and to allow a complete refolding of the proteins, the recombinant proteins were purified by affinity chromatography under non-denaturing conditions. During a successive step, salts were removed from the fractions containing recombinant proteins and a gel filtration chromatography step was performed. Finally, in order to remove endotoxin from the recombinant protein preparations (all reagents used for cell culture have to be endotoxin-free), the fractions containing the highest concentration of recombinant protein were subjected to the last purification step consisting of a Polymyxin B affinity chromatography.

The purified proteins were accurately quantified; the relative concentrations were measured using the image analysis system Chemidoc (Bio-Rad) and the Quantity One program (Fig. 13).

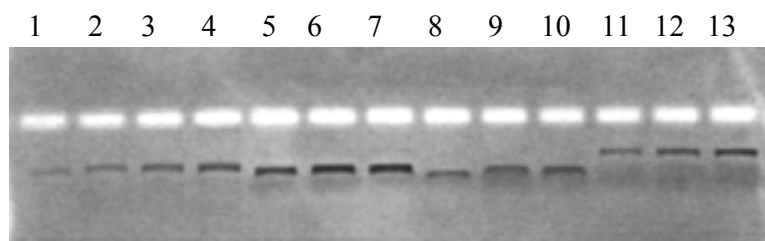


Fig. 13. Proteins eluted from detoxi-gel column were analyzed by SDS-PAGE gel stained with Coomassie blue. Lane 1: BSA 250 ng, lane 2: BSA 500 ng, lane 3: BSA 750 ng, lane 4: BSA 1000 ng; lanes 5-7: 3 μ l, 4 μ l and 5 μ l of rPar j 1, lanes 8-10: 3 μ l, 4 μ l and 5 μ l of rPar j 2; lanes 11-13: 3 μ l, 4 μ l and 5 μ l of rPjEDcys.

Recombinant proteins were tested for the endotoxin content using the multi-test limulus amoebocyte lysate (LAL) test. The endotoxin content in the wild-type allergens and hypoallergenic derivative rPjEDcys was less than 0.003 ng LPS/ μ g of recombinant protein.

Figure 14 shows the Coomassie Brilliant Blue-stained recombinant proteins, rPar j 1, rPar j 2 and rPjEDcys.

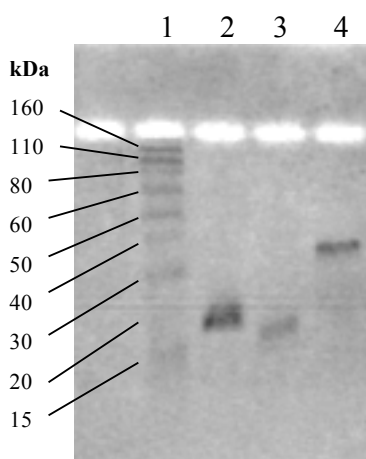


Fig. 14. Protein extracted from recombinant *E. coli* strains were analyzed by SDS-PAGE gel stained with Coomassie Brilliant blue. Lane 1: Novex Sharp Prestained Protein Standard, lane 2: rPar j 1, lane 3: rPar j 2, lane 4: rPjEDcys.

2. Human PBMC proliferation assay: innate and adaptive immune response to *Parietaria major* allergens and the hypoallergenic derivate

A recently published paper from Bonura et al. showed that rPjEDcys was able to stimulate CD3⁺ cell proliferation in allergic patients. Therefore, I wanted to investigate which cell subpopulation, within CD3⁺ cells, proliferated in response to the mixture of rPar j 1 and rPar j2 allergens and rPjEDcys hybrid.

To do this, PBMC from 10 allergic patients to *Parietaria judaica* pollen were labeled with CFDA-SE and cultured at 10⁶ cells/ml without any antigenic stimuli (negative control) or with the following stimuli: a mixture of recombinant allergen Par j 1 and Par j 2 (1 and 10 µg/ml) or rPjEDcys (1 and 10 µg/ml). After 7 days of incubation cells were collected and stained.

Initially, PBMC from the first 3 allergic patients were stained with anti-CD4 and anti-CD8. Our results have shown that rPjEDcys retains T cell reactivity, inducing a CD4⁺ proliferative response as the mixture of rPar j 1 and rPar j 2, but not CD8 proliferation. However, particularly interesting data emerged by analyses on CD4⁻ and CD8⁻ proliferating cells.

Although CD4⁻ cells did not proliferate after antigen-specific stimulation, CD8⁻ cells proliferated in response to the mixture of wild-type allergens and rPjEDcys hybrid. The role of the adaptive immune system in allergy has been well characterized but the role of the innate immune system in the development of allergies is a controversial area. Some papers describe how the innate immune system might contribute to the development of allergies [117-118]. Therefore, I wanted to investigate whether innate immune cells proliferate in response to the mixture of wild type allergens and to rPjEDcys. In order to do this, I decided to carry out a more detailed analysis that allowed me to characterize the CD8⁻ proliferating cells, highlighting a CD8⁻ proliferative response higher than CD4⁺ one. Therefore, in the other 7 allergic patients the staining was performed with anti-CD4, anti-CD8, anti-CD16 and anti-CD56. To study cell proliferation, analyzed cells were selected by a live lymphocyte gate based on the combination of forward (FSC) and side scatter (SSC) (data not shown). Background proliferation was measured in an unstimulated culture. In every experiment described in this thesis, we studied the immune response after antigen specific *in vitro* stimulation compared always to an unstimulated sample.

2.1 Cytofluorimetric analysis on CD4⁺CFSE⁺ cells

A detailed analysis on CD4⁺ cell proliferation in response to the mixture of allergens and rPjEDcys for a representative patient (patient 7) is shown in panels a-f of figure 15.

PBMC stimulation with two different antigen doses of the mixture of *Parietaria* allergens, 1 and 10 µg/ml, showed a dose-dependent increase of the percentage of CD4⁺ proliferating cells. The data reported in figure 15 also indicates that PBMC stimulation with rPjEDcys showed an increase in a percentage of CD4⁺ proliferating cells.

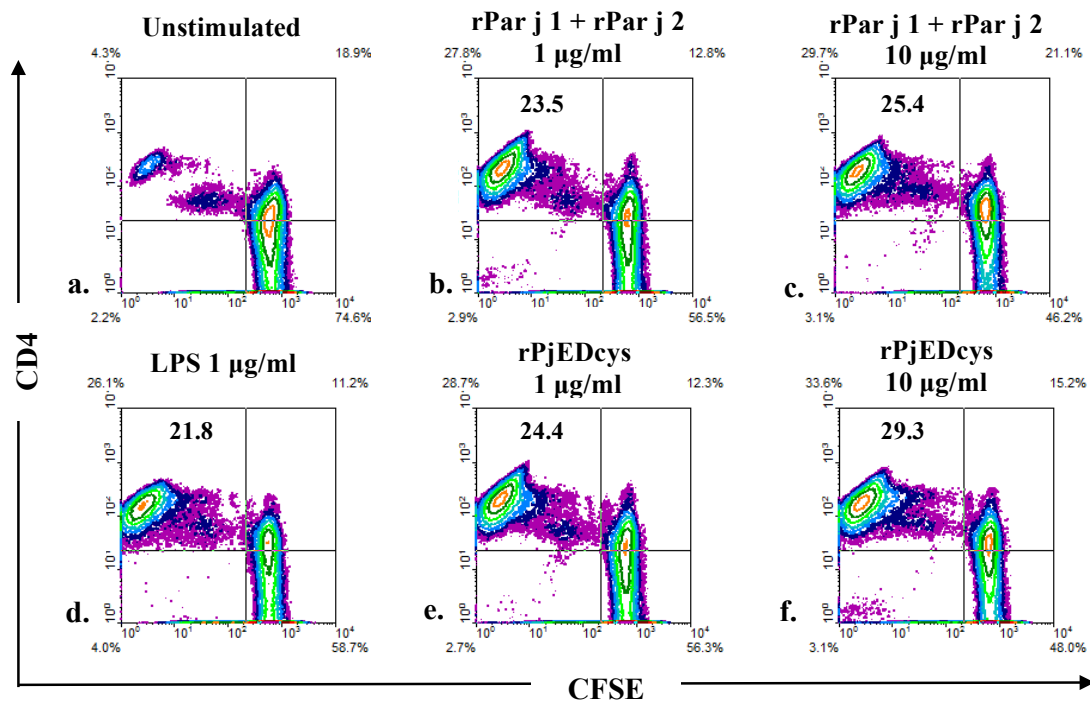


Fig. 15. Representative dot plot of CD4⁺CFSE⁺ proliferation assay. Panels show cytofluorimetric analyses on a representative patient (patient 7) Unstimulated CFSE-labeled PBMC (a.). CFSE-labeled PBMC stimulated with 1 and 10 µg/ml of a mixture containing the rPar j 1 and rPar j 2 allergens (b. and c.) and an equivalent concentration of rPjEDcys hybrid (e. and f.). CFSE-labeled PBMC stimulated with LPS (d.). Background proliferation was measured in unstimulated culture.

The data reported in figure 16 show the percentage of CD4⁺ proliferating cells in all the 10 *P. judaica* allergic patients after antigen-specific stimulation.

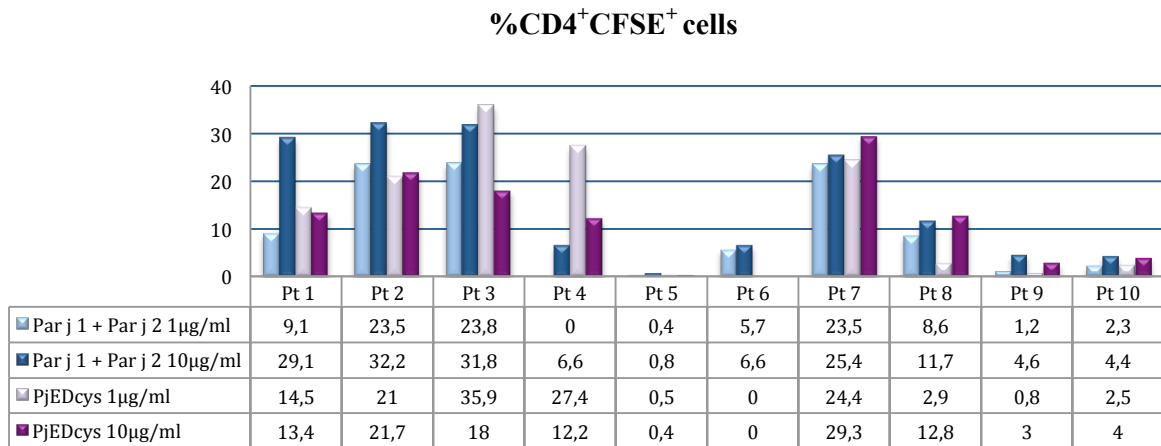


Fig. 16. Histograms of percentage of CD4⁺ Proliferating cells. Histogram and table show the percentage of CD4⁺ proliferating cells in response to the mixture of allergens and rPjEDcys. The values were subtracted of the proliferation obtained in the unstimulated culture.

In 1 out of the 10 analyzed patients (patient 4), the mixture of wild-type allergens, used at 1 µg/ml, did not show a higher percentage of proliferating CD4⁺ cells than the unstimulated sample. However, in all the other analyzed patients, PBMC stimulation with the two different antigen doses of Par j 1 and Par j 2 allergens, showed a dose-dependent increase in the percentage of CD4⁺ proliferating cells. All patients, except one (patient 6), showed an increase in percentage of proliferating CD4⁺ cells in response to rPjEDcys. In five out ten patients (patients 1-5) the percentage of CD4⁺ proliferating cells in response to rPjEDcys is higher when used at lower concentration.

2.2 Cytofluorimetric analysis on CD8⁺CFSE⁺ cells

Analyses about the CD8⁺ cell proliferative response to the mixture or the hypoallergenic hybrid, are shown in panels **a-f** of figure 17. This representative experiment (patient 7) highlighted that CD8⁺ cell population did not show a significant proliferation in response to both antigens.

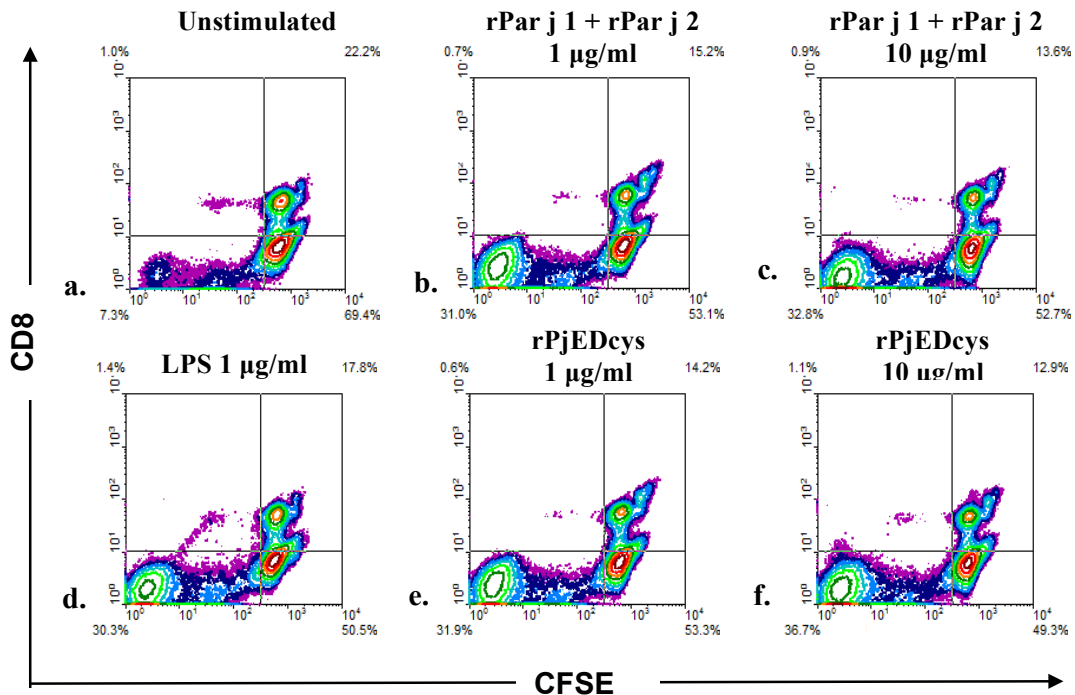


Fig. 17. Representative dot plot of CD8⁺CFSE⁺ proliferation assay. Panels show cytofluorimetric analyses on a representative patient (**patient 7**) Unstimulated CFSE-labeled PBMC (**a.**) CFSE-labeled PBMC stimulated with 1 and 10 µg/ml of a mixture containing the rPar j 1 and rPar j 2 allergens (**b.** and **c.**) and an equivalent concentration of rPjEDcys hybrid (**e.** and **f.**). CFSE-labeled PBMC stimulated with LPS (**d.**). Background proliferation was measured in unstimulated culture.

The data reported in figure 18 show the percentage of CD8⁺ proliferating cells in all the 10 P. judaica allergic patients after antigen-specific stimulation.

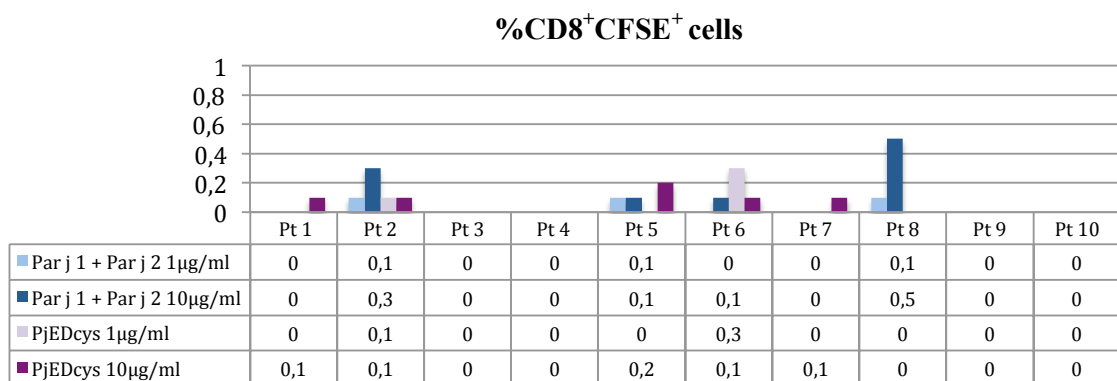


Fig. 18. Histograms of percentage of CD8⁺ Proliferating cells. Histogram and table show the percentage of CD8⁺ proliferating cells in response to the mixture of allergens and rPjEDcys. The values were subtracted of the proliferation obtained in the unstimulated culture.

2.3 Cytofluorimetric analysis on CD16⁺CFSE⁺ cells

Our data showed that other cells, not phenotypically characterized, proliferated in response to the mixture of *Parietaria* allergens and rPjEDcys hybrid. Therefore, I wanted to investigate which other cell subpopulation proliferated after the *in vitro* stimulation with different antigen doses of the rPar j 1 and rPar j 2 allergens and an equivalent concentration of the rPjEDcys hybrid.

I proposed to study the innate immune cell response to *Parietaria* major allergens and hypoallergenic derivative. In order to do this, PBMC were also stained with labeled antibodies against CD16 or CD56 (NK cell markers).

Panels a-f of figure 19 show a more detailed analysis on CD16⁺ cell proliferation in response to the mixture of allergens and to rPjEDcys for a representative patient (patient 7). Staining with anti-CD16 and cytofluorimetric analyses showed that CD16⁺ cells did not proliferate in response to the mixture or rPjEDcys.

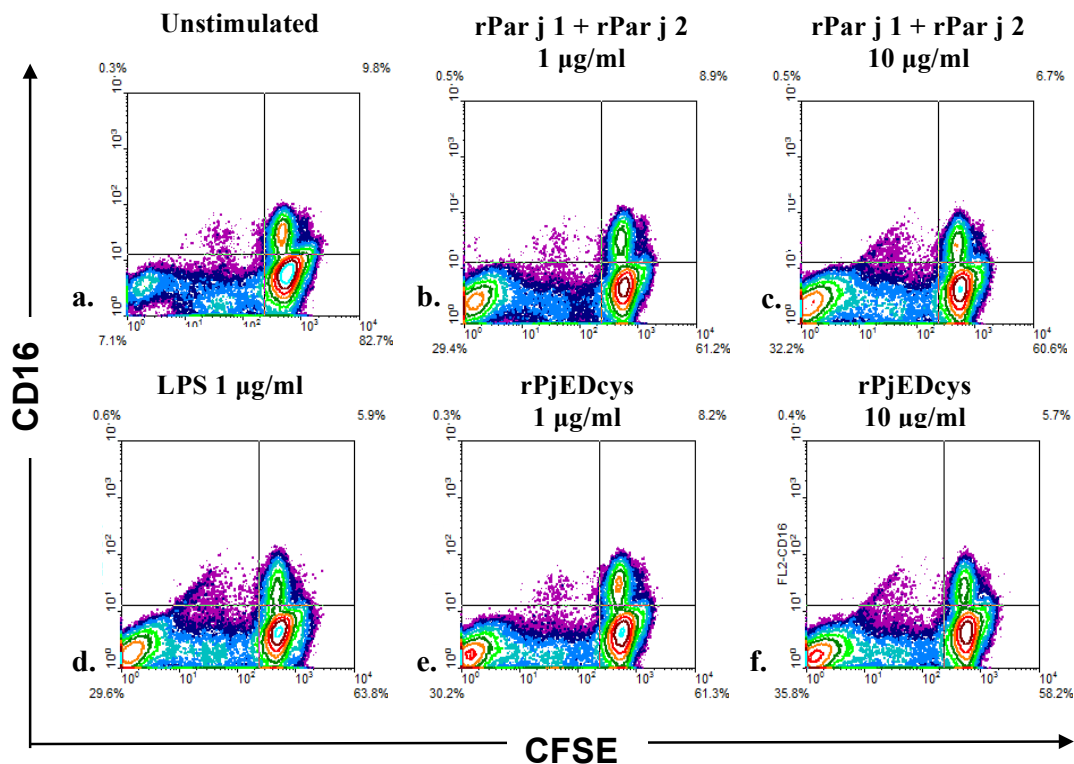


Fig. 19. Representative dot plot of CD16⁺CFSE⁺ proliferation assay. Panels show cytofluorimetric analyses on a representative patient (patient 7) Unstimulated CFSE-labeled PBMC (a.). CFSE-labeled PBMC stimulated with 1 and 10 µg/ml of a mixture containing the rPar j 1 and rPar j 2 allergens (b. and c.) and an equivalent concentration of rPjEDcys hybrid (e. and f.). CFSE-labeled PBMC stimulated with LPS (d.). Background proliferation was measured in unstimulated culture.

The data reported in figure 20, show the percentage in CD16⁺ proliferating cells in 7 allergic patients (from patient 3 to 10) after antigen-specific stimulation. Our analyses demonstrated that CD16⁺ cells did not proliferate if compared to an unstimulated culture.

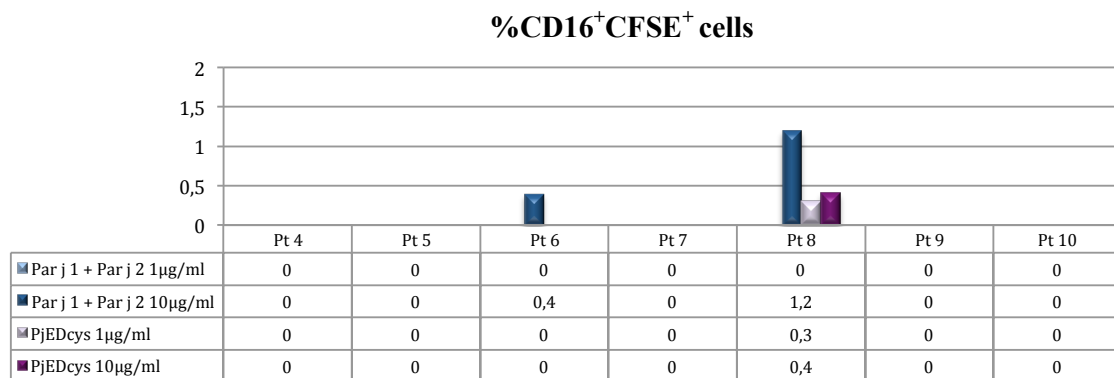


Fig. 20. Histograms of percentage of CD56⁺ proliferating cells. Histograms show the percentage of CD56⁺ proliferating cells in response to the mixture of allergens and rPjEDcys subtracted of the proliferation obtained in the unstimulated culture.

2.4 Cytofluorimetric analysis on CD56⁺CFSE⁺ cells

A detailed analysis on CD56⁺ cell proliferation in response to the mixture of allergens and rPjEDcys for a representative patient (patient 7) is shown in panels **a-f** of figure 21. Cell staining with anti-CD56 and cytofluorimetric analyses showed that allergen stimulation resulted in a low increase in percentage of CD56⁺ cells. The data reported in the panels of figure 21 indicates that PBMC stimulation with the two different antigen doses of the mixture induced a dose-dependent increase in percentage of CD56⁺ proliferating cells compared to an unstimulated cells. Moreover, also PBMC stimulation with rPjEDcys hybrid showed a dose-dependent increase in percentage of CD56⁺ proliferating cells compared to an unstimulated sample.

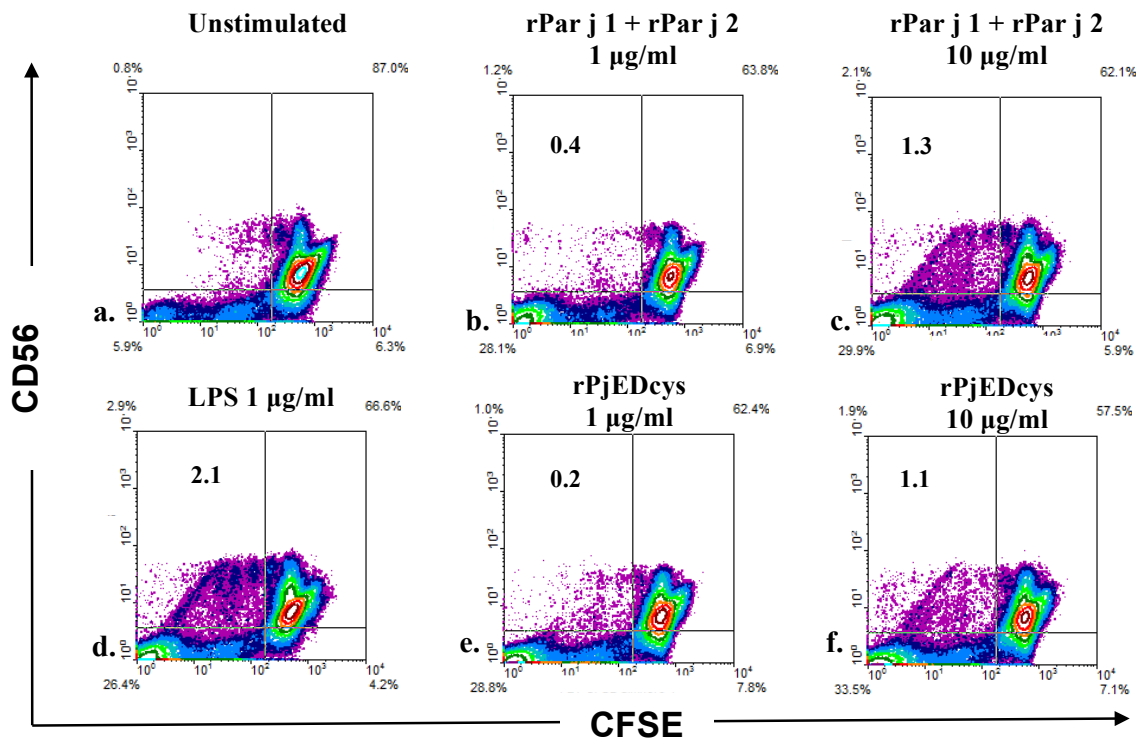


Fig. 21. Representative dot plot of CD56⁺CFSE⁺ proliferation assay. Panels show cytofluorimetric analyses on representative patient (patient 7) Unstimulated CFSE-labeled PBMC (a). CFSE-labeled PBMC stimulated with 1 and 10 µg/ml of a mixture containing the rPar j 1 and rPar j 2 allergens (b. and c.) and an equivalent concentration of rPjEDcys hybrid (e. and f.). CFSE-labeled PBMC stimulated with LPS (d.). Background proliferation was measured in unstimulated culture.

The data reported in figure 22, show the percentage in CD56⁺ proliferating cells in 7 allergic patients (from patient 3 to 10). These data demonstrate CD56⁺ cells proliferated after the stimulation with the mixture of allergens and to the hypoallergenic hybrid in a dose dependent manner even though at low levels (Fig. 22).

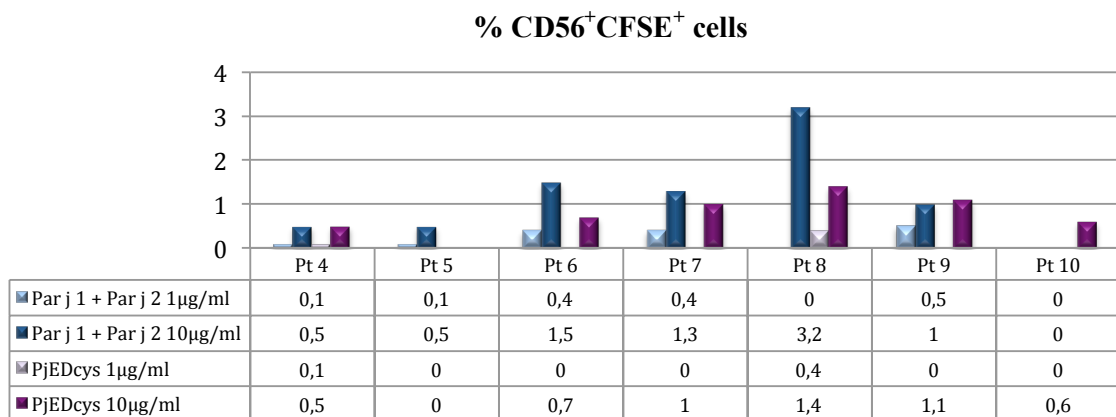


Fig. 22. Histograms of percentage of CD56⁺ proliferating cells. Histograms show the percentage of CD56⁺ proliferating cells in response to the mixture of allergens and rPjEDcys subtracted of the proliferation obtained in the unstimulated culture.

PBMC proliferation assays showed that CD4⁺ cells are clearly the major cell population proliferating in response to the mixture of wild-type allergens and to the rPjEDcys. Moreover, these studies, combined with other experimental data, showed that the mixture of allergens and rPjEDcys hybrid are able to induce proliferation of CD56⁺ cells and suggested that innate immune cells are associated with allergic response.

3. IL-5, IL-13, IL-10 and IFN- γ cytokine secretion assay after PBMC stimulation with wild type allergens and rPjEDcys

Our results showed that CD4⁺ cells are the main proliferating cell population in response to the mixture of Par j 1 and Par j 2 allergens. Furthermore, the hypoallergenic derivate rPjEDcys retained the ability to stimulate CD4⁺ cell proliferation. The recognition of antigens by memory CD4⁺ T cells initiates an immunological response characterized by the rapid secretion of several different cytokines. Therefore, I decided to study the cytokine production by CD4⁺ T cells after an antigen-specific *in vitro* stimulation.

I was interested in analyzing four different cytokines released by PBMC of allergic patients, in response to the mixture of wild type allergens and to the rPjEDcys hybrid.

I set up an experimental design that could allow me to investigate if there were any differences regarding the Th1/Th2 profile induced by rPjEDcys hybrid in comparison to the wild type allergens. In particular, I decided to look at some of the most important inflammatory and regulatory cytokines such as IL-5 and IL-13, which are Th2 cytokines that play a critical role in the development of allergy. IFN- γ , the main Th1 effector cytokine, and IL-10 which is an important cytokine with anti-inflammatory and suppressive functions.

To accomplish this experimental design, I chose to carry out a cytokine secretion assay.

PBMC from 10 allergic patients were stimulated *in vitro* for 16 hrs with a mixture of wild type allergens and rPjEDcys hybrid at the final concentration of 20 μ g/ml. The co-stimulatory antibodies (anti-CD28/49d) were added in all samples to enhance the T cell response. A negative control sample was treated exactly the same as the antigen-stimulated sample but without the addition of antigen; this control was included to evaluate the spontaneous cytokine secretion.

The cytokine secreting cells were stained with anti-CD4 and dead cells were excluded by staining with propidium iodide (PI), to reduce nonspecific background staining and increased sensitivity.

Moreover, for an optimal sensitivity I labeled monocytes with anti-CD14.

Therefore, analyzed cells were selected by a lymphocyte gate (R1) based on the combination of FSC and SSC (as shown in Fig. 23 A). An R2 gate selected cytokine-secreting cells, excluding dead cells and monocytes in accordance to PI and CD14 staining in a FL-2-cytokine versus an FL-3-CD14/PI plot (as shown in Fig. 23 B).

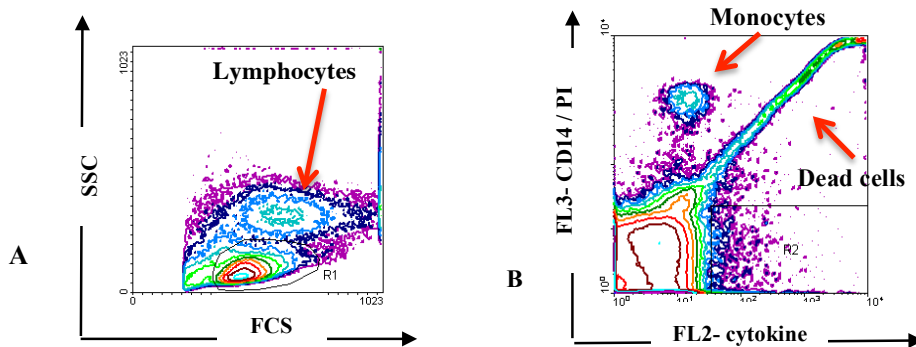


Fig. 23. Detection and isolation of cytokines secreting cells. A. Cells were selected by a lymphocyte gate based on the combination of forward (FSC) and side scatter (SSC) B. Cytokine secreting cells were selected by R2 gate and dead cells and monocytes were excluded according to PI and CD14 staining in a FL-2 versus FL-3 plot

Panels of figures 24-27 show data for a representative experiment (patient 10) of 10 analyzed patients. Background cytokine secretion by CD4⁺ cells was measured in an unstimulated culture. IL-5 secretion assay showed that PBMC stimulation with the mixture of wild type allergens or rPjEDcys hybrid induced an increase in IL-5 secreting allergen-specific T CD4⁺ cells of 22.1% and 1.5% respectively (Fig. 24 b and c).

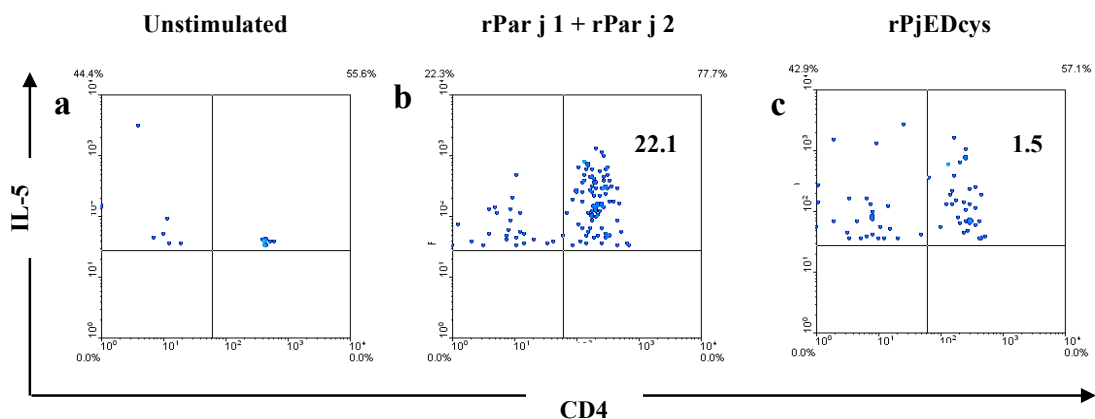


Fig. 24. Representative dot plot of IL-5 Secretion Assay. IL-5 secreting allergen-specific T CD4⁺ cell for a representative patient (patient 10) in unstimulated culture (a) and after a stimulation with a mixture containing the rPar j 1 and rPar j 2 allergens (b) and an equivalent concentration of rPjEDcys hybrid (c). Inside of dot plot b. and c. it is shown the difference between the percentage of IL-5 secreting T CD4⁺ cell after antigen-specific stimulation and the unstimulated culture.

Panels of figure 25 show the percentage of IL-13 secreting T CD4⁺ cells in patient 10. IL-13 secretion assay showed the mixture of wild type allergens induced an increase in percentage of IL-13 secreting T CD4⁺ cells of 9%, instead rPjEDcys hybrid did not induce an increase of IL-13 secreting T CD4⁺ cells (Fig. 25 b and c).

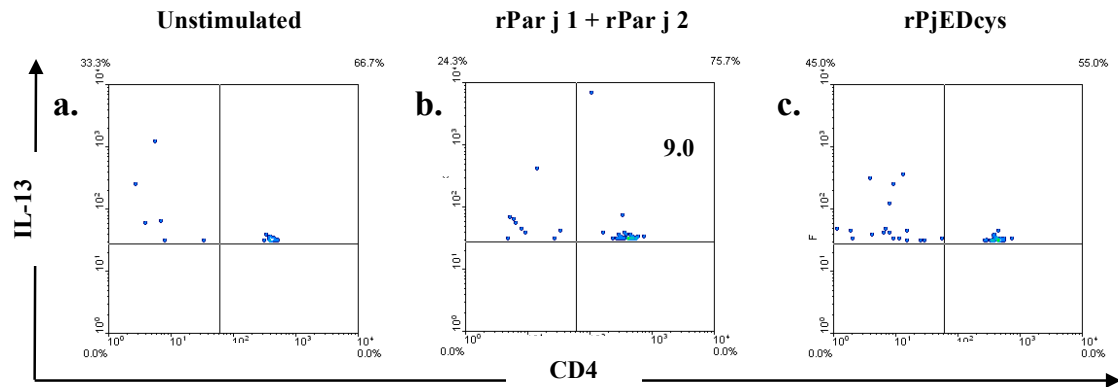


Fig. 25. Representative dot plot of IL-13 Secretion Assay. IL-13 secreting allergen-specific T CD4⁺ cell for a representative patient (**patient 10**) in unstimulated culture (**a**) and after a stimulation with a mixture containing the rPar j 1 and rPar j 2 allergens (**b**) and an equivalent concentration of rPjEDcys hybrid (**c**). Inside of dot plot b. and c. it is shown the difference between the percentage of IL-13 secreting T CD4⁺ cell after antigen-specific stimulation and the unstimulated culture.

Staining with anti-IFN- γ and following flow cytometric analysis, showed that CD4⁺ cell did not produce IFN- γ after stimulation with the mixture. On the contrary, this analysis showed that rPjEDcys hybrid induced a low increase of IFN- γ secreting T CD4⁺ cells of 0.6% (panels a-c of Fig. 26).

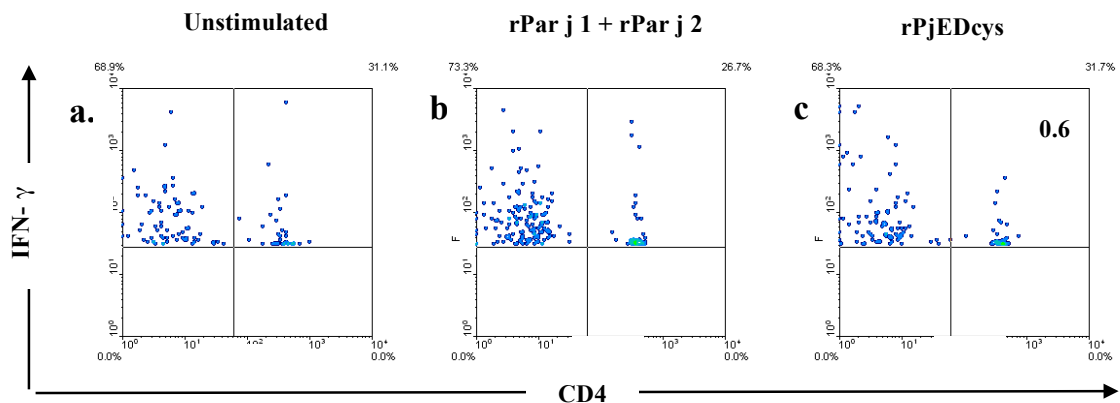


Fig. 26. Representative dot plot of IFN- γ Secretion Assay. IFN- γ secreting allergen-specific T CD4⁺ cell for a representative patient (**patient 10**) in unstimulated culture (**a**) and after a stimulation with a mixture containing the rPar j 1 and rPar j 2 allergens (**b**) and an equivalent concentration of rPjEDcys hybrid (**c**). Inside of dot plot b. and c. it is shown the difference between the percentage of IFN- γ secreting T CD4⁺ cell after antigen-specific stimulation and the unstimulated culture.

Finally, the IL-10 secretion assay, for this representative experiment (patient 10), showed that only PBMC stimulation with the mixture of wild type allergens induced an increased percentage of IL-10 secreting T CD4⁺ cells of 2.5% (panel b of Fig. 27).

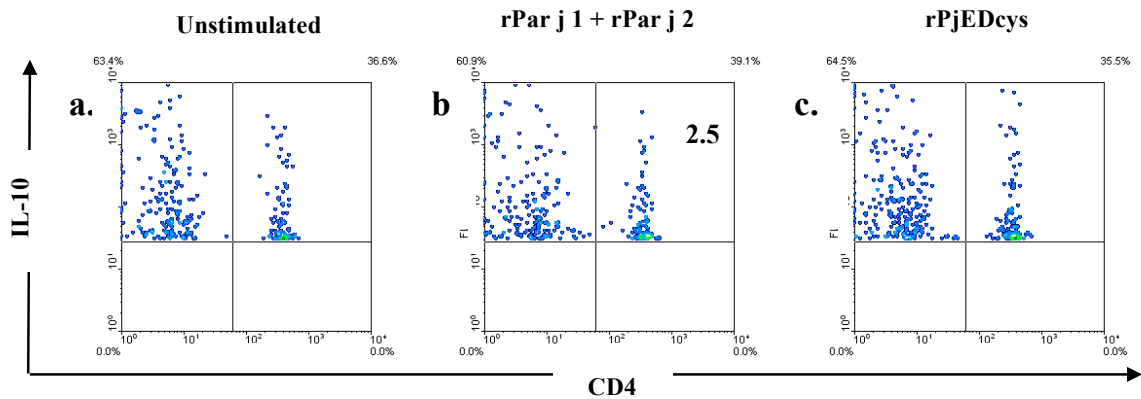


Fig. 27. Representative dot plot of IL-10 Secretion Assay. IL-10 secreting allergen-specific T CD4⁺ cell for a representative patient (**patient 10**) in unstimulated culture (**a**) and after a stimulation with a mixture containing the rPar j 1 and rPar j 2 allergens (**b**) and an equivalent concentration of rPjEDcys hybrid (**c**). Inside of dot plot b. and c. it is shown the difference between the percentage of IL- secreting T CD4⁺ cell after antigen-specific stimulation and the unstimulated culture.

A cytofluorimetric analysis using PBMC from this *P. judaica* allergic patient (patient 10) showed that PBMC stimulation with the hypoallergen induced a lower IL-5 and IL-13 secretion by CD4⁺ cells compared to the mixture. As regards the IFN- γ secretion, our cytofluorimetric analysis have shown that IFN- γ secretion by CD4⁺ cells did not increase in response to the mixture of *Parietaria* allergens, however a low increase of IFN- γ secreting T CD4⁺ cells was observed in response to rPjEDcys hybrid. Moreover, the analyses on patient 10 have indicated that only PBMC stimulation with the mixture induced an increased production of suppressive cytokine IL-10 by T CD4⁺ cells (2.5%).

The data reported in panels A-D of figure 28 show the results of the cytokine secretion assay for each cytokines studied. Each panel shows the percentage of cytokine-secreting CD4⁺ cell for 10 *P. judaica* allergic patients after antigen-specific stimulation subtracted of the cytokine background in the unstimulated culture.

% Cytokine-secreting CD4⁺ cells

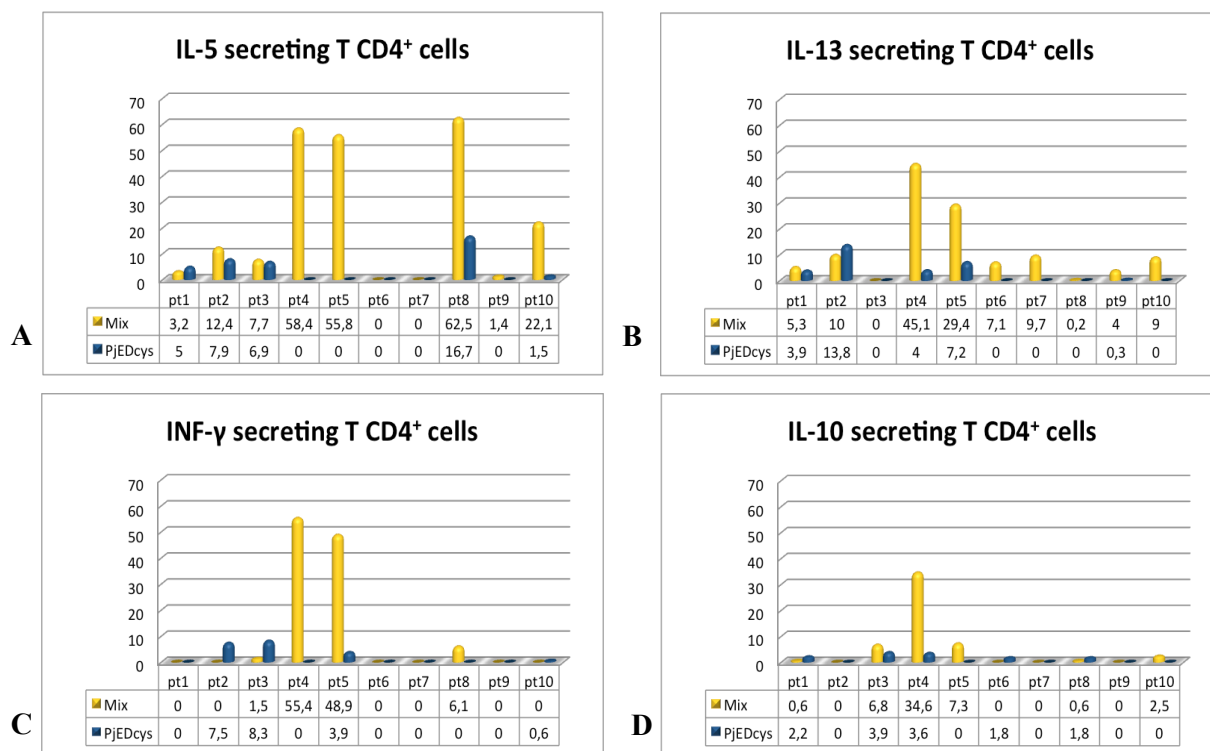


Fig. 28. Histograms show the percentage of cytokines secreting antigen-specific T CD4⁺ cells. Percentage of cytokine-secreting T CD4⁺ cells in response to the mixture of allergens and rPjEDcys subtracted of cytokines background obtained in the unstimulated culture. IL-5 secreting T CD4⁺ cells are showed in panel **A**, IL-13 secreting T CD4⁺ cells are showed in panel **B**, IFN- γ secreting T CD4⁺ cells are showed in panel **C**, IL-10 secreting T CD4⁺ cells are showed in panel **D**.

These results pointed out that in all 10 studied patients, PBMC stimulation with the mixture or with rPjEDcys hybrid showed some differences concerning the cytokine profile produced by CD4⁺ T cells.

In eight out of ten analyzed patients (patients 1-5, 8-10) PBMC stimulation with the mixture of allergens showed an increase in the percentage of IL-5 secreting CD4⁺ cells. In five out of ten analyzed patients (patients 1-3, 8-10) PBMC stimulation with rPjEDcys showed an increase in the percentage of IL-5 secreting CD4⁺ cells, but it is generally lower than that induced by the mixture. While patients 6 and 7 did not show an increase in IL-5 secreting T CD4⁺ cells after the *in vitro* stimulation with the two different antigens. These patients showed a generally weak cytokine secretion by CD4⁺ cells (Fig. 28 A). Panel B of figure 28, shows the percentage of IL-13 secreting T CD4⁺ cells in all ten patients. In nine out of ten analyzed patients (all patients except number 3),

PBMC stimulation with the mixture of wild type allergens induces an increase in percentage of IL-13 secreting T CD4⁺ cells. In five out of ten analyzed patients (patients 1, 2, 4, 5 and 9), also PBMC stimulation with rPjEDcys increases the secretion of IL-13 by CD4⁺ cells, which is less pronounced than that induced by the mixture.

In summary, these cytokine secretion assays have shown that PBMC *in vitro* stimulation with rPjEDcys induces a lower secretion of IL-5 and IL-13 by T CD4⁺ cells than the wild type allergens. These results are very interesting because IL-5 and IL-13 are Th2 cytokines with a critical role in the development of allergy and actually many studies identify them as a primary therapeutic target for the improvement of allergic symptoms [63-66].

Differences between the cytokine secretion in response to the mixture and PjEDcys were tested using the Wilcoxon test and have been described as p-value (Fig. 29).

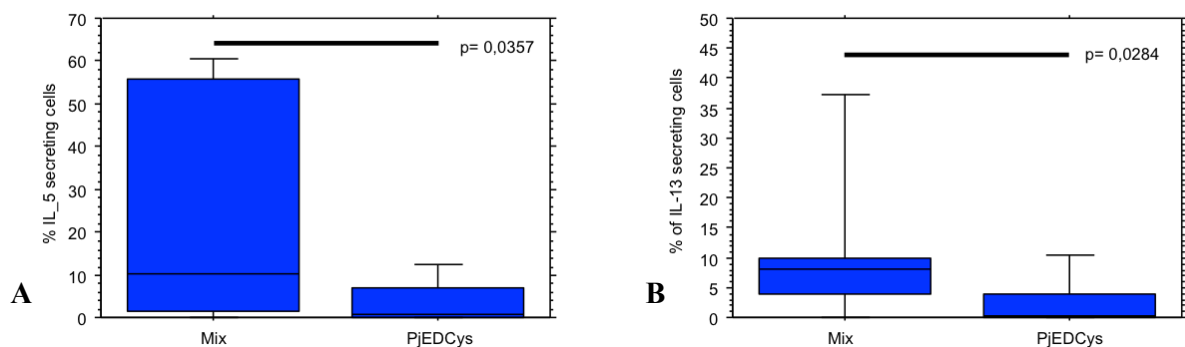


Fig. 29. Cytokine secretion in total patients. Comparison of % IL-5(A)- and IL-13 (B)-secreting CD4⁺ cells from PBMC cultures stimulated with the mixture of wild type allergens and PjEDcys. Statistically significant are the differences between the mixture and PjEDcys ($p < 0.05$).

Published data showed that allergen specific-immunotherapy induces an immune deviation in T helper cytokine phenotype from Th2 towards a protective Th1 response and triggers a tolerogenic response. Sure enough, SIT treated patients revealed increases in IFN- γ expressing cells.

Therefore IFN- γ and IL-10 secretion by CD4⁺ cells was investigated. These cytokines are particularly relevant since they may represent a redirection of the allergen-specific Th2 response towards a protective Th1 immune response and establish an allergen-specific immune tolerance. They can produce cascades, enhance or suppress the production of each other and often influence their actions [119-122].

Our analysis on IFN- γ and IL-10 secretion assays were performed only on cytokines-secreting CD4⁺ cells and showed a high individual variability. CD4⁺ cells are not the only source of IFN- γ and IL-10 and it would be interesting to perform further analyses in order to study IFN- γ and IL-10 secretion in response to *Parietaria* allergens and hypoallergenic derivate.

It is known that a wide range of cellular types produce IFN- γ and IL-10. IFN- γ is the main cytokine produced by Th1 cells but these cells are not the only source of IFN- γ . Several studies have identified additional IFN- γ secreting cell types, including activated Natural Killer (NK) cells, CD8⁺ T cytotoxic cells $\gamma\delta$ T cells, NKT cells, macrophages, dendritic cells and B cells.

IFN- γ is known to be a pleiotropic cytokine that induces and modulates many different immune responses, it also exerts a suppressive effect on allergic disease by various mechanism one of which is by direct inhibitory effects on Th2 cytokines, reducing the levels of IL-4, IL-5 and IL-13 production. Many different cell populations can produce IL-10, depending on stimulation and environmental conditions.

T CD4⁺ cells could mediate their suppressive activity by secreting a high level of IL-10.

Additional IL-10-secreting cell types have been identified as monocyte, NK cells, DC and B cells. Starting from the awareness that some cytokines could be produced by many cell types and besides one cytokine often influences the synthesis of other ones, I decided to look at IFN- γ and IL-10 production by CD4⁺ T cells especially on the patients 6, 7 and 9, who had not shown an increase in percentage of cytokine-secreting CD4⁺ cells. The data reported in panels of figure 29 show the results of cytokine secretion assay for IFN- γ and IL-10 in one out of these three patients (patient 7).

Panels a-c of figure 30 show the results of IFN- γ secretion assay, while panels d-f show the results of IL-10 secretion assay.

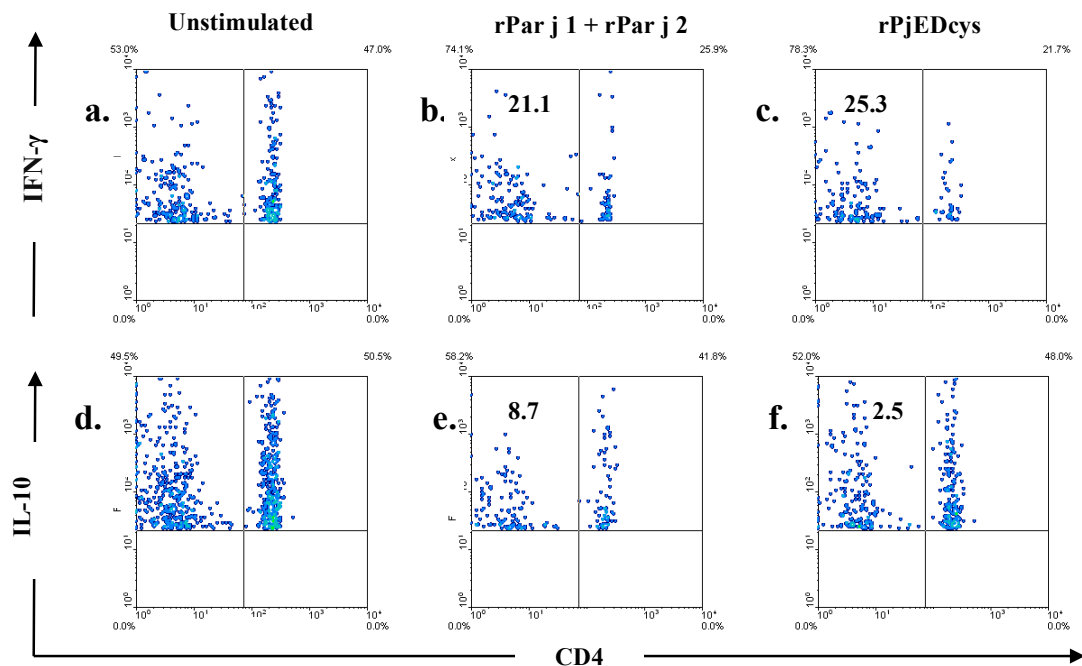


Fig 30. Representative dot plot of IFN- γ and IL-10 Secretion Assay on CD4⁺ cells. IFN- γ secretion assay (a-c) and IL-10 secretion assay (d-f) for a representative patient (**patient 7**) in unstimulated culture (a and d) and after a stimulation with a mixture containing the rPar j 1 and rPar j 2 allergens (b and e) and an equivalent concentration of rPjEDcys hybrid (c and f). Inside of dot plot b. and c. it is shown the difference between the percentage of IL-10 secreting T CD4⁺ cell after antigen-specific stimulation and the unstimulated culture.

Our analyses on patient 7 have shown that *in vitro* stimulation with the mixture or rPjEDcys induced a high increase in IFN- γ secreting CD4⁺ cells, of 21.1% and 25.3% respectively. In addition, the percentage of IL-10 secreting CD4⁺ cells increased of 8.7% and 2.5% in response to the mixture and rPjEDcys respectively.

The data reported in panels A-B of figure 31 show the results of the cytokine secretion assay for IFN- γ and IL-10 by CD4⁺ cells in patients 6, 7 and 9.

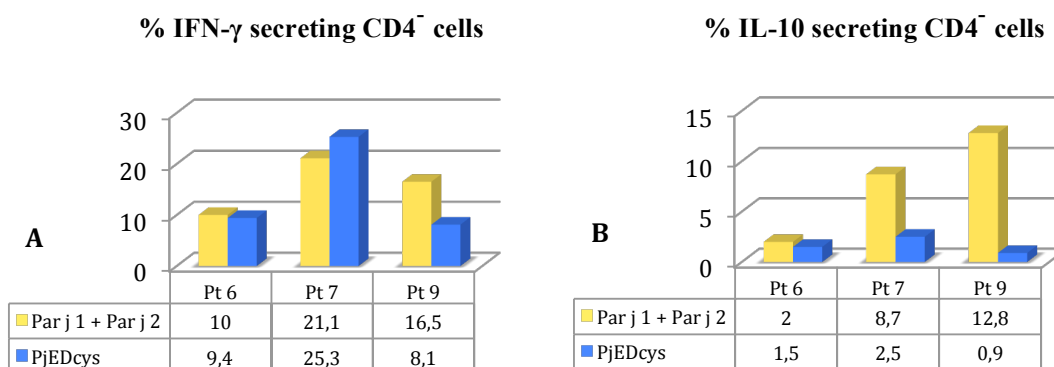


Fig. 31. Histograms of percentage of IFN- γ and IL-10 secreting antigen-specific T CD4⁺ cells. Percentage of cytokine-secreting T CD4⁺ cells in response to the mixture of allergens and rPjEDcys subtracted of cytokines background obtained in the unstimulated culture. IFN- γ secreting T CD4⁺ cells are showed in panel A and IL-10 secreting T CD4⁺ cells are showed in panel B.

Cytofluorimetric analysis on CD4⁺ cells allowed us to investigate further the immunological response activated after the *in vitro* stimulation with the mixture of wild type allergens and hypoallergenic hybrid.

The analyses on patient 6, 7 and 9 suggested that presumably the missed or low IL-5 and/or IL-13 secretion by CD4⁺ cells after the *in vitro* stimulation with the mixture of wild type allergens could be due to an increase in production of IFN- γ and/or IL-10 by different cells, some of which did not express CD4 on their cell surface. The identity of these cells remained unknown and it would be interesting characterize them. Surely, other studies have to be performed to further investigate the role of these cytokines in the immune response both to the mixture of *Parietaria* major allergens and rPjEDcys.

4. Modulation of CD25 expression on CD4⁺ cells in response to *Parietaria* major allergens and rPjEDcys

During recent years there was increasing evidence on the development of allergy, as a mechanism controlled by several populations of Tregs. Studies on immune response to allergens in healthy non-atopic individuals showed that Tregs play a crucial role in preventing Th2 response to allergens and in maintaining the peripheral tolerance.

It is accepted that Tregs play their role during the sensitization phase, and therefore the targeting of Tregs could be an advantageous therapy for the prevention and/or treatment of pathogenesis of allergy [81].

In order to study the selection of a putative allergen-specific subset of Tregs, I evaluated the modulation of CD4 and CD25 expression on PBMC from allergic patients by means of immunophenotyping.

CD25 is the interleukin-2–receptor α -chain that is expressed by Tregs and also by activated effector T cells. Nevertheless, the density of CD25 expression allows the distinction between regulatory and activated effector T cells.

PBMC isolated from heparinized peripheral blood from 4 allergic patients were stimulated for a time course of 8 days valuated each day for the expression of CD4 and CD25 by multiparametric flow cytometry (see Materials and Methods).

Therefore, analyzed cells were selected by a live lymphocyte gate based on the combination of FSC and SSC.

Figure 32 shows a flow cytometric analysis from a representative allergic patient (patient 4).

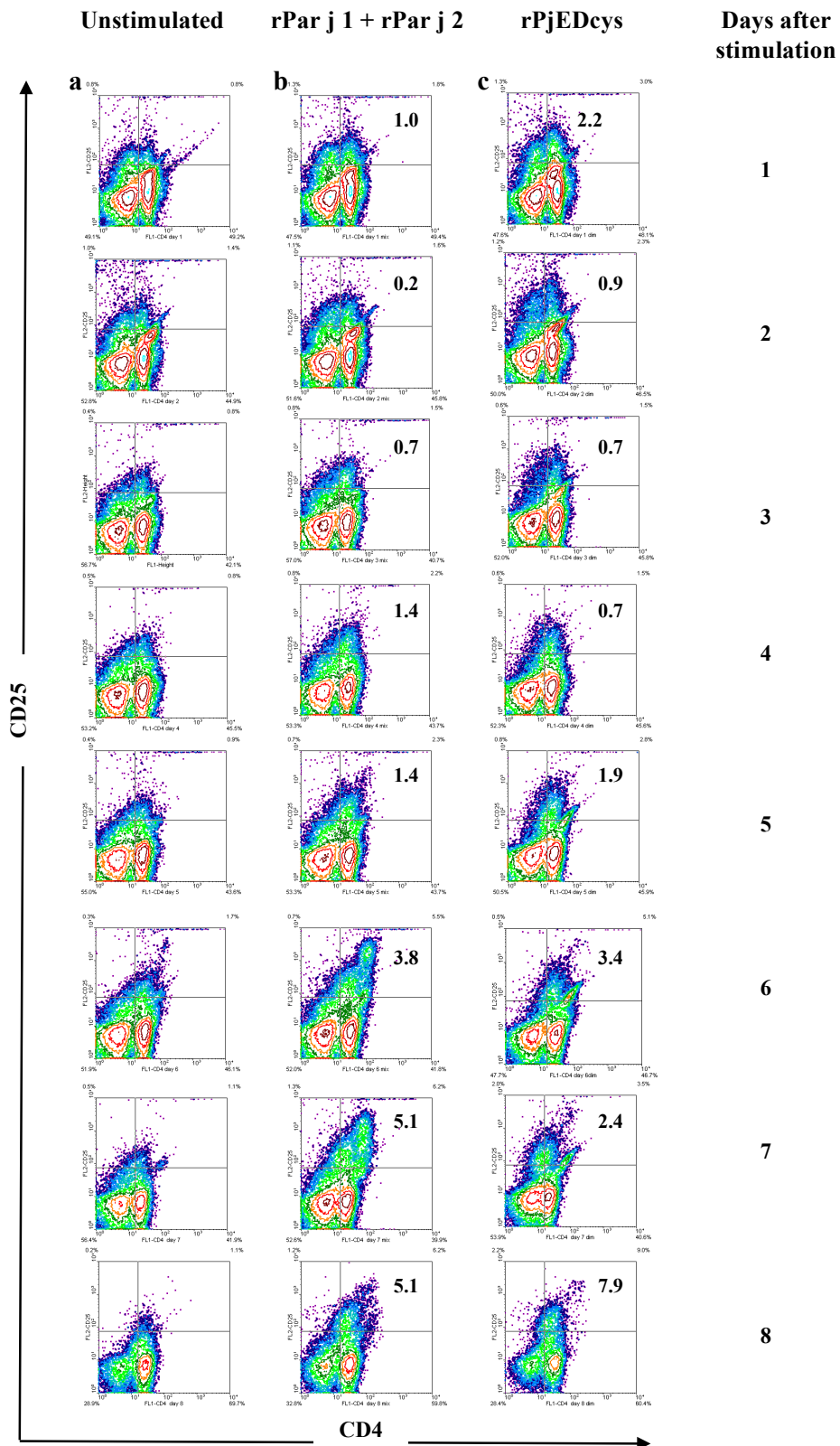


Fig. 32. Representative flow cytometric Dot Plot analysis of CD4⁺ CD25⁺ cells. PBMC from a representative allergic patient (patient 4) were cultured with the mixture of wild type allergens (b) and hypoallergenic hybrid (c) for 8 days. (a): negative control. Numbers inside the graphs indicate the percentage of CD4⁺CD25⁺ cells compared to an unstimulated culture.

From these analyses we observed that CD4⁺CD25⁺⁺ population started to increase from day 6 and reached its peak at day 8 after the *in vitro* stimulation. These analyses highlight that PBMC stimulation with the mixture of wild type allergens or rPjEDcys modulate the CD25 expression on CD4⁺ cell surface.

This pattern of activation was observed in almost all analyzed patients with some differences during the time course of stimulation (Fig. 33). The pattern of activation reached the peak between day 7 and 8 in all patients.

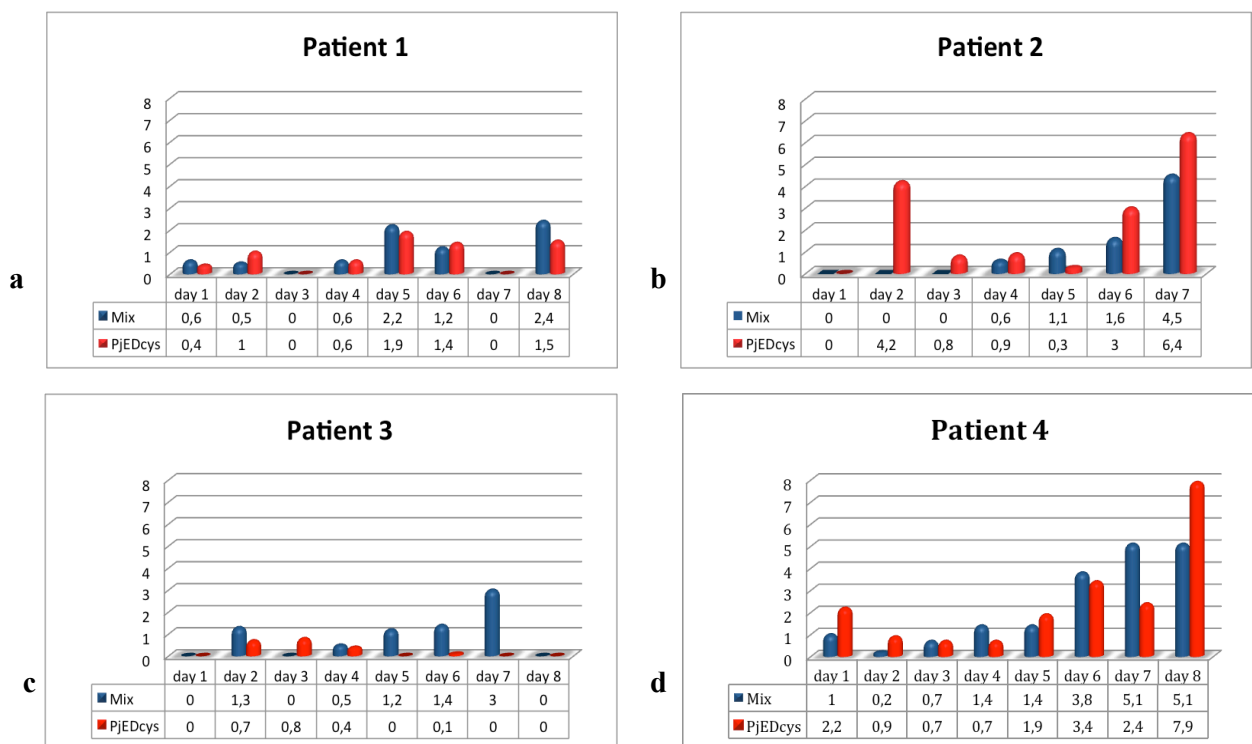


Fig. 33. Histograms of percentage of CD4⁺CD25⁺⁺. Graphic show the percentage of activated CD4⁺CD25⁺⁺ cells after *in vitro* stimulation with the mixture of wild type allergens and rPjEDcys, subtracted of the background in the unstimulated culture.

5. Study of allergen-specific CD4⁺ CD25⁺⁺ CD127⁺ Tregs after PBMC stimulation with *Parietaria major* allergens and hypoallergenic hybrid

As previously shown PBMC stimulation with the mixture of wild type allergens rPar j 1 and rPar j 2 and the rPjEDcys hybrid for a time course of 8 days induced an increase in CD4⁺CD25⁺⁺ cells from day 6 and this population reached its peak on day 7 or 8.

Starting from these preliminary data, we wanted to further investigate whether an allergen-specific Treg subset was activated after the *in vitro* stimulation with the mixture of wild type allergens or the hybrid. Different regulatory cell subsets have been described and the best characterized in pathology of allergic disease are CD4⁺CD25⁺⁺ CD127⁻ Tregs. These cells are characterized by the constitutive expression of high levels of CD25 (IL-2 receptor α chain) and the low or no expression of CD127 [80-81].

Although CD4 and CD25 identify a Tregs subset, activated effector T cells also express the two antigens. Nevertheless, the density of CD25 expression allows us to discriminate between human regulatory and activated effector T cells, but other markers may be helpful in this discrimination. CD127, the α -chain of the IL-7 receptor, is expressed on most mature T cells and plays an important role in their proliferation and differentiation. CD127 is absent on Tregs, thus CD127 can be used as an additional marker to discriminate between Tregs and activated effector T cells [123-124]. In order to characterize whether an allergen-specific Tregs subset was activated after the *in vitro* stimulation with the mixture of wild type allergens or the rPjEDcys hybrid, PBMC from six allergic patients were cultured in presence of 10 μ g/ml of antigens for 8 days and analyzed looking at two outputs:

- Immunophenotyping experiment: PBMC were analyzed at day 6, 7 and 8 for the expression of CD4, CD25 and CD127 by flow cytometry.
- Tregs isolation and characterization: PBMC at day 8 were magnetically labeled for the isolation of CD4⁺CD25⁺⁺CD127⁻.

Therefore, cells were selected by a live lymphocyte gate (R1) based on the combination of FSC and SSC (as shown in Fig. 34 A) and were selected also by a R2 gate based on the expression of CD4 and CD25 (as shown in Fig. 34 B).

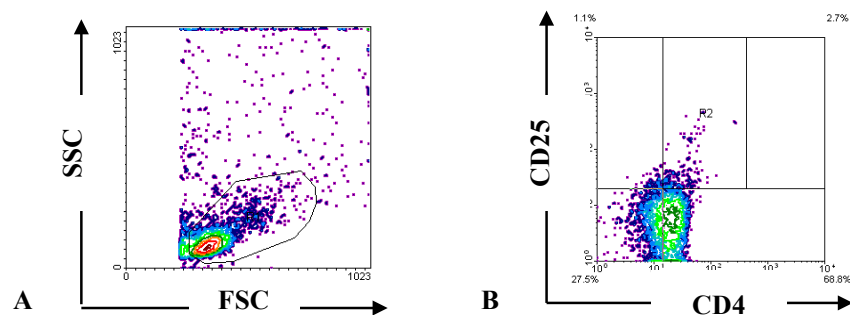


Fig. 34. Gating strategy: **A.** Dot plot of forward scatter (FSC) versus side scatter (SSC), analyzed cells were selected by a lymphocyte gate based on the combination FSC and SSC **B.** Dot plots of CD4 versus CD25 fluorescence; analyzed cells were selected by R2 gate based on the expression of CD4 and CD25.

Figure 35 shows a flow cytometric analysis from a representative patient (patient 5).

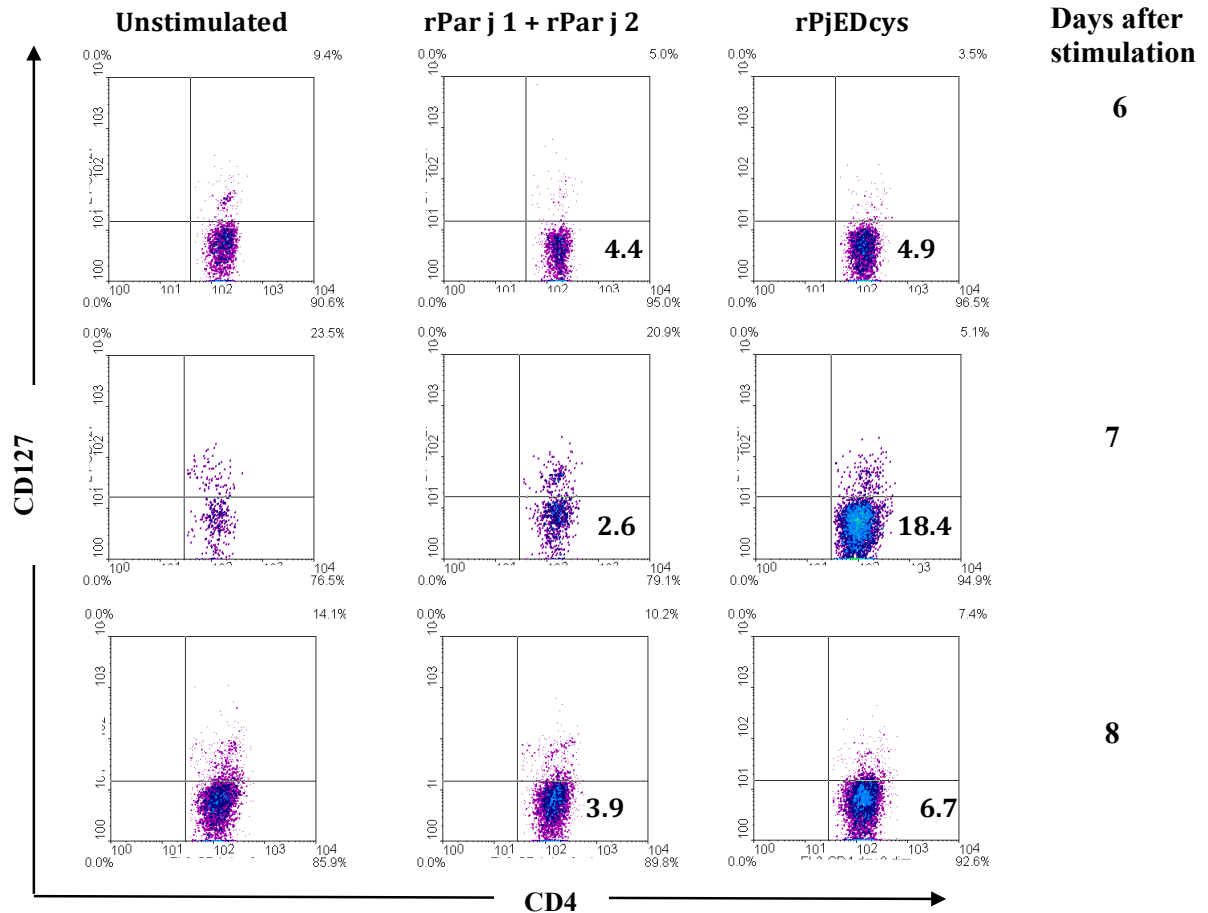


Fig. 35 Representative flow cytometric Dot Plot analysis of CD4⁺ CD25⁺⁺ CD127⁻ cells. PBMC from an allergic patient (patient 5) were cultured with the mixture of wild type allergens and hypoallergenic hybrid for 8 days. Numbers inside the graphs indicate the percentage of CD4⁺ CD25⁺⁺ CD127⁻ cells subtracted of the background in the unstimulated culture.

These analyses highlight that PBMC stimulation with the mixture of wild type allergens or rPjEDcys activated CD4⁺ CD25⁺⁺ cells. Moreover, these cells express high level of CD25 (data not shown) but not CD127 on their cell surface.

Our analysis on patient 5 have shown that the stimulation with a mixture of rPar j 1 and rPar j 2 allergens induced an increase in the percentage of activated CD4⁺ CD25⁺⁺ CD127⁻, after 6, 7 and 8 days in culture, of 4.4%, 2.6% and 3.9% respectively. This increase is more significative after the *in vitro* stimulation with rPjEDcys. In fact, the hypoallergenic derivate induced an increase in the percentage of activated CD4⁺ CD25⁺⁺ CD127⁻ of 4.9%, 18.4% and 6.7%, at 6, 7 and 8 days

respectively. In this representative patient, the percentage of CD4⁺ CD25⁺⁺ CD127⁻ cells reached its peak on day 6 in response to the mixture and on day 7 in response to rPjEDcys. Moreover, the hypoallergen induced a more significant increase in the percentage of activated CD4⁺CD25⁺⁺CD127⁻ compared to the mixture of wild type allergens. These results suggested the activation of Treg subsets in response to the mixture of rPar j 1 and rPar j 2 and hypoallergenic hybrid. The data reported in panels A-E of figure 36 show the results of the immunophenotyping for each analyzed patient.

% CD4⁺CD25⁺⁺CD127⁻ cells

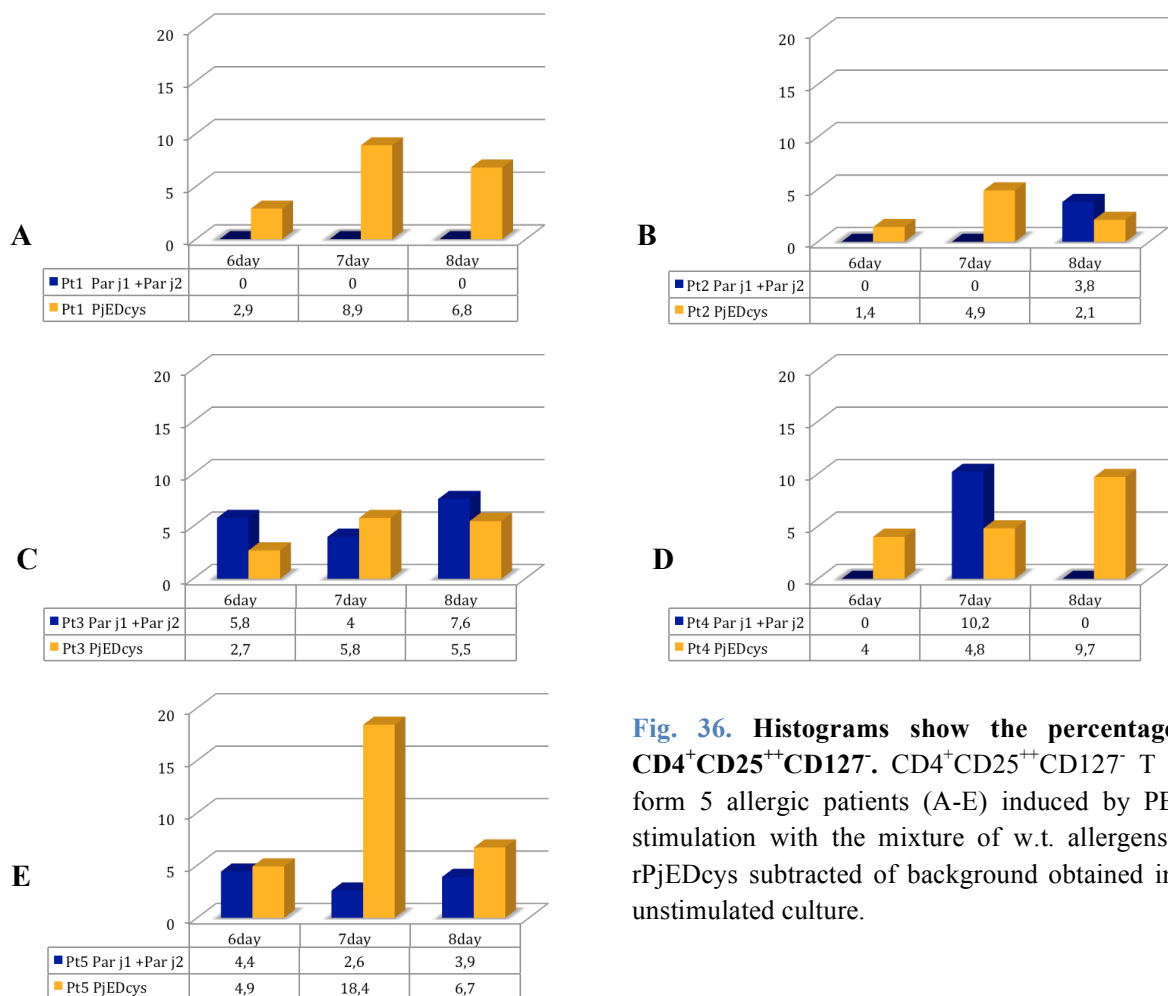


Fig. 36. Histograms show the percentage of CD4⁺CD25⁺⁺CD127⁻. CD4⁺CD25⁺⁺CD127⁻ T cells from 5 allergic patients (A-E) induced by PBMC stimulation with the mixture of w.t. allergens and rPjEDcys subtracted of background obtained in the unstimulated culture.

In these experimental settings, we were able to demonstrate that PBMC stimulation with the mixture of wild type allergens and the rPjEDcys induced the activation of a putative subset of Tregs, defined as CD4⁺CD25⁺⁺ CD127⁻. Our analyses showed that PBMC stimulation with the

rPjEDcys induce a generally more significant increase in percentage of CD4⁺CD25⁺⁺ CD127⁻ T cells compared to the mixture.

These results strongly suggested the activation of Treg subsets by hypoallergenic hybrid.

6. Magnetic separation of CD4⁺ CD25⁺⁺ CD127⁻ Tregs after stimulation with *Parietaria major* allergens and hypoallergenic hybrid

To further characterize putative Treg subset activate in response to the mixture of wild type allergens and rPjEDcys PBMC from six allergic patients were cultured for 8 days and magnetically labeled for the isolation of CD4⁺CD25⁺⁺CD127⁻ (Tregs).

The magnetic activated cell sorting was used in order to isolate CD4⁺CD25⁺⁺CD127⁻ Tregs and to perform a phenotypic and molecular characterization. The isolation of CD4⁺CD25⁺⁺CD127⁻ Tregs from human PBMC was performed using a two-step magnetic purification MACS procedure. The method can be divided into three phases and each phase has its own results.

1) Before magnetic separations, the PBMC were evaluated for the expression of CD4 and CD25 by multiparametric flow cytometry. [Analysis gate was set on live lymphocyte gate based on the combination of FSC and SSC]. Our analysis showed that the *in vitro* stimulation with the mixture of *Parietaria major* allergens and the hypoallergenic hybrid induced an increase of CD4⁺ CD25⁺ cell percentage of 3.5% and 2.1% respectively (Fig. 37).

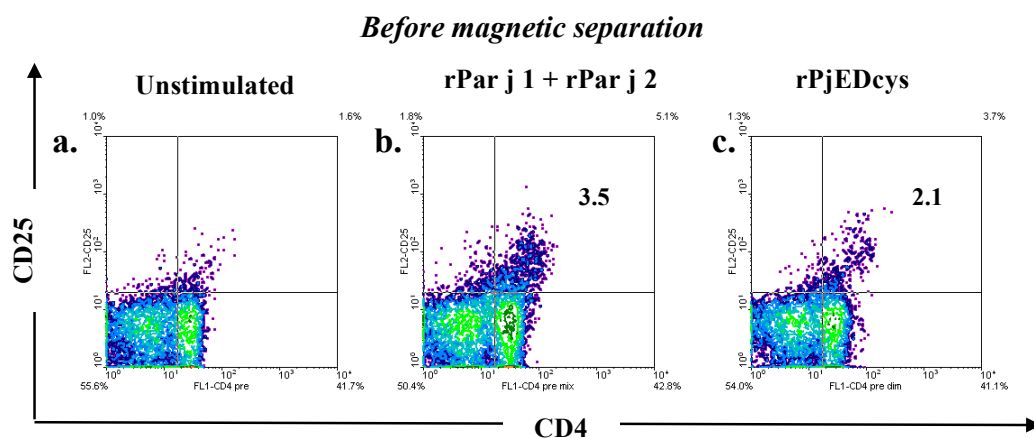


Fig. 37. Flow cytometric analysis on CD4⁺ CD25⁺⁺ cells. PBMC from *Parietaria* allergic patient were cultured with the mixture of wild type allergens (b) and hypoallergenic hybrid (c) for 8 days. A negative control sample was treated as the antigen-stimulated sample but without addition of antigen (a). Cells were collected after 8 days and before magnetic separation these cells were labeled with an anti-CD4 and anti-CD25 mAbs and analyzed by flow cytometry. Numbers inside the graphs indicate the percentage of CD4⁺ CD25⁺⁺ cells compared to an unstimulated culture.

2) With the aim of enriching for CD4⁺ T cells expressing high levels of CD25, the remaining part of the samples was magnetically labeled and isolated using two consecutive enrichment steps.

During the first magnetic purification, all PBMC were labeled with a cocktail of biotin-conjugated antibodies that allowed the labeling of all PBMC except for CD4⁺CD127⁻ cells. Then, PBMC were indirectly magnetically labeled with anti-biotin monoclonal antibodies conjugated to Micro Beads, as secondary labeling reagent.

The magnetically labeled cell suspension was loaded onto a LD MACS column, which was placed into the magnetic field of a MACS Separator. Therefore, non-CD4⁺ T cells were retained in the column, while the unlabeled, CD4⁺ CD127⁻ T cells flowed through and were collected (negatively selected cell fraction). Then, columns were removed from the magnetic field and the retained non-CD4⁺ T cells were eluted and collected (the positively selected cell fraction).

An aliquot of each positively and negatively selected cell fraction was fluorescently stained with anti-CD4 and anti-CD25 antibodies. These samples were analyzed for the CD4 and CD25 expression, in order to evaluate the percentage of enrichment and/or depletion of the different cell populations. Figure 38 shows the two fractions obtained after the first magnetic separation: the magnetically labeled fraction, containing the non-CD4⁺ cells and the flow-through fraction, containing the pre-enriched CD4⁺ CD127⁻ cells.

**Depletion of non-CD4⁺ cells with LD Column
from total PBMC**

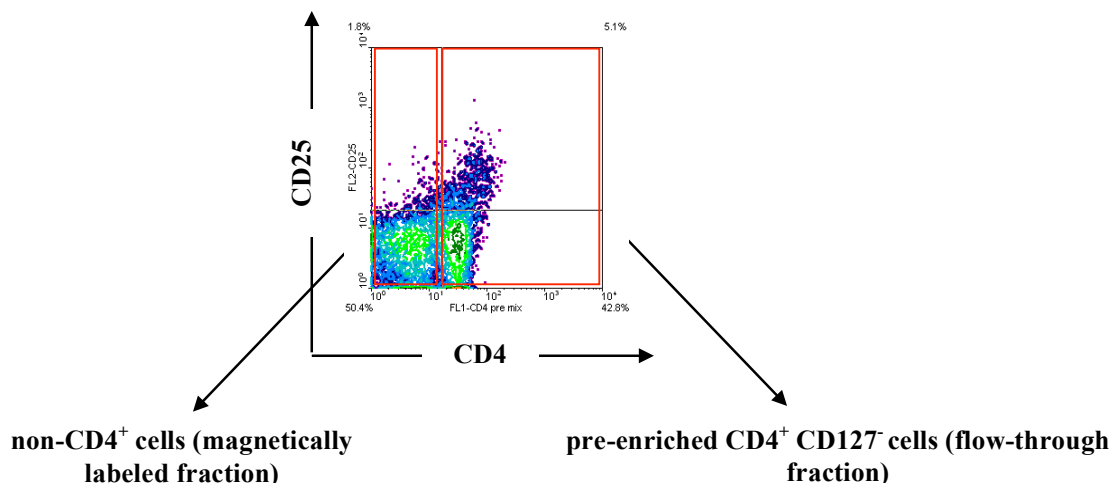


Fig. 38. Schematic representation of depletion of non-CD4⁺ cells. The first of the two-steps of magnetic purification allows to obtain two different fractions: the magnetically labeled fraction containing the non-CD4⁺ cells and the flow-through fraction containing the pre-enriched CD4⁺ CD127⁻ cells. During the depletion phase, undesired cells (all non-CD4⁺ cells) are retained in a MACS column and are eluted after removing the column from the magnetic separator while the unlabeled cells pass through the MACS column and collected as pre-enriched fraction.

The data reported within figure 39 show the cytometric analysis performed on the two fractions obtained from the first magnetic separation, showing that, in all three analyzed samples, an enrichment of the CD4⁺ population was obtained.

Depletion of non-CD4⁺ cells with LD Column

Non-CD4⁺ cells (magnetically labeled fraction)

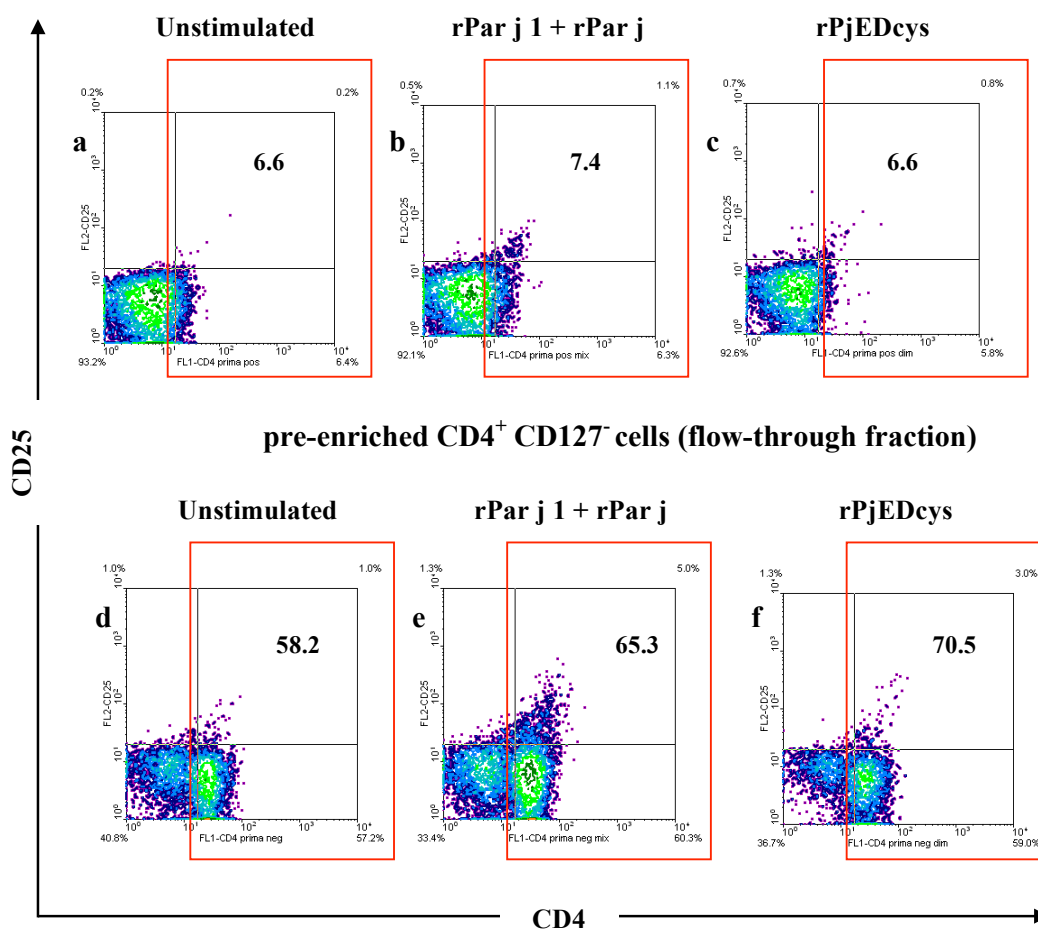


Fig. 39. Flow cytometric Dot Plot analysis of CD4⁺ CD25⁺⁺ cells. Cytometric analysis was performed on the two fractions obtained from the first magnetic separation: 1) magnetically labeled fraction containing the non-CD4⁺ cells in unstimulated culture (a) and after *in vitro* stimulation with a mixture containing the rPar j 1 and rPar j 2 allergens (b) and an equivalent concentration of rPjEDcys hybrid (c) 2) the flow-through fraction containing the pre-enriched CD4⁺ CD127⁻ cells in unstimulated culture (d) and after *in vitro* stimulation with a mixture containing the rPar j 1 and rPar j 2 allergens (e) and an equivalent concentration of rPjEDcys hybrid (f). Numbers inside the graphs indicate the percentage of CD4⁺ CD25⁺⁺ cells compared to an unstimulated culture.

3) The third phase of the experiment allows the selection of $CD4^+CD25^{++}CD127^-$ Tregs based on the intensity of fluorescence of the surface molecule CD25.

Pre-enriched $CD4^+CD127^-$ cell fractions (flowed-through fractions obtained from the first magnetic separation) were magnetically labeled with anti-CD25 Micro Beads.

In this second step, the $CD4^+CD25^{++}CD127^-$ Tregs were isolated by a positive selection from the pre-enriched $CD4^+CD127^-$ T cell fraction by means of the separation over a MS MACS Column, which was placed into the magnetic field of a MACS Separator.

Figure 40 shows the two fractions obtained after the second magnetic separation: the magnetically labeled fraction containing the enriched $CD4^+ CD25^{++} CD127^-$ cells and the flow-through fraction containing the $CD4^+ CD25^- CD127^-$ cells.

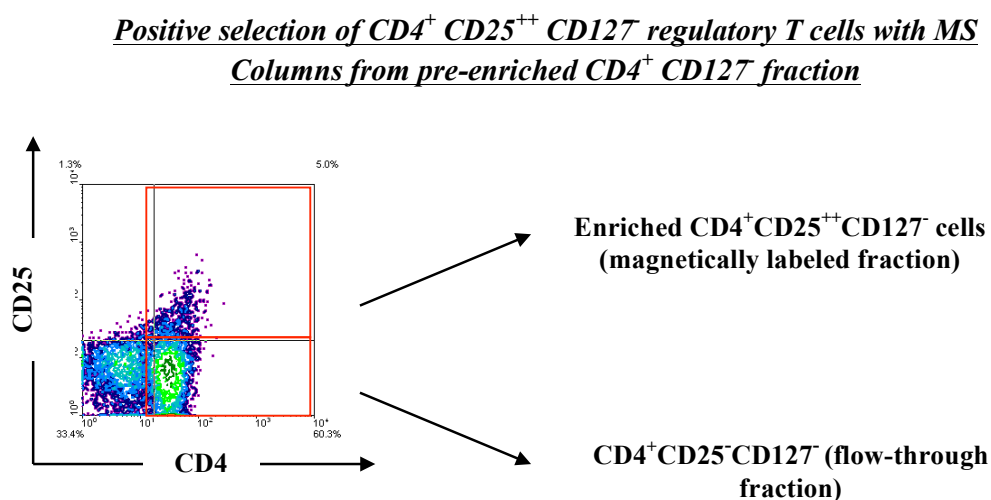


Fig. 40. Schematic representation of positive selection. The second step of magnetic purification allows two obtain two different fractions: the magnetically labeled fraction, containing the enriched $CD4^+CD25^{++}CD127^-$ cells, and the flow-through fraction, containing the $CD4^+ CD25^- CD127^-$ cells. During the positive selection phase, target cells are retained in the MACS column and are eluted after removing the column from the magnetic separator, while the unlabeled cells pass through the MACS column and are collected as negative fraction.

Therefore, during this second purification, target cells (magnetically labeled $CD4^+CD25^{++}CD127^-$ T cells) were retained in the column and the unlabeled $CD4^+CD25^- CD127^-$ T cells run through and were collected as the negatively selected cell fraction. After removing the column from the

magnetic field, the magnetically retained CD4⁺CD25⁺⁺CD127⁻ Tregs were eluted as the positively selected cell fraction.

In order to evaluate the percentage of enrichment and/or depletion of the different cell populations, aliquots of the positively and of the negatively selected cell fractions were fluorescently stained with anti-CD4 and anti-CD25 antibodies and analyzed for the expression of the two cell surface antigens.

The second step of magnetic separation allowed us to remove the non-CD4⁺ cells still present in the pre-enrichment fraction and allowed us to further enrich the CD4⁺ population in all samples.

As expected, the percentage of CD4⁺CD25⁺⁺CD127⁻ purified in each sample was very low, because antigen-specific Tregs are very rare.

Figure 41 shows the cytofluorimetric analysis performed on the two fractions obtained from the second magnetic separation: the magnetically labeled fraction containing the enriched CD4⁺CD25⁺⁺CD127⁻ Tregs and the flowed-through fraction containing the CD4⁺CD25⁻CD127⁻ cells.

These data have shown that in an unstimulated samples, 1% of cells retained in the column and eluted as positive fraction were CD4⁺CD25⁺⁺ cells (panel **a**), but some of these cells identified as CD4⁺CD25⁺⁺ passed through the column and were collected in the negative fraction: these represent 0.4% of harvested cells (panel **d**).

In samples stimulated with the mixture of *Parietaria* major allergens, 2.9% of the cells retained in the column and eluted as positive fraction were the CD4⁺CD25⁺⁺ cells, (panel **b**) although 2.3% of the cells collected in negative fraction were the CD4⁺CD25⁺⁺ cells (panel **e**).

In the samples stimulated with rPjEDcys, 2.7% of the cells retained in the column and eluted as positive fraction were CD4⁺CD25⁺⁺ cells (panel **c**), while 0.8% of the cells that passed through the column and collected in flow-through fraction were CD4⁺CD25⁺⁺ (panel **f**).

Positive selection of CD4⁺CD25⁺⁺ regulatory T cells with MS Columns

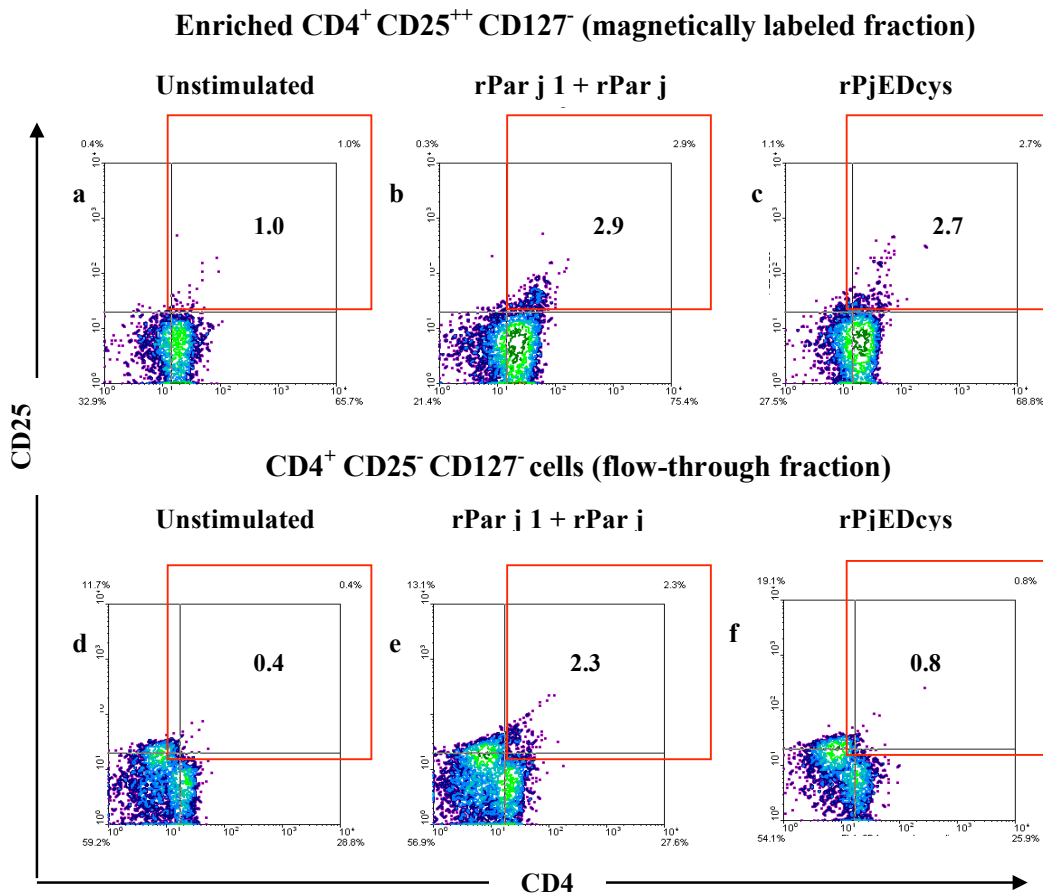


Fig 41. Flow cytometric analysis on CD4⁺CD25⁺⁺ cells. Cytofluorimetric analysis was performed on the two fractions obtained from the first magnetic separation: 1) magnetically labeled fraction containing the CD4⁺CD25⁺⁺CD127⁻ cells in unstimulated culture (a) and after *in vitro* stimulation with a mixture containing the rPar j 1 and rPar j 2 allergens (b) and an equivalent concentration of rPjEDcys hybrid (c) 2) the flow-through fraction containing the CD4⁺CD25⁻CD127⁻ cells in unstimulated culture (d) and after *in vitro* stimulation with a mixture containing the rPar j 1 and rPar j 2 allergens (e) and an equivalent concentration of rPjEDcys hybrid (f). Numbers inside the graphs indicate the percentage of CD4⁺CD25⁺⁺ cells.

In summary, these data demonstrated that using the two-step magnetic purification we obtained an enrichment of CD4⁺CD25⁺⁺CD127⁻ Tregs from human PBMC.

The analyses on enriched CD4⁺CD25⁺⁺CD127⁻ have shown that the stimulation with a mixture of rPar j 1 and rPar j 2 allergens and rPjEDcys induced an increase in the percentage of activated CD4⁺CD25⁺⁺ of 1.9% and 1.7% respectively.

The most significant results emerged from the analysis of the expression of CD25 on CD4⁺CD25⁺⁺CD127⁻ fraction.

Figure 42 shows the histograms related to the intensity of CD25 expression in $CD4^+CD25^{++}CD127^-$ cell fraction after 8 days in culture. The number of events per intensity of fluorescence in the mixture of wild type allergen-treated (solid blue histogram) and rPjEDcys-treated (solid violet histogram) was compared to an untreated sample (solid light blue histogram).

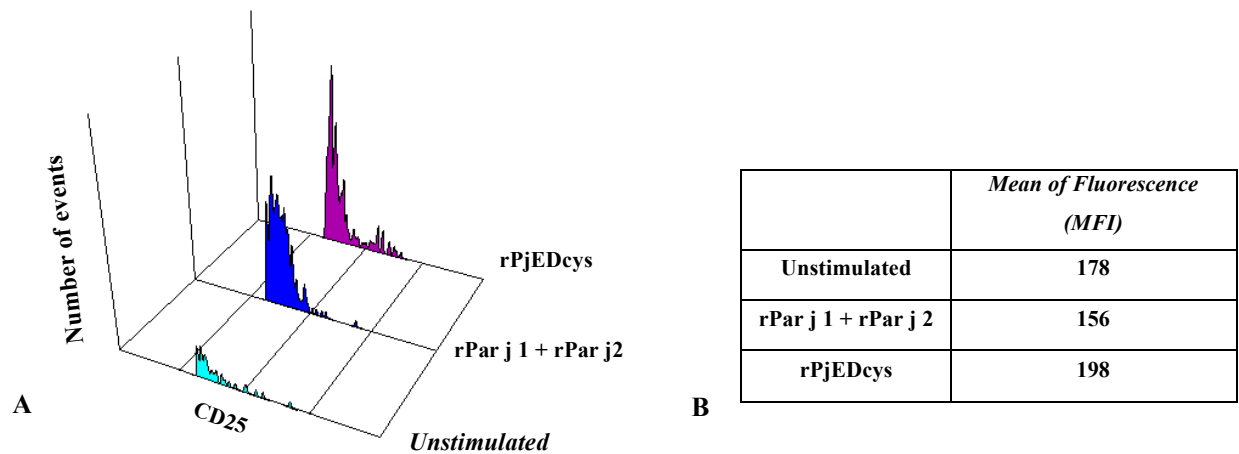


Fig. 42. Histograms of the CD25 expression intensity on $CD4^+CD25^{++}$ cells. Histograms in 3D (A) and Mean of fluorescence of CD25 expression (B) in $CD4^+CD25^{++}CD127^-$ cell fraction after 8 days in culture. The intensity of fluorescence in mixture of wild type allergens-treated (blue line) and rPjEDcys-treated (violet line) was compared to untreated sample (solid light blue histogram).

The histograms have shown that at day 8, the *in vitro* stimulation with wild type allergens and hypoallergenic hybrid rPjEDcys induced an increase of cells expressing high CD25 on their cell surface.

As previously said CD25 is expressed by Tregs and also by activated effector T cells, but the density of CD25 expression allows us to discriminate between these two T cell populations. Therefore, a more interesting data emerged by the analysis on the number of events that showed high-density expression of CD25. In particular, our analysis showed that only after the *in vitro* stimulation with hypoallergenic hybrid, the Mean Fluorescence Intensity (MFI) of CD25 expression in $CD4^+$ cells increased compared to an unstimulated culture. The MFI measure the shift in fluorescence intensity of a population of cells. The described experiments have demonstrated that PBMC stimulation with hypoallergenic hybrid for 8 days induced a significant increase of $CD4^+CD25^{++}CD127^-$ cell population.

7. Molecular characterization of allergen-specific CD4⁺ CD25⁺⁺ CD127⁻ T Regulatory Cell

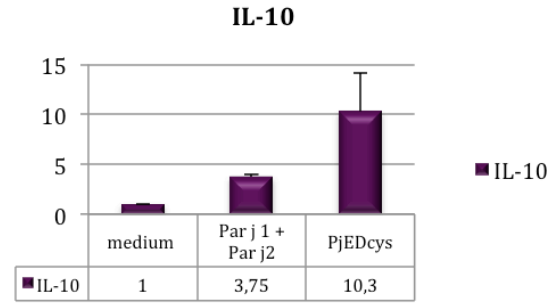
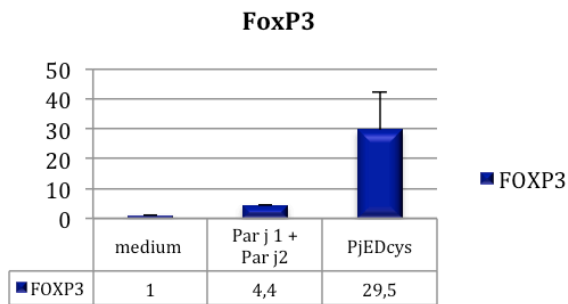
PBMC isolated from each patient were contextually cultured in presence of 10 µg/ml of antigens for 8 days in order to isolate and characterize the CD4⁺CD25⁺⁺CD127⁻ Tregs using a two-step magnetic purification MACS procedure, as previous described.

I performed reverse transcription and quantitative PCR experiments on RNA extracted from the CD4⁺CD25⁺⁺CD127⁻ cell fractions, obtained after the magnetic separation, in order to characterize which Tregs subset was activated after the *in vitro* stimulation with the mixture or the hybrid.

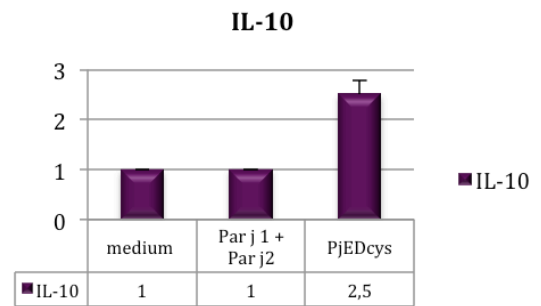
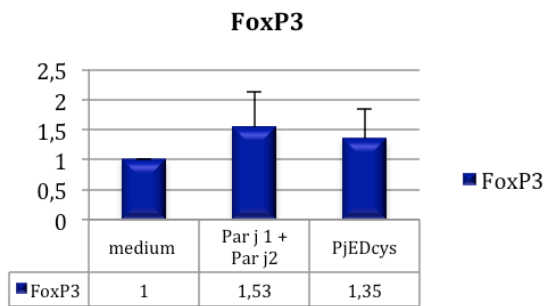
The cDNA obtained from 6 patients were used as a template for Real Time PCR reactions.

We looked at the expression levels of four genes encoding for proteins essential for the development and function of Tregs. Using RT-qPCR we measured the FoxP3, IL-10, GARP and TGF-β mRNA levels in the enriched CD4⁺CD25⁺⁺CD127⁻ cells after an *in vitro* stimulation. The transcription factor FoxP3 was described as the “master regulator” Treg [31, 39]. But, not all Tregs express FoxP3, there are other types of Treg cells that can be induced from *naïve* T cells in the periphery which are FoxP3⁻. These different subsets have been identified and show different phenotype and suppressive activity [125]. The mRNA levels of TGF-β cytokine, immunosuppressive cytokines involved in regulatory pathway of Tregs, were also analyzed. Recently the expression of a gene encoding for a transmembrane proteins called GARP was identified on human Tregs activated only after TCR stimulation [126-129]. FoxP3, IL-10, TGF-β and GARP expression were standardized using the human housekeeping 18S rRNA. The experiment was repeated in triplicate with similar results, as shown in figure 43. Panels A-F of figure 43 show the results of Real Time PCR on FoxP3 and IL-10 mRNA levels for each patient.

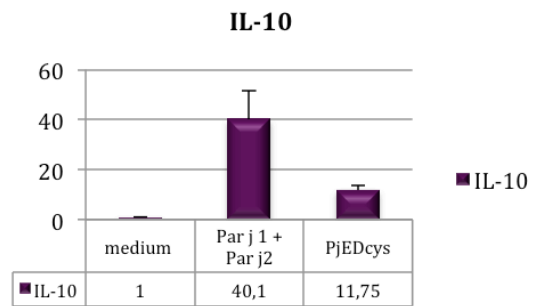
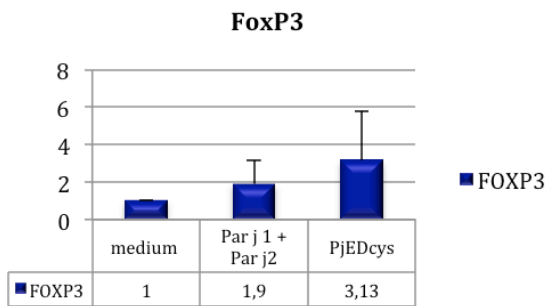
A. Patient 1



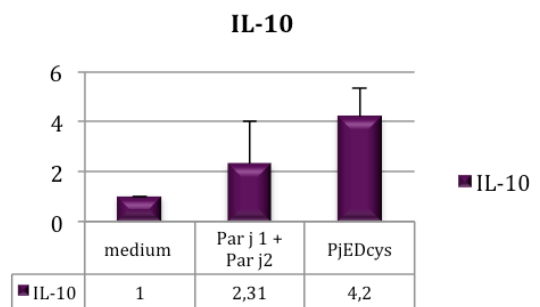
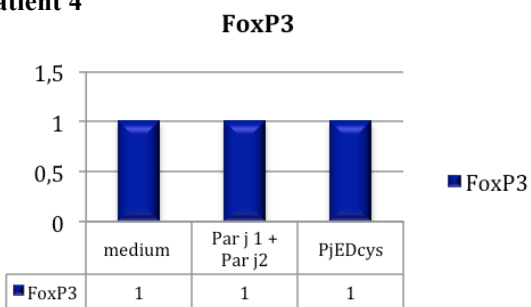
B. Patient 2



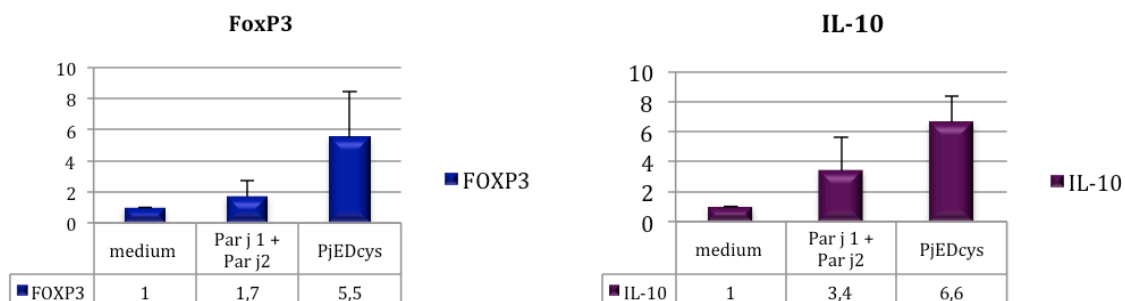
C. Patient 3



D. Patient 4



E. Patient 5



F. Patient 6

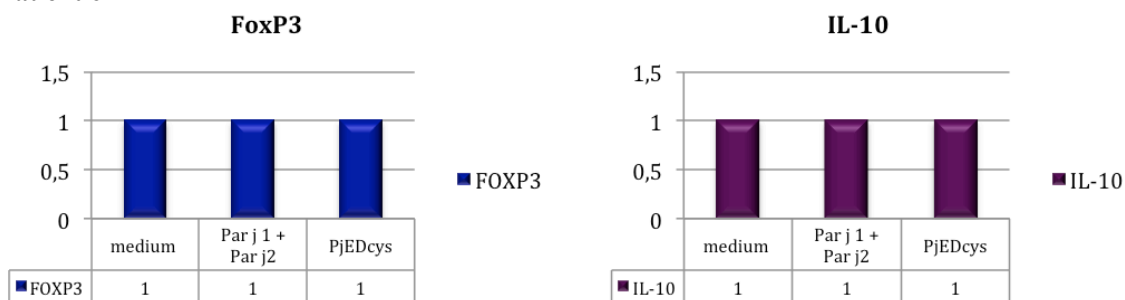


Fig. 43. Histograms of percentage of gene expression. Real-time PCR analysis of FoxP3 and IL-10 mRNA expression in enriched CD4⁺ CD25⁺⁺ CD127⁻ cell fraction treated with the mixture of wild type allergens or with the rPjEDcys compared to the unstimulated sample.

Analysis of FoxP3 gene expression on enriched CD4⁺CD25⁺⁺CD127⁻ cell fractions showed that in four out of six analyzed patients (patients 1-3 and 5) the PBMC stimulation with the mixture of wild type allergens or rPjEDcys induced an increase of FoxP3 mRNA levels. In three out of these four patients (patients 1, 3 and 5) RTq-PCR revealed significantly higher expression levels of the relevant FoxP3 mRNA in isolated CD4⁺CD25⁺⁺ CD127⁻ cells in response to rPjEDcys as compared to the mixture of *Parietaria* allergens.

In two out of six analyzed patients (patients 4 and 6) RTq-PCR did not revealed higher expression levels of the FoxP3 mRNA in isolated CD4⁺CD25⁺⁺ CD127⁻ cells in response to both antigens.

Our results about the analysis of IL-10 expression on enriched CD4⁺ CD25⁺⁺ CD127⁻ cell fraction showed that in five out of six analyzed patients (patients 1-5) IL-10 mRNA levels increase in the sample stimulated with both antigens.

Moreover, in four out of six analyzed patients (patients 1, 2, 4 and 5) quantitative PCR revealed that the rPjEDcys induced a significantly higher expression levels of the relevant IL-10 mRNA in CD4⁺CD25⁺⁺CD127⁻ cells than the mixture of *Parietaria* allergens.

Patient 6 was the only one where the PBMC stimulation with the mixture of allergens or rPjEDcys did not induce an increase of mRNA levels for each genes.

In summary, our analyses on FoxP3 and IL-10 gene expression showed a similar increasing trend in response to the mixture and the hypoallergenic hybrid. On the contrary, our analyses on TGF- β mRNA levels expression did not show an increase in response to antigen-specific stimulation (data not shown). Our analyses on GARP mRNA levels showed high variability within and between patients, therefore our analyses confirmed the data published in literature, which suggest the almost undetectable GARP mRNA level in CD4⁺ T cells and a post-transcriptional regulation of GARP expression [38, 129]. On the contrary, our analyses on TGF- β mRNA levels expression did not show an increase in response to antigen-specific stimulation (data not shown).

8. Analysis of GARP and LAP expression on CD4⁺CD25⁺⁺ cells in response to Parietaria major allergens and rPjEDcys

In recent years, GARP expression was found to be highly expressed on activated T regulatory cells. Recent studies suggested the possibility of using GARP as a marker to detect activated T reg cells with suppressor functions. However, Unutmaz et al. (the first one to identify GARP mRNA into Treg cells) showed that GARP was expressed only after TCR stimulation of Treg [126].

In addition, GARP mRNA expression was defined undetectable in freshly isolated CD4⁺ T cells; on the contrary the GARP protein accumulates on Treg cell surface post activation [36-37, 128-129].

Thanks to the discovery of GARP function, a new interest was given to TGF- β on Treg cell biology. GARP function is associated with latent transforming growth factor- β (proTGF- β) on Treg cell surface and regulates Treg activation [130].

GARP was in fact identified as a receptor for latent TGF- β providing an explanation for the detection of membrane-bound TGF- β on Treg cells, as reported by several groups [130-131]

To study the expression of GARP and LAP on CD4⁺CD25⁺⁺ cell surface induced by *in vitro* stimulation with the mixture or rPjEDcys, we stimulated PBMC from four allergic patients for a time course of 6 days (as shown in Materials and Methods).

The negative control sample was treated, exactly the same as the antigenstimulated sample, but without addition of antigens. Cells were recovered at 2h, 4h, 6h, 18h, 24h and then every 24 hours until day 6 and analyzed for the expression of CD4, CD25, GARP or LAP by multiparametric flow cytometry.

Therefore, analyzed cells were selected by a live lymphocyte gate based on the combination of FSC and SSC and were selected also by a gate on the expression of CD4 and CD25 (as shown before).

Figure 44 shows a flow cytometric analysis on CD4⁺CD25⁺⁺GARP⁺ cells from a representative patient (patient 1).

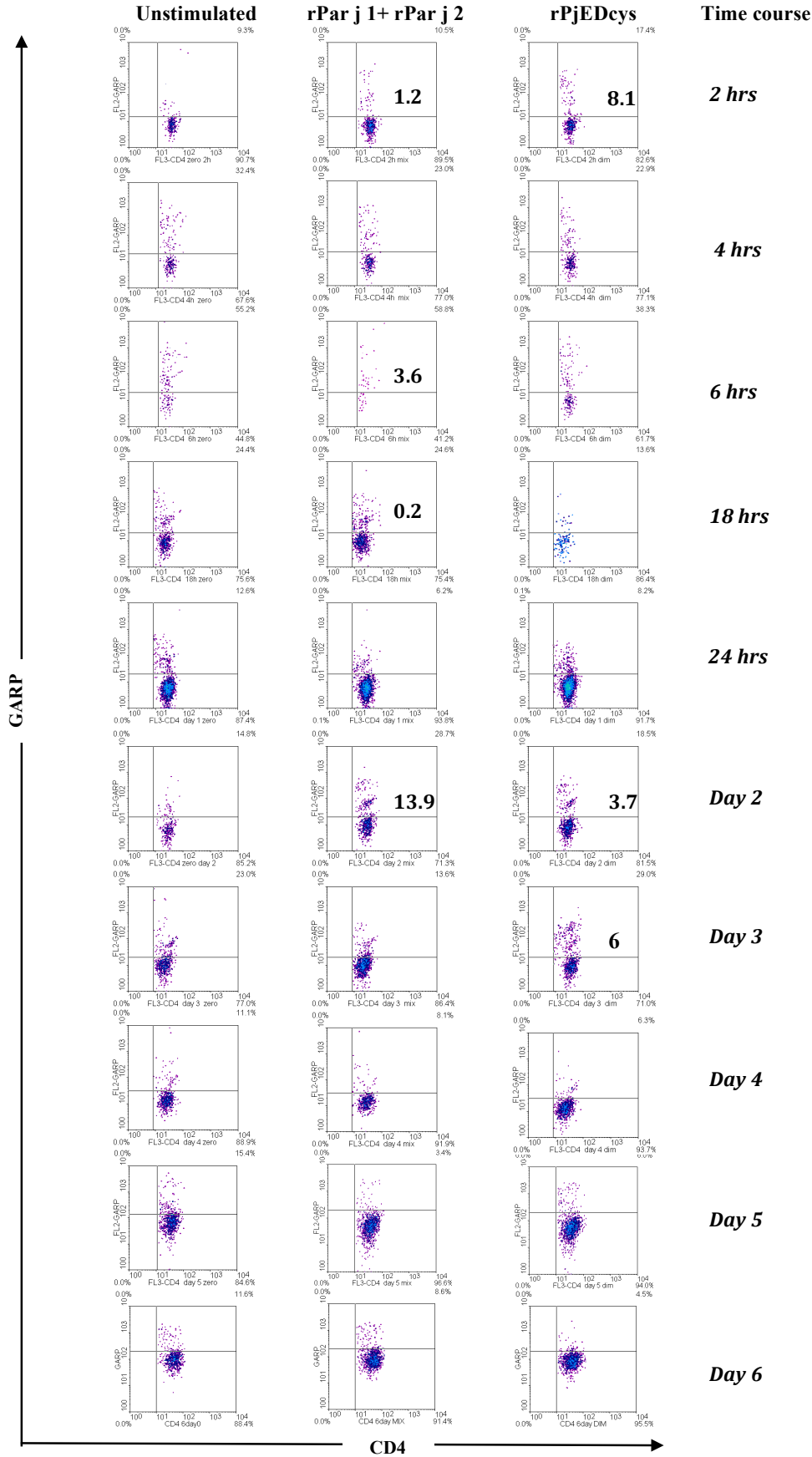


Fig. 44. Representative flow cytometric Dot Plot analysis of CD4⁺CD25⁺⁺GARP⁺ cells. Panels show cytometric analyses of CD4⁺ CD25⁺⁺ GARP⁺ cells in a representative patient (patient 1). Numbers inside the graphs indicate the percentage of CD4⁺ CD25⁺⁺ cells after background subtraction (unstimulated culture).

The results obtained from the analyses of GARP expression on CD4⁺ CD25⁺⁺ cells showed that PBMC stimulation with the wild type allergens or rPjEDcys induce an increase in the percentage of CD4⁺ CD25⁺⁺ GARP⁺ cells. Furthermore, we observed that CD4⁺ CD25⁺⁺ population started to express the GARP protein on cell surface 2h after the *in vitro* stimulation and the expression of GARP was higher in response to rPjEDcys than to the mixture, (8.1% and 1.2% respectively).

After 6h, an increase in percentage of CD4⁺ CD25⁺⁺GARP⁺ cells was exclusively observed in the PBMC stimulated with the mixture. Our preliminary data show that the *in vitro* stimulation of human PBMC from allergic patients with both the w.t. antigens and the derivative induced an increase of CD4⁺ CD25⁺⁺ GARP⁺ cells after 2 days. Moreover, GARP expression increases until day 3 only in response to rPjEDcys.

In this experimental set up we also analyzed the expression of LAP on CD4⁺CD25⁺⁺ cells after *in vitro* stimulation with the mixture or rPjEDcys. For this reason, cells were selected by a lymphocyte gate (R1) based on the combination of FSC and SSC and were also selected by a R2 gate based on the expression of CD4 and CD25 (as shown before).

Figure 45 shows a flow cytometric analysis on CD4⁺ CD25⁺⁺ LAP⁺ cells from patient 1.

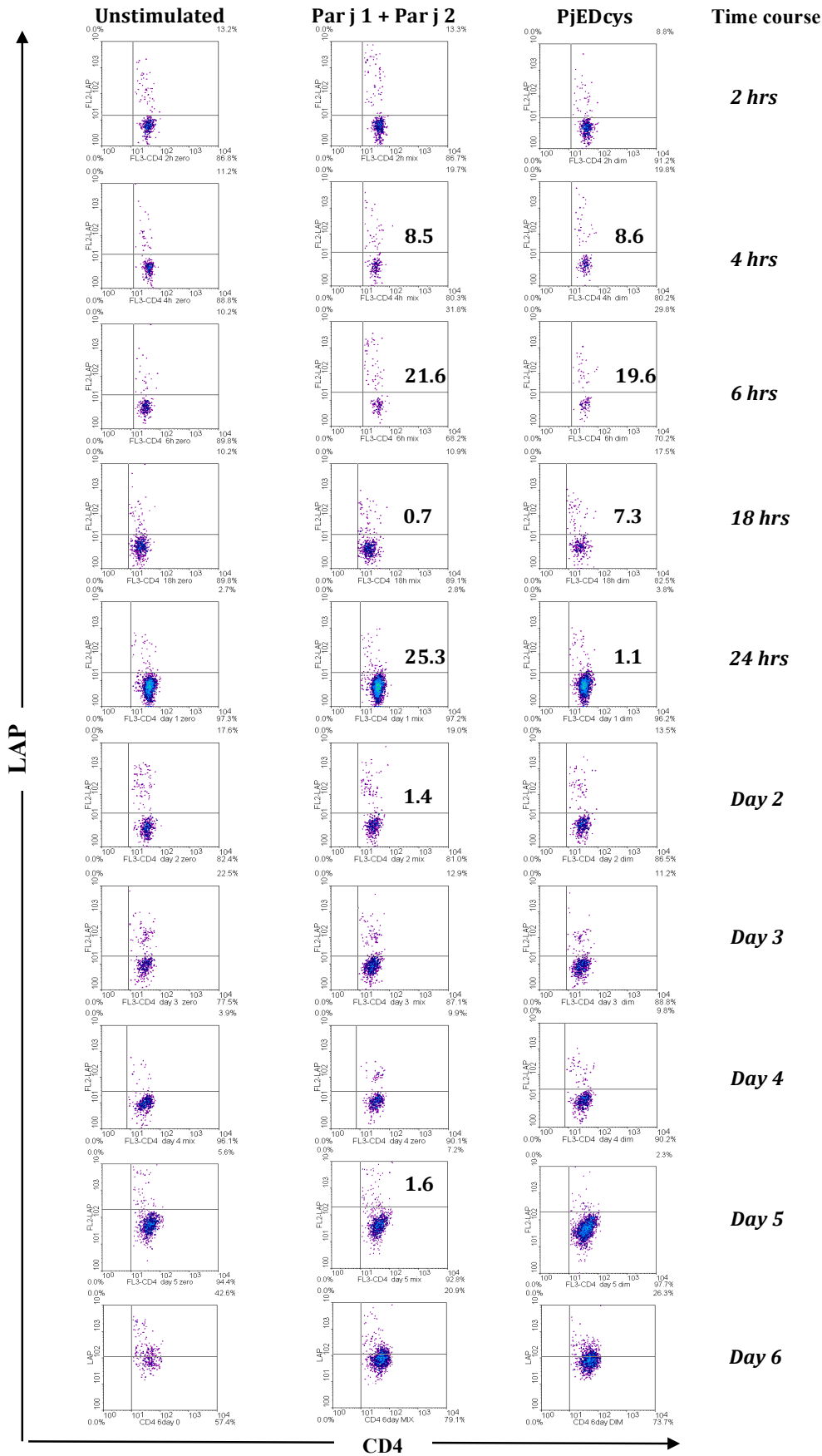


Fig. 45. Representative flow cytometric Dot Plot analysis of CD4⁺CD25⁺⁺LAP⁺ cells. Panels show cytometric analyses of CD4⁺CD25⁺⁺LAP⁺ cells in a representative patient (patient 1). Numbers inside the graphs indicate the percentage of CD4⁺CD25⁺⁺ cells after background subtraction (unstimulated culture).

The results obtained from the analyses of LAP expression on CD4⁺ CD25⁺⁺ showed that the PBMC stimulation with the wild type allergens or rPjEDcys increase the percentage of CD4⁺ CD25⁺⁺ LAP⁺ cells. We observed that the LAP expression on CD4⁺ CD25⁺⁺ cells started to increase 4h after the *in vitro* stimulation with the mixture or rPjEDcys (8.5% and 8.6% respectively).

In the sample stimulated with the mixture of *Parietaria* allergens, LAP protein is expressed on CD4⁺CD25⁺⁺ cells until day 2. The percentage of CD4⁺ CD25⁺⁺ LAP⁺ cells increased at 4h after PBMC stimulation with the mixture and reached the peak after 6h. Afterwards, the percentage of CD4⁺ CD25⁺⁺ LAP⁺ strongly increased 24h after PBMC stimulation.

Our analysis showed that the percentage of CD4⁺ CD25⁺⁺ LAP⁺ cells increased within 2h after *in vitro* stimulation with hypoallergenic hybrid, compared to an unstimulated culture. The percentage of CD4⁺ CD25⁺⁺ LAP⁺ cells reached the peak at 6h and then progressively decreased until 24h.

The histograms reported in panels A-B of figure 46 show the results concerning to the GARP and LAP expression.

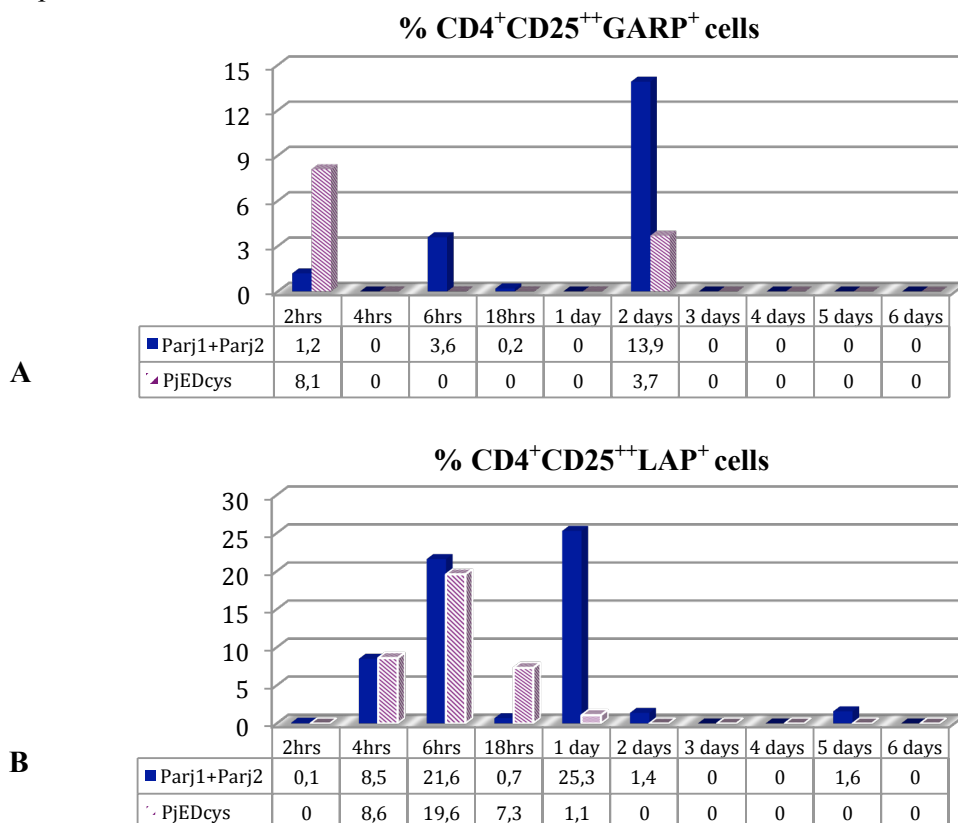


Fig. 46. Histograms of percentage of CD4⁺CD25⁺⁺GARP⁺ and CD4⁺CD25⁺⁺LAP⁺ cells. Graphics show the percentage of activated CD4⁺CD25⁺⁺GARP⁺ cells (A) and CD4⁺CD25⁺⁺LAP⁺ (B) in a representative patient (patient 1), after *in vitro* stimulation with the mixture of wild type allergens and rPjEDcys subtracted of the background in the unstimulated culture.

Starting from this preliminary experiment and data in literature, we decided to perform another set of experiments in order to study the CD4, CD25, GARP and LAP expression [36, 129]. In the successive experiments the PBMC were stimulated for a time course of 3 days. In fact, from the first experiment we observed that cell surface expression of either GARP or LAP was not detected after 3 days in culture except for a low increase in percentage of CD4⁺CD25⁺⁺LAP⁺ on day 5. Studies in literature showed that the level of GARP mRNA increased during the first 6 hrs after TCR stimulation. Moreover, the levels of GARP and LAP proteins were analyzed until 24 hrs after cell stimulation [129]. The data reported in panels A-D of figure 47 shows the histograms concerning to the GARP expression in all four analyzed patients.

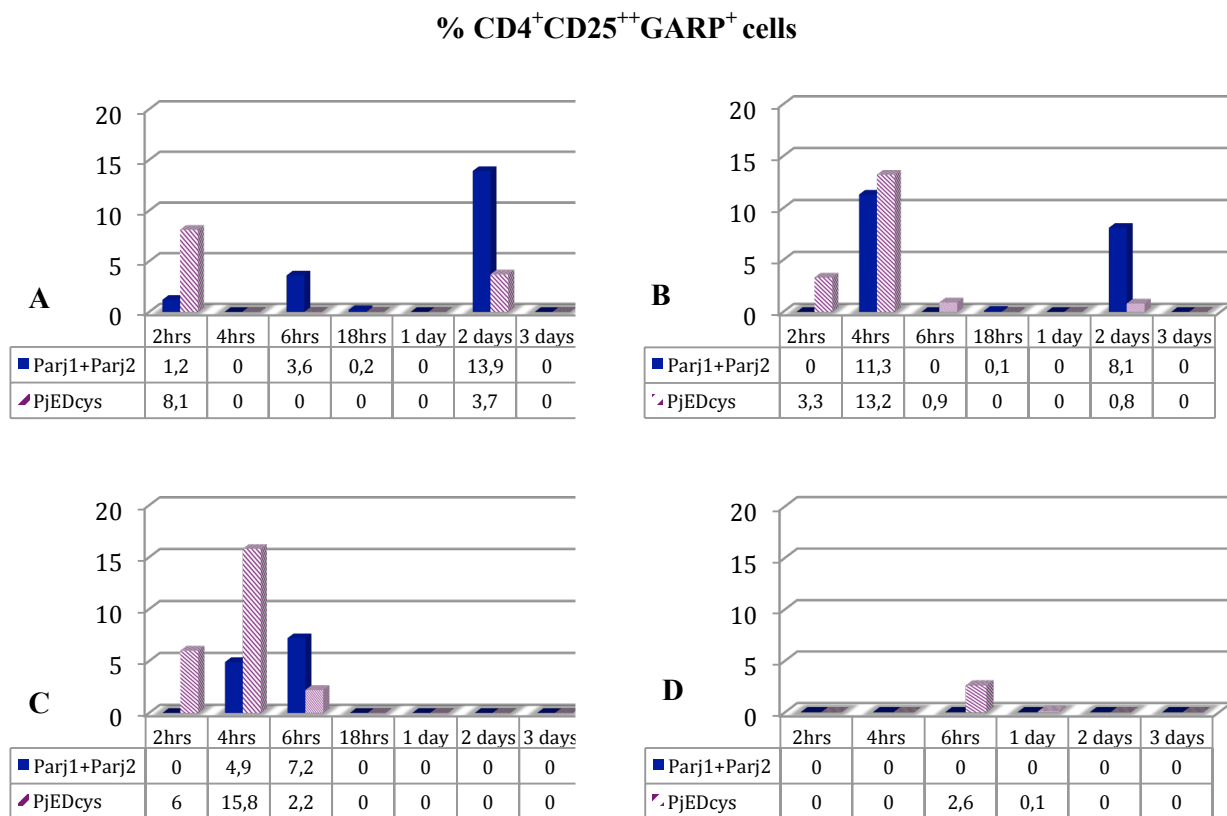


Fig. 47. Histograms of percentage of CD4⁺CD25⁺⁺GARP⁺. Graphics show the percentage of activated CD4⁺CD25⁺⁺GARP⁺ cells in all four analyzed patients (A-D) after *in vitro* stimulation with the mixture of wild type allergens and rPjEDcys, subtracted of the background in the unstimulated culture.

Our analyses on four *Parietaria* allergic patients showed a rapid GARP expression on CD4⁺CD25⁺⁺ cells in response to both antigens. GARP expression increased 2-6 hrs post PBMC

stimulation with rPjEDcys in all four analyzed patients. On the contrary, PBMC stimulation with the mixture of rPar j 1 and rPar j 2 showed that GARP expression started later than that one in rPjEDcys stimulated sample (4-6 hrs post-stimulation). In two out of four analyzed patients (patients 1 and 2) GARP showed a late expression at day 2 and day 3 post-stimulation in response to both antigens. However, in all patients GARP expression in response to the hypoallergenic hybrid and the mixture appears to have a early kinetic of expression.

The histogram reported in panels A-D of figure 48 show the results concerning to the LAP expression in four analyzed patients.

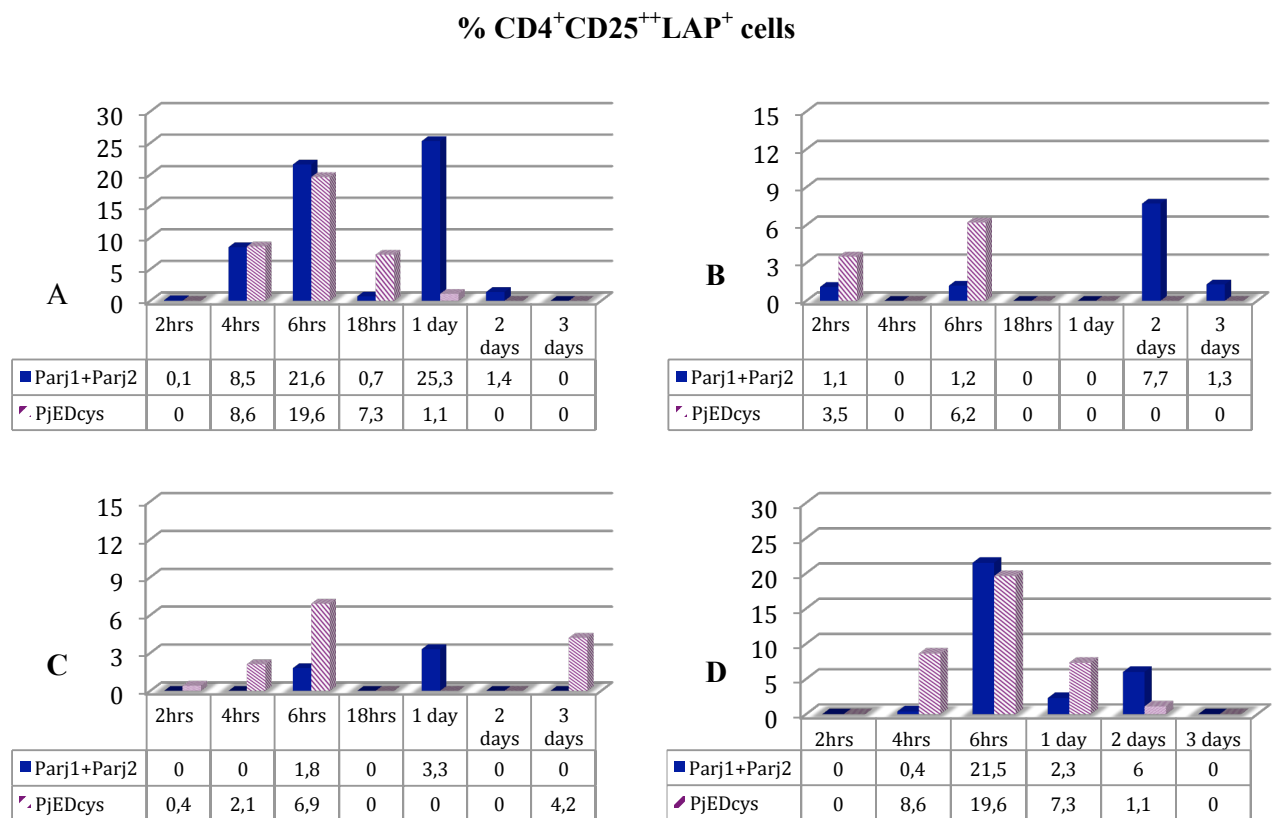


Fig. 48. Histograms of percentage of CD4⁺CD25⁺⁺LAP⁺ cells. Graphics show the percentage of activated CD4⁺CD25⁺⁺ LAP⁺ in four analyzed patients (A-D) after *in vitro* stimulation with the mixture of wild type allergens and rPjEDcys, subtracted of the background in the unstimulated culture.

Our analyses on four *Parietaria* allergic patients showed a rapid LAP expression on CD4⁺CD25⁺⁺ cell surface. LAP expression increased from 4 hrs to 24 hrs post PBMC stimulation with rPjEDcys in all four analyzed patients, and it reached its peak at 6hrs.

Taking together our analyses on LAP and GARP expression on four *Parietaria* allergic patients on CD4⁺CD25⁺⁺ cell surface it appears that the two antigens have a different temporal regulation with an early GARP expression followed by the LAP expression.

The kinetic of LAP expression in response to the mixture of wild type allergens is less definite. Moreover, these results show that PBMC stimulation with rPjEDcys induces an higher percentage of CD4⁺CD25⁺⁺GARP⁺ and CD4⁺CD25⁺⁺LAP⁺ cells than the mixture of wild type allergens.

9. Study of GARP and LAP co-expression on CD4⁺CD25⁺⁺ cells after PBMC stimulation with the mixture of wild type allergens and the hypoallergenic derivate

As, previously shown, PBMC stimulation with the mixture of *wild type* allergens and the hypoallergenic hybrid induced the modulation of GARP and LAP expression on CD4⁺CD25⁺⁺ cells. Starting from our preliminary data, by means of a multiparametric flow cytometry analysis we wished to study the kinetics of co-expression of GARP and LAP, simultaneously to the expression of CD4 and CD25.

To do this, we purified human PBMC from heparinized peripheral blood of three *Parietaria judaica* allergic patients. PBMC were stimulated until 6 hrs with *Parietaria* major allergens and rPjEDcys. Negative control sample was treated without addition of antigens.

Cells were recovered at 2 hrs and 6 hrs, based on results of previous experiments mentioned above, and analyzed for the co-expression of CD4, CD25, GARP and LAP.

Therefore, analyzed cells were selected by a lymphocyte gate (R1) based on the combination of FSC and SSC and were selected also by a R2 gate based on CD4⁺CD25⁺⁺ cell population (CD4/CD25).

Figure 49 shows a flow cytometric analysis on CD4⁺CD25⁺⁺GARP⁺LAP⁺ cells from the representative patient (patient 1).

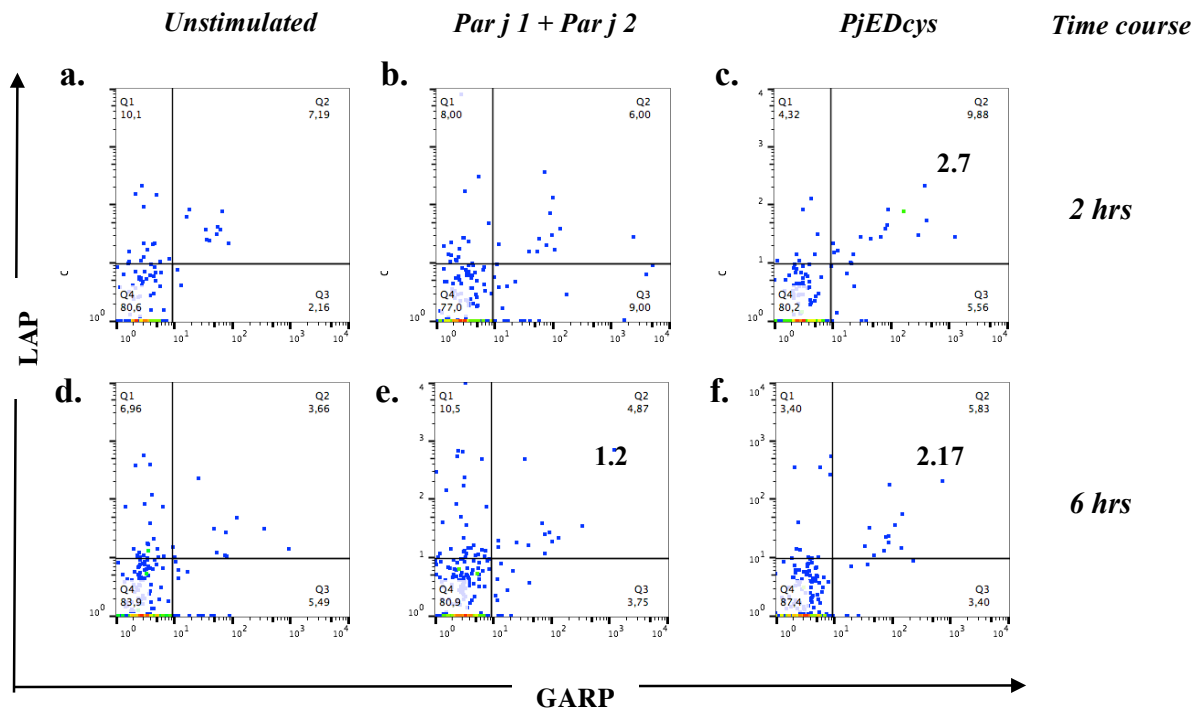


Fig. 49. Representative flow cytometric Dot Plot analysis of CD4⁺ CD25⁺⁺ GARP⁺ LAP⁺ cells. Panels show cytometric analyses of CD4⁺CD25⁺⁺GARP⁺LAP⁺ cells in a representative patient (**patient 1**) after 2 hrs (**a.- c.**) and 6 hrs (**d.- f.**) PBMC *in vitro* stimulation with the mixture of wild type allergens and rPjEDcys. Numbers inside the graphs indicate the percentage of CD4⁺CD25⁺⁺GARP⁺LAP⁺ cells compared to the unstimulated culture.

The results obtained from our analyses show that only the PBMC stimulation for 2 hrs with PjEDcys induced a 2.7% increase in the percentage of CD4⁺CD25⁺⁺GARP⁺LAP⁺ cells compared to unstimulated sample. PBMC stimulation for 6 hrs with both the *wild type* allergens and rPjEDcys induced an increase of GARP and LAP co-expression of 1.7% and 1.8 % respectively. The data reported in panels A-C of figure 50 show the results concerning the GARP and LAP co-expression in all three analyzed patients.

% CD4⁺CD25⁺⁺GARP⁺LAP⁺ cells

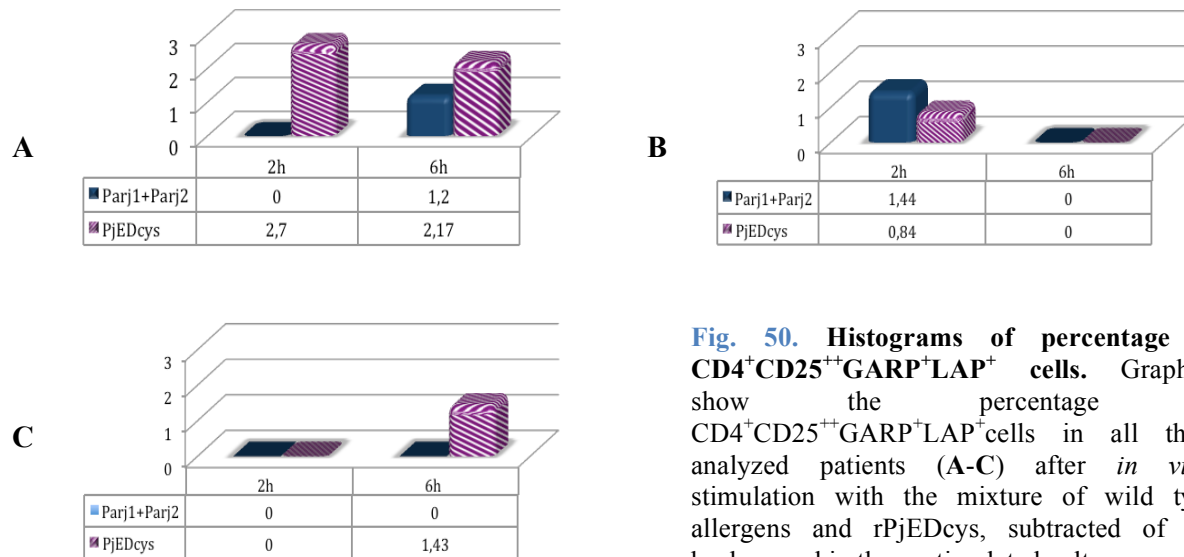


Fig. 50. Histograms of percentage of CD4⁺CD25⁺⁺GARP⁺LAP⁺ cells. Graphics show the percentage of CD4⁺CD25⁺⁺GARP⁺LAP⁺ cells in all three analyzed patients (A-C) after *in vitro* stimulation with the mixture of wild type allergens and rPjEDcys, subtracted of the background in the unstimulated culture.

Our analyses showed that in two out of three analyzed patients (patients 1 and 2), PBMC stimulation with the mixture induced an increase in percentage of CD4⁺CD25⁺⁺ cells co-expressing GARP and LAP molecule compared to the unstimulated sample. In all three analyzed patients, PBMC stimulation with rPjEDcys induced an increase in percentage of CD4⁺CD25⁺⁺GARP⁺LAP⁺ compared to the unstimulated sample.

Taking together our analyses on LAP and GARP co-expression on CD4⁺CD25⁺⁺ cells in all three *Parietaria* allergic patients showed that PBMC stimulation with hybrid increases always the GARP and LAP co-expression at 2hrs or 6hrs depending on the individual response. On the contrary, PBMC stimulation with the mixture induced the co-expression of GARP and LAP on CD4⁺CD25⁺⁺ cells in two out of three patients (patients 1 and 2).

10. Analysis of CD39 expression on CD4⁺ cells in response to *Parietaria* major allergens and rPjEDcys

In the previous experiments we studied the selection of Tregs after PBMC stimulation with the mixture of *wild type* allergens and rPjEDcys. At the beginning, we evaluated the modulation of CD25 and CD127 expression on CD4⁺ cells from allergic patients by means of

immunophenotyping. A major limitation in the study of regulatory T cells is a lack of unique cell surface markers. Recent paper suggest to study the human Treg cells based on the expression of functional markers [127-129, 132]. We already studied the kinetic expression of two functional markers, as GARP and LAP on CD4⁺CD25⁺⁺ cells. Since Treg cells employ multiple immunoregulatory mechanisms, I decided to study also the adenosine pathway.

In fact, a growing body of evidence supports an important role for the Treg activities mediated by CD39/CD73 adenosine pathway in regulating immune response.

CD39 is a cell surface ecto-enzyme that regulate levels of ATP and adenosine in extracellular microenvioroment [132].

This ecto-enzyme mediates principally anti-inflammatory effects by dephosphorylating ATP into ADP and then into AMP. AMP produced by CD39 is then converted into adenosine by another ecto-enzyme, as CD73 [133]. CD39 is uniformly and highly expressed by CD4⁺CD25⁺⁺ T cells with suppression activities and it may participate in the immunoregulatory role of Treg cells.

CD39 has been considered critical in the generation of immunosuppressive microenvironments through adenosine production as shown in several diseases, such as autoimmunity, cancer, allergy and allograft rejection. In the immune system, adenosine inhibits functions of innate and adaptive immune cells and it is considered to be a crucial anti-inflammatory factor [133-136].

In order to study the modulation of CD39 on CD4⁺ cell induced by *in vitro* stimulation with the mixture or rPjEDcys, PBMC were stimulated for a time course of 8 days (as shown in Materials and Methods). As usual, the negative control sample was treated without addition of antigens. Cells were recovered at 2h, 4h, 6h, 24h and then every 24 hours until day 8 and analyzed for the expression of CD4 and CD39 by flow cytometry.

Therefore, analyzed cells were selected by a live lymphocyte gate based on the combination of FSC and SSC and were selected also by a gate based on the expression of CD4 (SSC/CD4⁺) (data no shown).

Figure 51 shows a flow cytometric analysis on CD4⁺ CD39⁺ cells from a representative patient (patient 1).

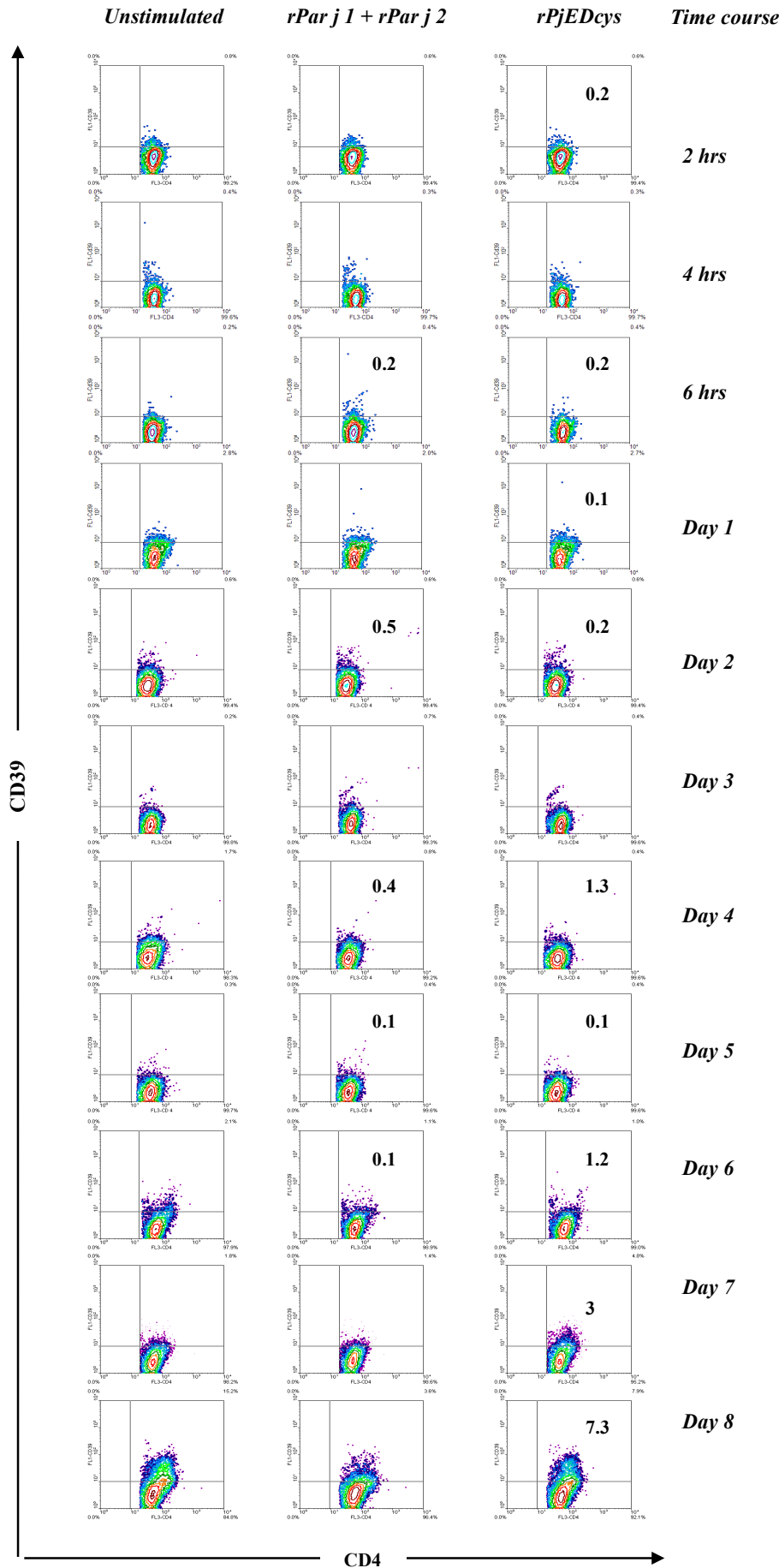


Fig. 51. Representative flow cytometric Dot Plot analysis of CD4⁺ CD39⁺ cells. Panels show cytometric analyses of CD4⁺CD39⁺ cells in a representative patient (**patient 1**) after *in vitro* stimulation with the mixture of wild type allergens and rPjEDcys. Numbers inside the graphs indicate the percentage of CD4⁺CD39⁺ cells compared to the unstimulated culture.

From these analyses we showed that CD4⁺CD39⁺ population started to increase from day 4 and reached its peak at day 8, only after PBMC stimulation with rPjEDcys. These analyses highlight that PBMC stimulation with the mixture of wild type allergens did not induce an increase in percentage of CD4⁺CD39⁺ compared to unstimulated sample.

This pattern of activation was observed in two analyzed patients with some differences during the time course of stimulation (Fig. 52). The pattern of activation reached the peak at day 8, in both analyzed patients.

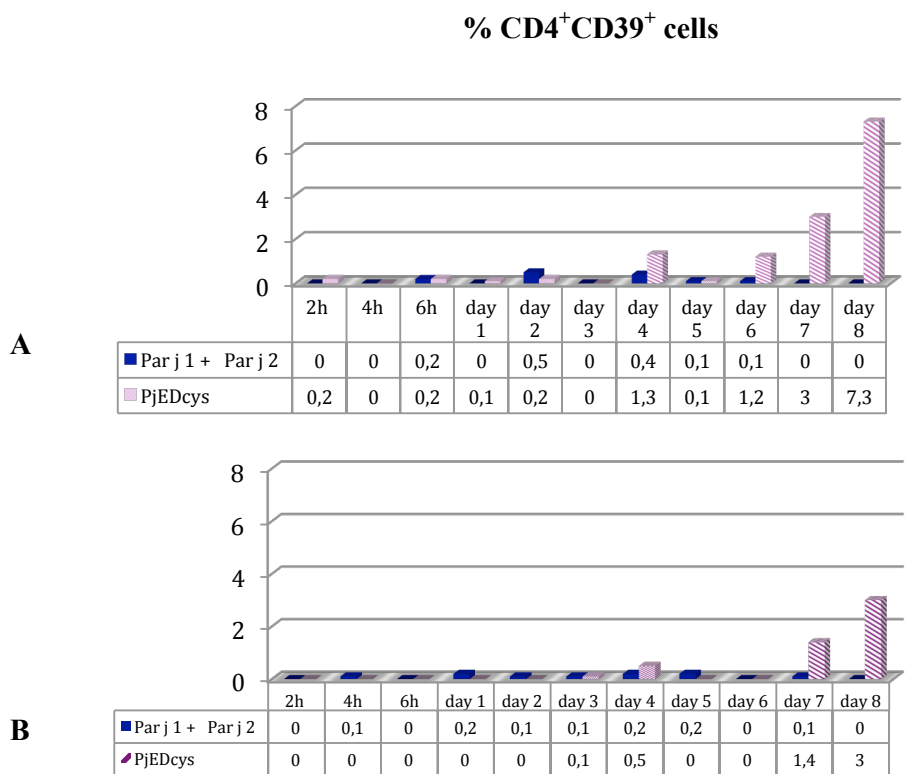


Fig. 52. Histograms of percentage of CD4⁺CD39⁺ cells. Graphics show the percentage of CD4⁺CD39⁺ cells in two analyzed patients (A-B) after *in vitro* stimulation with the mixture of wild type allergens and rPjEDcys, subtracted of the background in the unstimulated culture.

11. Evaluation of putative cross-talk between two suppressive pathways of human Treg cells

The expression of CD39 and CD73 on Treg cells and the capacity to convert ATP into adenosine has been suggested as one of Treg suppressive actions. Recent data indicated a possible cross-talk between regulatory pathways, as the connection between the TGF- β and the adenosine pathways [137-138]. Mouse studies have shown that the addition of TGF- β to cultures of activated splenic T cells demonstrated a clear effect on CD39 and CD73 expression. In fact, the up-regulation of both antigens was enhanced in the presence of TGF- β ; possibly leading to the amplification of the adenosine pathway in a microenvironments enriched with this cytokine [137]. In 2015, Mann et al confirmed the connection between the two suppressive pathways in human; a significant proportion of CD4⁺ T cells co-express CD73 with GARP and LAP [138].

Therefore, I wanted to investigate if the PBMC stimulation with the mixture of *Parietaria judaica* major allergens or the hypoallergenic derivate induced the cross-talk between the two regulatory pathways. In particular, we were interested in studying the co-expression of CD39, the first enzyme involved in adenosine pathway, with GARP and LAP molecules on the surface of CD4⁺ T cells. To do this, PBMC from two *Parietaria judaica* allergic patients were stimulated for a time course of 8 days (as shown in Materials and Methods). The negative control sample was treated without addition of antigens. Cells were recovered at 2 hrs, 4 hrs, 6 hrs, 24 hrs and then every 24 hours until day 8 and analyzed for the expression of CD4, CD39, GARP or LAP by flow cytometry.

Therefore, analyzed cells were selected by a live lymphocyte gate based on the combination of FSC and SSC and were selected also by a gate based on the expression of CD4 (SSC/CD4⁺) and a gate based on the expression of GARP or LAP (SSC/GARP or SSC/LAP⁺).

Figure 53 shows a flow cytometric analysis on CD4⁺ CD39⁺ GARP⁺ cells from a representative patient (patient 1) during the PBMC early stimulation, from 2 hrs until 3 days. This is the same time course followed—during the GARP and LAP expression analysis on CD4⁺CD25⁺⁺ cells, as suggested in literature [128].

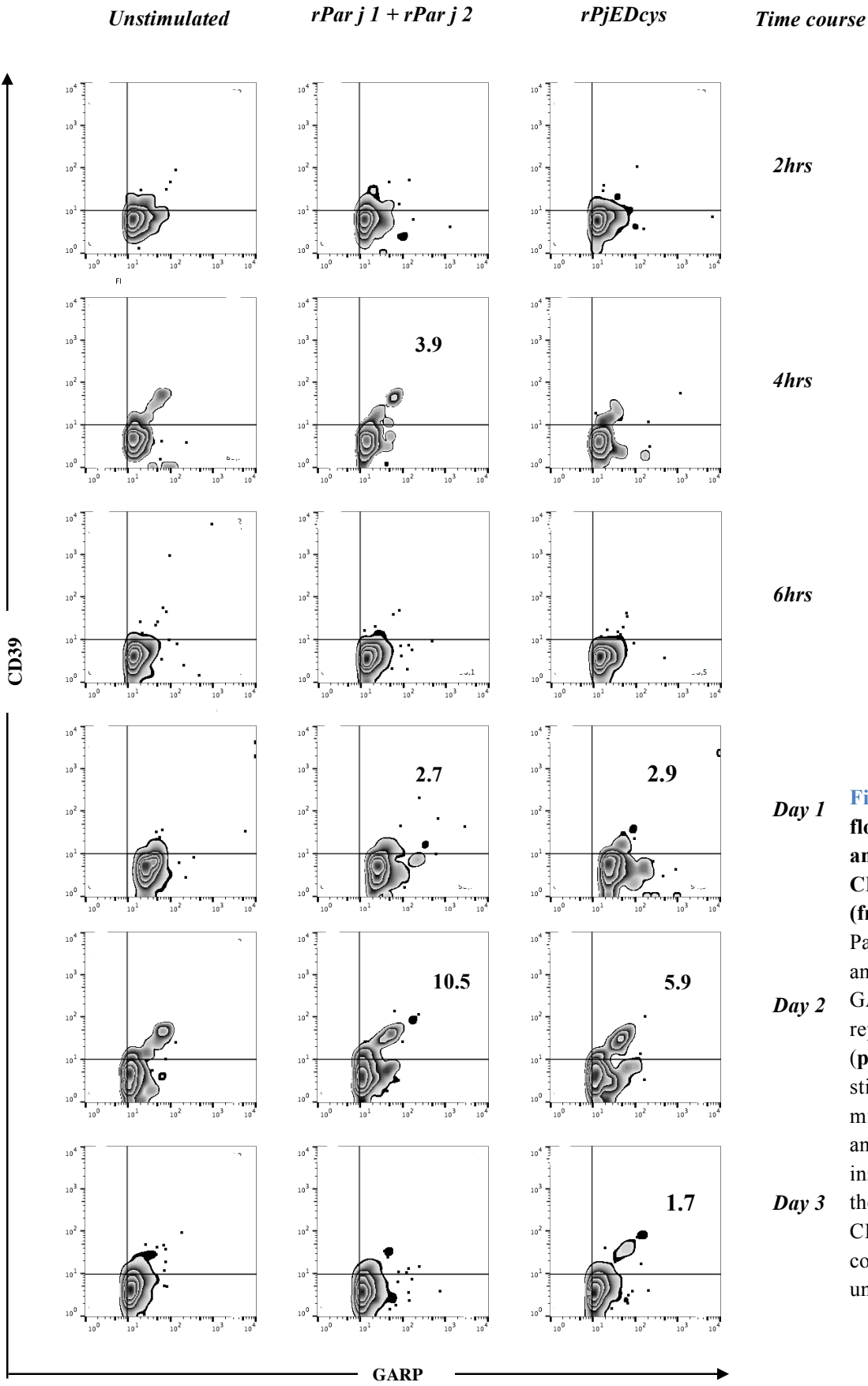


Fig. 53. Representative flow cytometric Dot Plot analysis of CD4⁺CD39⁺GARP⁺ cells (from 2 hrs until day 3). Panels show cytometric analyses of CD4⁺CD39⁺GARP⁺ cells in a representative patient (patient 1) after *in vitro* stimulation with the mixture of w.t. allergens and rPjEDcys. Numbers inside the graphs indicate the percentage of CD4⁺CD39⁺GARP⁺ cells compared to an unstimulated culture.

The analyses on early PBMC stimulation for this representative patient have shown that the PBMC stimulation with the mixture of w.t. allergens and the rPjEDcys increase the percentage of cells co-expressing CD39 and GARP. We observed a low increase in percentage of cells co-expressing CD39 and GARP molecule after 2hrs of stimulation in the sample stimulated with the mixture and 1 and 3 days after PBMC stimulation with both mixture and rPjEDcys.

However, data in literature define CD39 as activation marker expressed only by activated Treg cells [139]. Moreover, our data showed that CD4⁺CD39⁺ population started to increase from day 4 and reached its peak at day 8 after PBMC *in vitro* stimulation with rPjEDcys.

Therefore, we decided to analyze the co-expression of GARP and CD39 also for a longer time-course of stimulation, in fact our analyses were continued until day 8.

Panels of figure 54 show the cytofluorimetric analyses of CD4⁺CD39⁺GARP⁺ cells after *in vitro* stimulation with the mixture of wild type allergens or the hypoallergenic rPjEDcys and in an unstimulated culture, from day 4 until day 8.

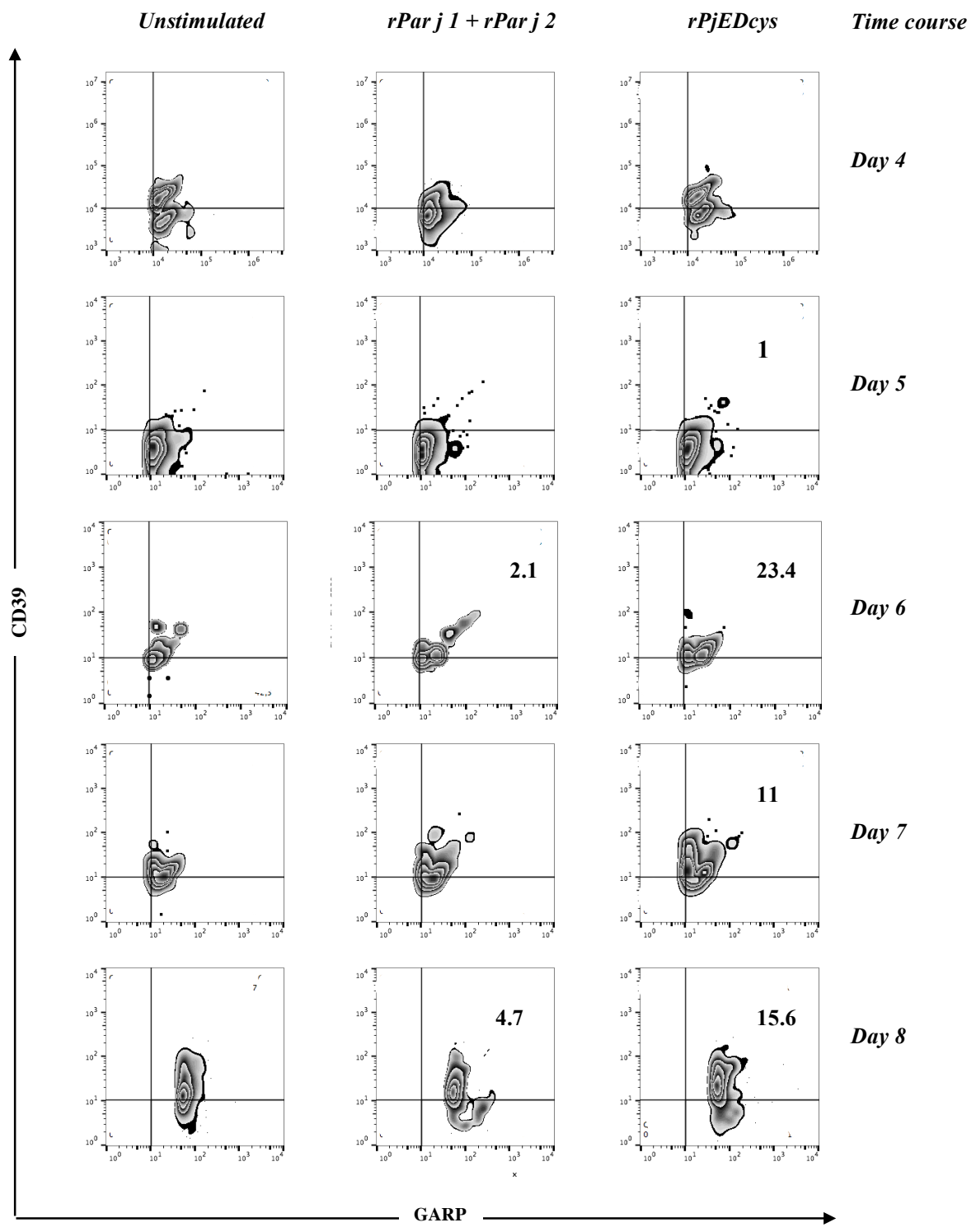


Fig. 54. Representative flow cytometric Dot Plot analysis of CD4⁺ CD39⁺ GARP⁺ cells (from day 4 through 8). Panels show cytometric analyses of CD4⁺ CD39⁺ GARP⁺ cells in a representative patient (patient1) after *in vitro* stimulation with the mixture of w.t. allergens and rPjEDcys. Numbers inside the graphs indicate the percentage of CD4⁺ CD39⁺ GARP⁺ cells compared to an unstimulated culture.

Long-term PBMC stimulation with the mixture and rPjEDcys significantly increased the percentage of CD4⁺ cells co-expressing both CD39 and GARP molecules compared to an unstimulated sample. From these analyses we observed that CD4⁺CD39⁺GARP⁺ population began to increase from day 6 through day 8. These analyses highlight that the co-expression of CD39 and GARP is more significant in the sample stimulated with rPjEDcys compared to the *wild type* allergen stimulation.

The histograms reported in panels A-B of figure 55 show the data concerning the percentage of CD4⁺ cells co-expressing GARP and CD39 molecule in two analyzed patients.

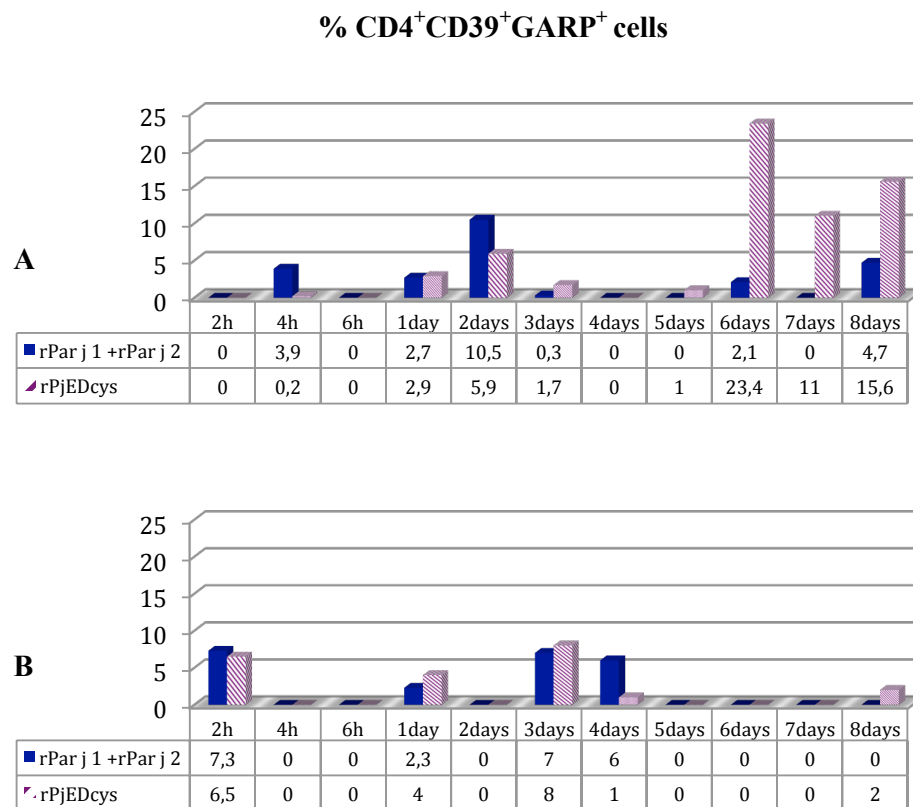


Fig. 55. Histograms of percentage of CD4⁺CD39⁺GARP⁺ cells. Graphics show the percentage of activated CD4⁺CD39⁺GARP⁺ cells in two analyzed patients (A-B) after *in vitro* stimulation with the mixture of wild type allergens and rPjEDcys, subtracted of the background in the unstimulated culture.

Our analyses show that in one out of two patient (patient 1) PBMC stimulation with the mixture of w.t. allergens induces the co-expression of GARP and CD39 on CD4⁺ cells compared to unstimulated sample. The percentage of cells co-expressing both molecules is induced at 4hrs and at 24 and 48hrs after stimulation and it increases again at day 6. Analysis on patient 1 showed that PBMC stimulation with the hypoallergenic derivate, induces a significant increase in percentage of CD4⁺CD39⁺ GARP⁺ cells which starts at day 1 until day 3 after *in vitro* stimulation. Moreover, this increase reach a more significative value from day 6 to day 8. Nevertheless, the co-expression of both molecules is higher and more significant in the sample stimulated with rPjEDcys compared to the mixture.

The analyses on patient 2 have showed that PBMC stimulation, at 2hrs and then between day 1 and day 4, with both the mixture and the rPjEDcys induced a high increase in percentage of CD4⁺CD39⁺ GARP⁺ compared to unstimulated sample. On the contrary, at day 8, only PBMC stimulation with rPjEDcys induced an increase of GARP and CD39 co-expression on CD4⁺ cells. Also in patient 2 PBMC stimulation with rPjEDcys induced an higher percentage of CD4⁺CD39⁺ GARP⁺ cells than those induced by the mixture.

Furthermore, we also analyzed the co-expression of CD39 and LAP molecules on CD4⁺ cells.

Figure 56 shows a flow cytometric analysis on CD4⁺ CD39⁺ LAP⁺ cells from the same representative patient (patient 1) in the early time course, from 2 hrs until day 3.

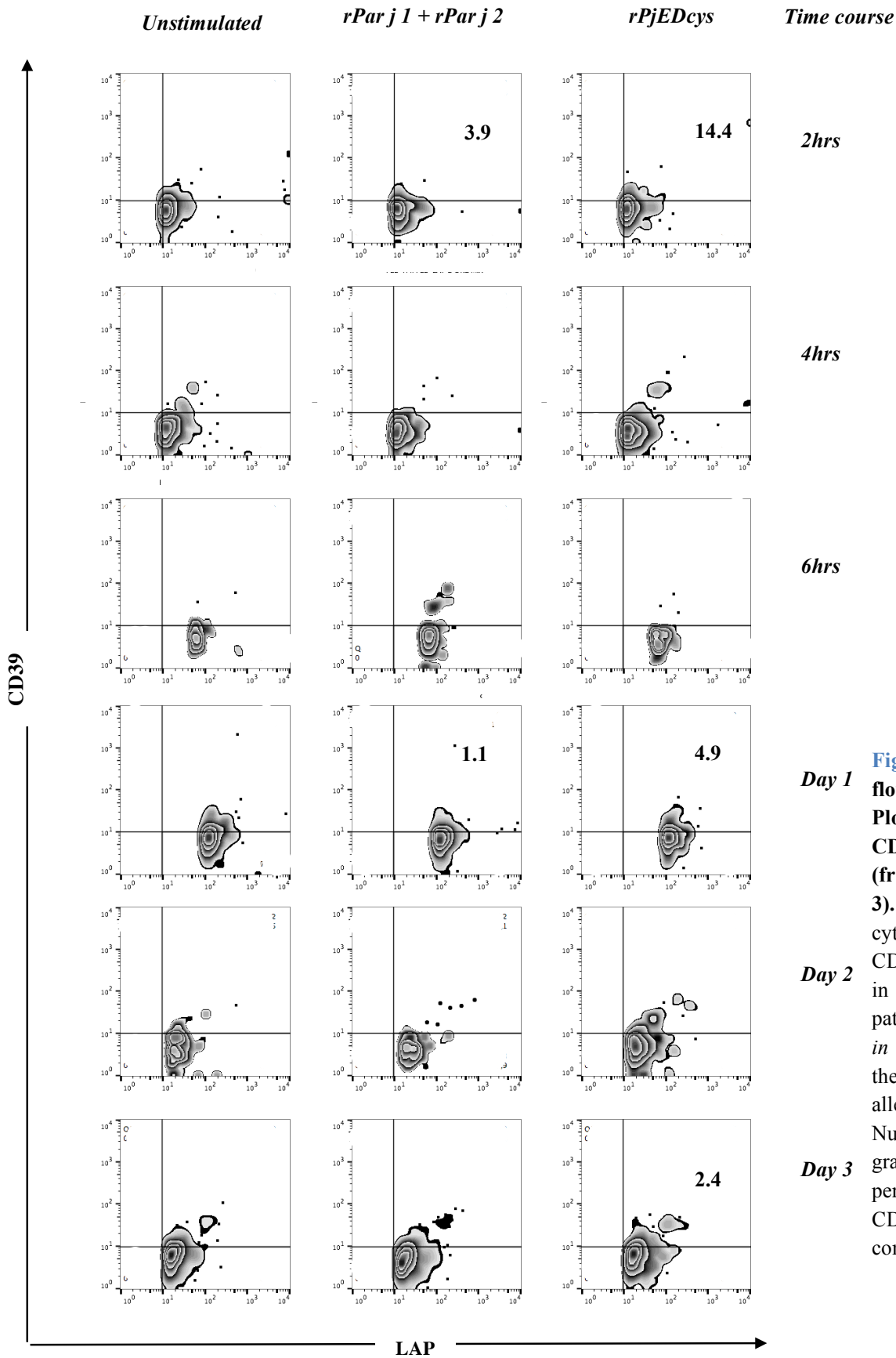


Fig. 56. Representative flow cytometric Dot Plot analysis of CD4⁺CD39⁺LAP⁺ cells (from 2 hrs until day 3). Panels show cytometric analyses of CD4⁺ CD39⁺ LAP⁺ cells in a representative patient (**patient 1**) after *in vitro* stimulation with the mixture of w.t. allergens and rPjEDcys. Numbers inside the graphs indicate the percentage of CD4⁺CD39⁺ LAP⁺ cells compared to an

Cytofluorimetric analyses of CD39 and LAP co-expression on CD4⁺ cells have shown that the co-expression of both these functional markers is induced at significant levels in an early PBMC stimulation with the mixture or rPjEDcys. Moreover, it is higher in the sample stimulated with the hypoallergenic hybrid. PBMC stimulation with the rPjEDcys increased the percentage of CD4⁺CD39⁺LAP⁺ cells 1 day after stimulation compared to an unstimulated sample (1.1% and 4.9% respectively). At day 3, only PBMC stimulation with rPjEDcys induced an increase of LAP and CD39 co-expression on CD4⁺ cells.

The LAP and CD39 co-expression on CD4⁺ cells was also investigated in a longer PBMC stimulation time with both antigens. Figure 57 shows a flow cytometric analysis on CD4⁺CD39⁺LAP⁺ cells from the same representative patient (patient 1) from 4 until 8 days after PBMC stimulation.

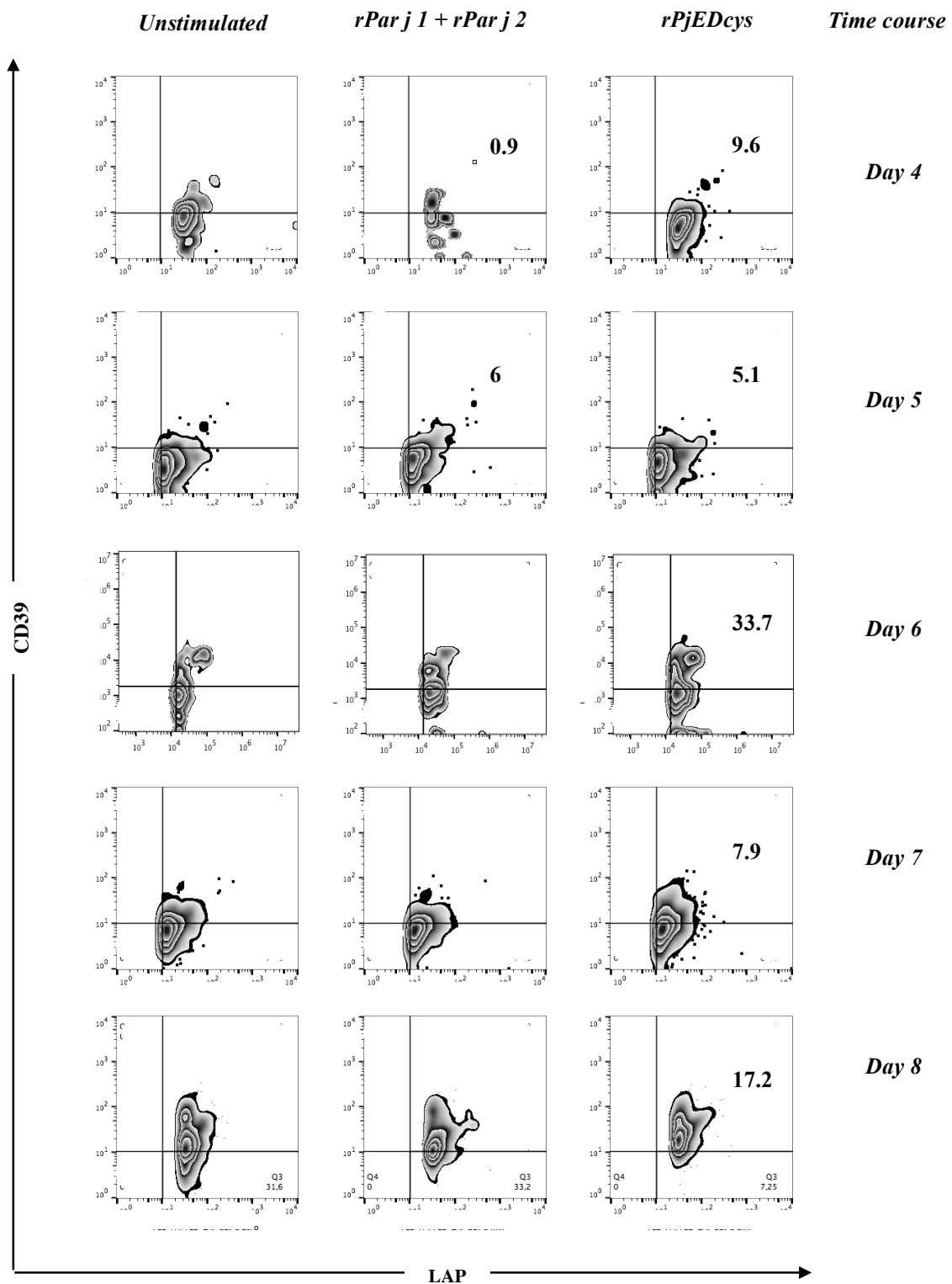


Fig. 57. Representative flow cytometric Dot Plot analysis of CD4⁺ CD39⁺ LAP⁺ cells (from day 4 through 8). Panels show cytometric analyses of CD4⁺ CD39⁺ LAP⁺ cells in a representative patient (patient 1) after *in vitro* stimulation with the mixture of w.t. allergens and rPjEDcys. Numbers inside the graphs indicate the percentage of CD4⁺CD39⁺ LAP⁺ cells compared to an unstimulated culture.

Our analyses for this representative patient (patient 1) have shown that the PBMC stimulation with the mixture of w.t. allergens and the rPjEDcys increased the percentage of cells co-expressing CD39 and LAP. These analyses highlight that PBMC stimulation with the mixture of w.t. allergens induced an increase in percentage of CD4⁺CD39⁺LAP⁺ cells in comparison to unstimulated sample. This increase was observed 4 days after PBMC stimulation until day 5. On the other hand, rPjEDcys was able to induce a more significative increase in the percentage of CD4⁺ cells co-expressing CD39 and LAP from day 4 through day 8.

The histograms reported in panels A-B of figure 58 shows the data concerning to the percentage of CD4⁺ cells co-expressing LAP and CD39 molecule in two analyzed patients.

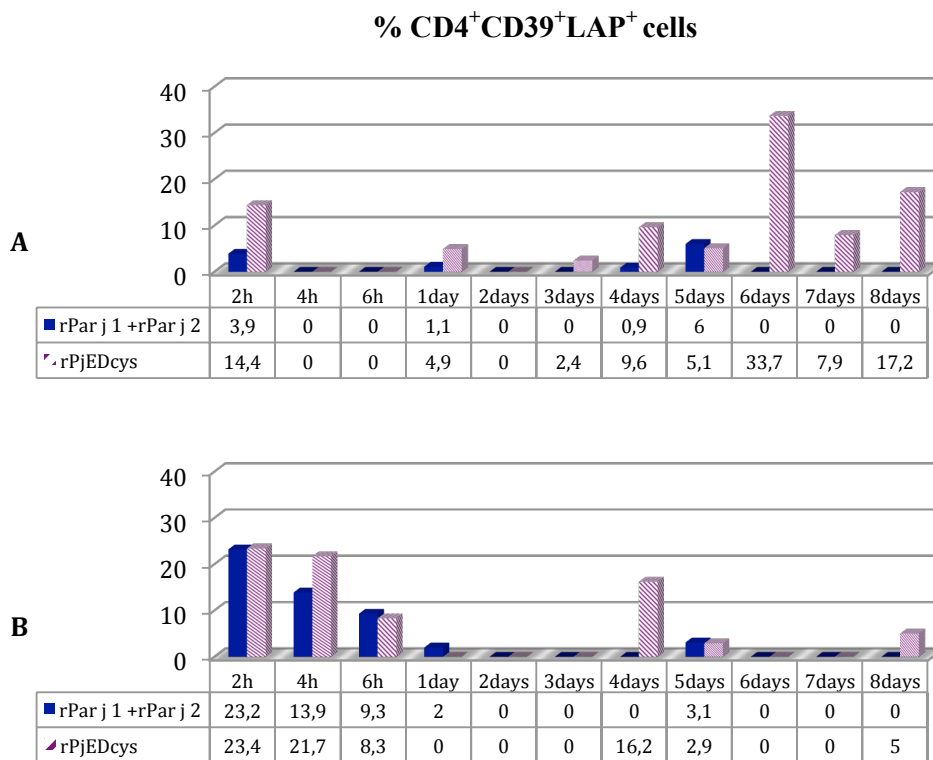


Fig. 58. Histograms of percentage of CD4⁺CD39⁺LAP⁺ cells. Graphics show the percentage of activated CD4⁺CD39⁺LAP⁺ cells in two analyzed patients (A-B) after *in vitro* stimulation with the mixture of wild type allergens and rPjEDcys, subtracted of the background in the unstimulated culture.

Our analysis of LAP and CD39 co-expression on CD4⁺ cells showed that in one out of two analyzed patients (patient 1) the PBMC stimulation for 2hrs with both mixture and the hypoallergen induced an increase in percentage of CD4⁺CD39⁺LAP⁺. At day 5, PBMC

stimulation with the mixture induce an increase in the percentage of these cells. However, from day 3 until day 8, only PBMC stimulation with rPjEDcys induced a significant increase of LAP and CD39 co-expression on CD4⁺ cells. Analyses on patient 2 have shown an early increase of LAP CD39 co-expression, between 2 and 6 hrs, which was higher in response to the rPjEDcys. The data on two patients demonstrated that PBMC stimulation with both the mixture and the hypoallergenic derivative induced an increase of CD39 and LAP co-expression on CD4⁺ cells compared to unstimulated sample. However, the co-expression of both molecules is higher and more significant in the sample stimulated with rPjEDcys compared to the mixture.

12. Analysis of PD1 expression on CD4⁺ CD25⁺⁺ cells in response to Parietaria major allergens and rPjEDcys

It has been reported that T reg play an important role in allergic diseases. Modulation of functions of Tregs may provide a novel strategy to prevent and treat allergic diseases. In recent years, it is recognized that Tregs cells play different inhibitory pathways to prevent hypersensitive immune responses and the underlying sensitization to allergens. Recent studies are just beginning to elucidate the role of PD1 pathway in allergy and asthma. PD1 is an immunoinhibitory receptor that belongs to the CD28 family with a critical role in the regulation of T cell activation. It has also been shown that PD-1 is up-regulated on T cells upon activation, and its ligands have distinct expression patterns, PD-L1 is expressed much more abundantly than PD-L2. In order to study the modulation of PD1 on CD4⁺ cell induced by *in vitro* stimulation with the mixture or rPjEDcys, PBMC from two allergic patients were stimulated for a time course of 8 days. Cells were recovered at 2h, 4h, 6h, 24h and then every 24 hours until day 8 and analyzed for the expression of CD4 and CD39 by flow cytometry. Therefore, analyzed cells were selected by a live lymphocyte gate based on the combination of FSC and SSC and were selected also by a gate based on the expression of CD4 and CD25 (CD4⁺/CD25⁺) (data not shown).

Figures 59 and 60 show the cytofluorimetric analyses related to the PD1 expression on CD4⁺CD25⁺⁺ cells.

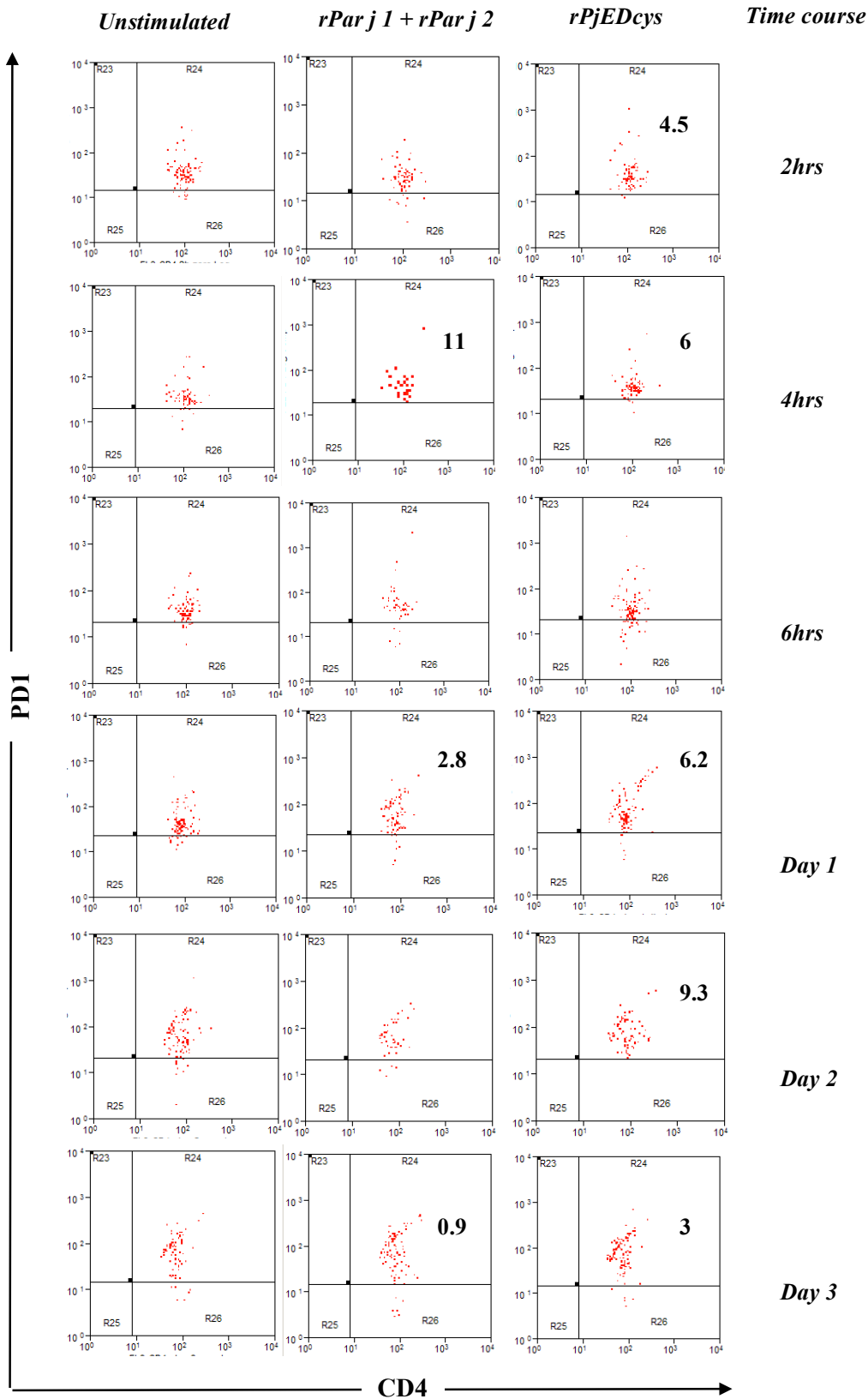


Fig. 59. Representative flow cytometric Dot Plot analysis of CD4⁺CD25⁺⁺PD1⁺ cells (from 2hrs until day 3). Panels show cytometric analyses of CD4⁺CD25⁺⁺PD1⁺ cells in a representative patient (**patient 1**) after *in vitro* stimulation with the mixture of w.t. allergens and rPjEDcys. Numbers inside the graphs indicate the percentage of CD4⁺CD25⁺⁺PD1⁺ cells compared to an unstimulated culture.

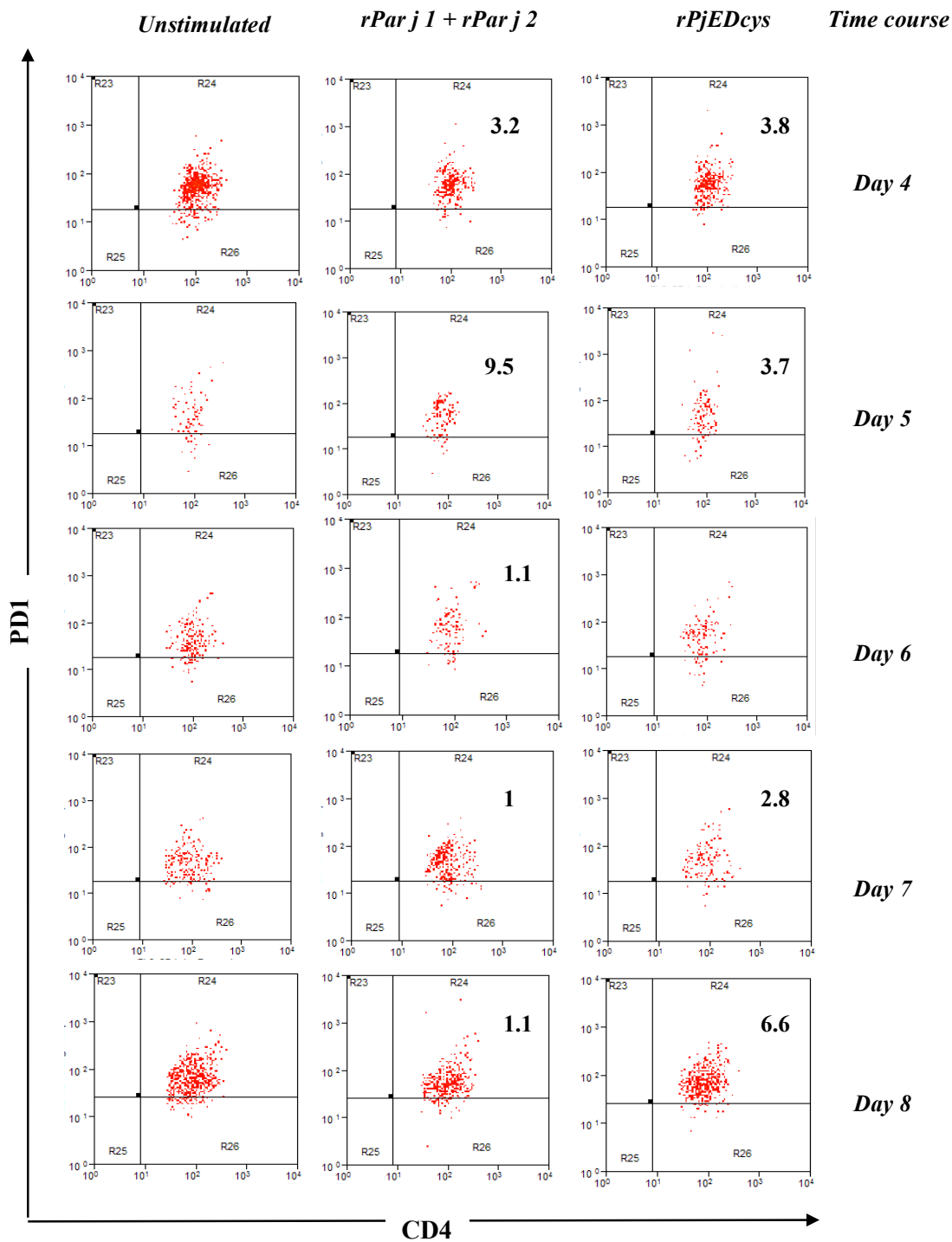


Fig. 60. Representative flow cytometric Dot Plot analysis of CD4⁺CD25⁺⁺PD1⁺ cells (from day 4 through 8). Panels show cytometric analyses of CD4⁺CD25⁺⁺PD1⁺ cells in a representative patient (patient 1) after *in vitro* stimulation with the mixture of w.t. allergens and rPjEDcys. Numbers inside the graphs indicate the percentage of CD4⁺CD25⁺⁺PD1⁺ cells compared to an unstimulated culture.

We observed an increase in the percentage of PD-1-expressing cells in CD4⁺CD25⁺⁺ cells after PBMC stimulation with the mixture and rPjEDcys. This increase was observed both in short than long time course of stimulation and it is generally higher after the PBMC stimulation with the hybrid rPjEDcys than wild type allergens.

The histograms reported in panels A-B of figure 61 shows the data concerning to the percentage of CD4⁺CD25⁺⁺PD1⁺ cells in two analyzed patients.

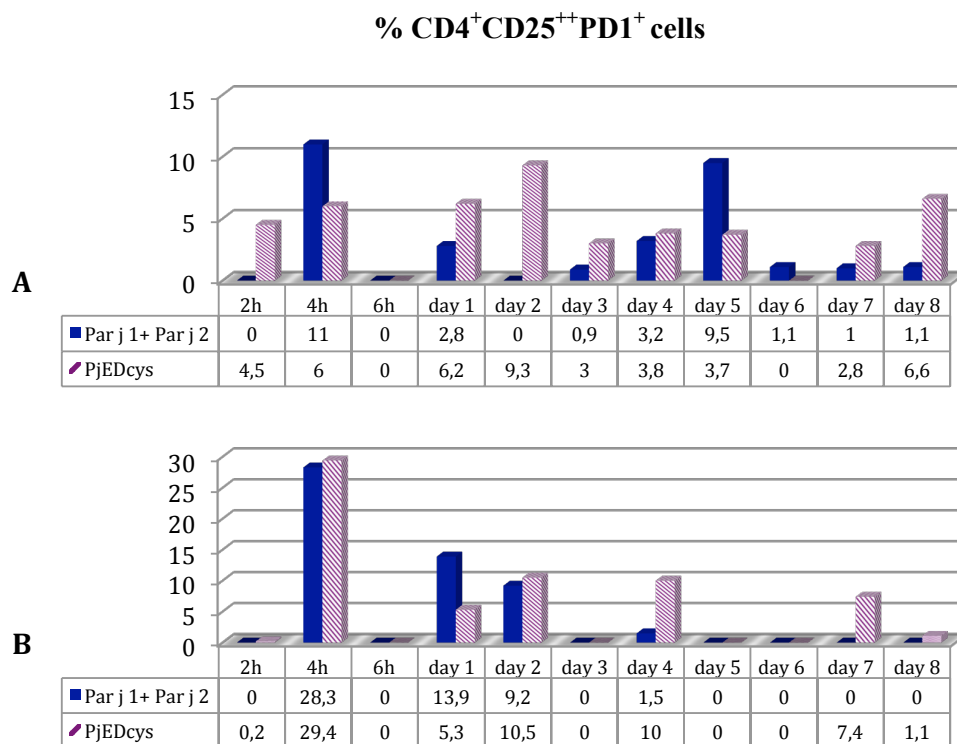


Fig. 61. Histograms of percentage of CD4⁺CD25⁺⁺PD1⁺ cells. Graphics show the percentage of activated CD4⁺CD25⁺⁺PD1⁺ cells in two analyzed patients (A-B) after *in vitro* stimulation with the mixture of wild type allergens and rPjEDcys, subtracted of the background in the unstimulated culture.

The increase in percentage of CD4⁺CD25⁺⁺PD1⁺ cells after PBMC stimulation with both antigens has been observed in two analyzed patients; this was higher in response to rPjEDcys.

Starting from the evidence that the number of CD4⁺CD25⁺⁺PD1⁺ cells induced after PBMC stimulation with the mixture and rPjEDcys is very low, only 0.1 to 0.5% of the total cells, we decided to measure the Mean Fluorescence Intensity (MFI) of PD1 expression in CD4⁺CD25⁺⁺ cells.

Figure 61 shows the histograms related to the intensity of PD1 expression in CD4⁺ CD25⁺⁺ cells in two analyzed patients.

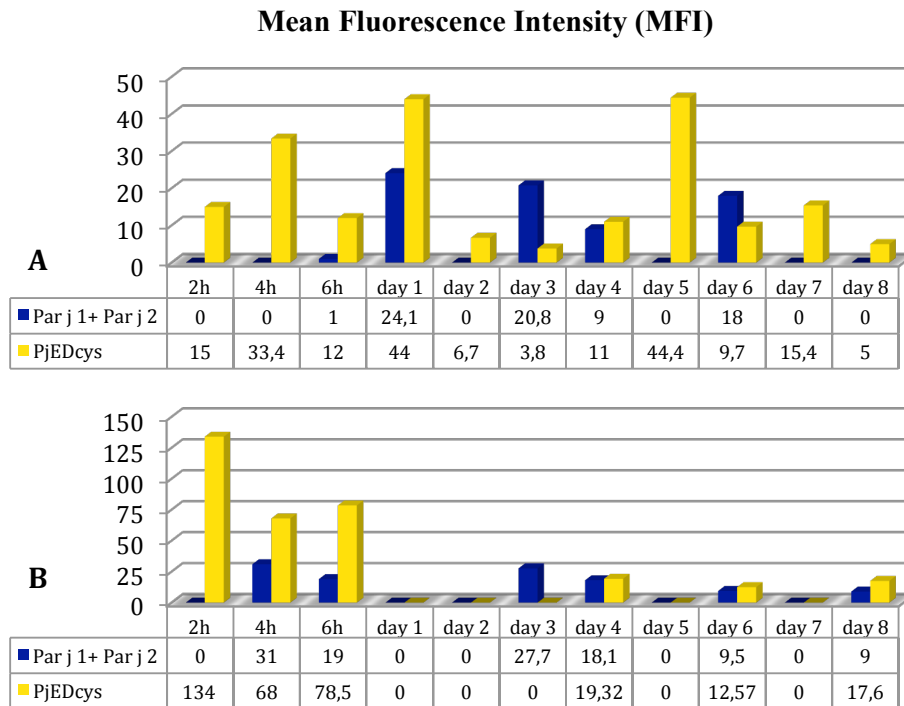


Fig. 62. Histograms of value of MFI of PD1 expression in CD4⁺CD25⁺⁺ cells. Graphics show the value of MFI of PD1 expression in CD4⁺CD25⁺⁺ cells in two analyzed patients (A-B) after *in vitro* stimulation with the mixture of wild type allergens and rPjEDcys, subtracted of the background in the unstimulated culture.

In these experiments we show an increase in number of CD4⁺ CD25⁺⁺ PD1⁺ cells after PBMC stimulation with rPjEDcys than w.t. allergens. Moreover, the MFI of PD1 expression in these cells after the *in vitro* stimulation with the rPjEDcys is significantly higher.

We used the coefficient of variation (CV), also known as relative standard deviation to determine the statistical significance of PD1 expression on CD4⁺ CD25⁺⁺ (CV < 5).

Discussion

Allergy is an immunological disorder affecting about 30% of the population living in the industrialized countries. The etio-pathogenesis of allergy is complex and influenced by many and different factors, including the genetic variation among individuals, the allergen dose and time of exposure and the structural characteristics of the allergens. Several clinical treatments could

alleviate the symptoms arising from the allergens; however, allergen immunotherapy is the only valid treatment able to modify the underlying pathological mechanisms of immune response and to have a long-lasting effect. SIT is a form of immunotherapy for allergic disorders in which the patient is vaccinated with gradually increasing doses of an allergen with the aim of inducing immunological tolerance. The molecular mechanisms involved in successful immunotherapy are not fully understood and several factors seem to influence the immune response such as the concentration of the allergen, the type of antigen-presenting cell and the type of adjuvant used for the formulation of vaccine. Several clinical evidences suggest the advantages of using recombinant allergens for immunotherapy [99]. Recombinant allergens can be produced as molecules with the same properties to their native counterparts or natural allergens (i.e., recombinant wild-type allergens); or as modified variants with advantageous properties such as reduced allergenic activity and increased immunogenicity; for example, as hybrid molecules resembling the epitopes of several different allergens to include the relevant epitopes of complex allergen sources. Moreover, recombinant allergens offer the possibility to use well-defined molecules with consistent pharmaceutical quality defined in mass units.

Parietaria judaica is the main allergenic source in the Mediterranean area. It has a long period of pollination, starting in the spring and lasting up to the autumn. Par j 1 and Par j 2 were classified as the two major allergens of *Parietaria* pollen and display some cross-reactive and independent IgE epitopes. Therefore the generation of a recombinant single protein comprising the major allergens of *Parietaria* pollen and containing their main B-cell and T-cell epitopes in one pharmaceutical product may reduce the production costs, providing a therapy that has the aim of modifying allergen-specific immune response towards the two major allergens of *Parietaria* pollen. This recombinant hybrid was previously produced and its low allergenic activity was demonstrated by Bonura et al. [118].

In order to test other properties of this protein, the immunogenicity of the hypoallergenic hybrid was studied *in vitro*, looking at the main immunological mechanisms involved in allergic inflammation and immune tolerance after specific immunotherapy (SIT).

We studied the PBMC proliferative response after the *in vitro* stimulation with a mixture containing the two recombinant (wild-type) *Parietaria* allergens (rPar j 1 and rPar j 2) and the hypoallergenic hybrid, rPjEDcys. Our data showed CD4⁺ cells are clearly the major cell population proliferating in response to the mixture of wild-type allergens and to the rPjEDcys. The percentage of CD4⁺ proliferating cells in response to rPjEDcys is higher when used at lower than

high concentration. Furthermore, the hypoallergenic hybrid scored a percentage of CD4⁺ proliferating cells higher than the wild type allergens when used at the lower concentration.

A few reports demonstrated that allergens are inducers of both the innate and acquired immune systems. We were able to demonstrate that components of innate immune system (CD56⁺ cells) are activated both by the mixture of *Parietaria* allergens and by the rPjEDcys hybrid, leading to the proliferation of these cells in allergic patients.

Starting from these results, we decided to look at the cytokines produced by T CD4⁺ cells upon an antigen-specific stimulation. In fact, once activated, CD4⁺ T cells play a main role in the regulation of immune response through the secretion of specific cytokines.

Allergic individuals generally show a Th2 biased immune response and several studies have shown that immune response to allergens in healthy individuals is characterized by the Treg (IL-10 secreting) and Th0/Th1 immune polarization [80, 140-141]. Healthy and allergic individuals display the same allergen-specific T cell subsets (Th1, Th2 and Tregs) but with different proportions. The main difference between healthy and allergic individuals is the nature of the immune response, which is characterized by a different balance between allergen-specific T regulatory cells and allergen-specific Th2 cells [141].

In these experimental settings, we were able to demonstrate that PBMC from *Parietaria* allergic patients stimulated with the wild type allergen mixture was predominantly of the Th2 type (IL-5 and IL-13). IL-5 secretion assays showed that PBMC from seven out of ten patients stimulated with rPjEDcys induced a lower percentage of IL-5 secreting T CD4⁺ cells compared to wild type allergen stimulation. These results are very interesting because IL-5 has been shown to play an important role in allergic diseases, including allergic rhinitis and asthma. Actually many studies identify IL-5 as a primary therapeutic target for the improvement of the treatment of allergy.

Moreover, in eight out of the ten patients analyzed, rPjEDcys induces a lower percentage of IL-13 secreting CD4⁺ cells, compared to that induced by wild type allergens.

This is another encouraging result because IL-13 has been proposed to be a cytokine mainly involved in the chronicity of allergic inflammation, and because in vivo studies in mouse model of allergic disorders showed that inhibition of IL-13 could be therapeutically beneficial.

The data on INF- γ and IL-10 secretion assays performed on CD4⁺ cells have shown a high individual variability and surely other studies have to be performed to further investigate the role of these cytokines in the immune response to the mixture of *Parietaria* major allergens and rPjEDcys.

These cytokines are particularly relevant since they may represent a redirection of the allergen-specific Th2 response towards a protective Th1 immune response and establish an allergen-specific immune tolerance [56, 121-122]. It is known that a wide range of cellular types produce IFN- γ and IL-10. Moreover, these cytokines regulate negatively the production of IL-5 and IL-13 by CD4⁺ cells [108, 120].

In recent years, numerous studies about the mechanism of immune tolerance have been performed in order to define new therapeutic protocols for the prevention and the treatment of several diseases including the allergic inflammation. The pivotal role of Tregs in inducing and maintaining peripheral immune tolerance and more generally in the control of both innate and adaptive immune response has been demonstrated in several immune diseases [19, 28, 79, 145]. These cells are important in maintaining protection against tumors, viral infections, allergen inflammations and transplantations. Defects in their function can induce immunological disorders and therefore the possibility to study Treg function could have many therapeutic potential applications. Many studies have been carried out to characterize Treg phenotypes and biological activity. Recent evidences have shown that allergen specific immunotherapy is the only valid treatment that leads to the reversal of the allergic disease and to the induction of a long tolerance and restoration of the normal immune response to allergens [80]. The induction of T regulatory cells with a T regulatory phenotype seems to be a critical event in a successful allergen specific immunotherapy. These cells use several modes of immune regulation to achieve tolerance: cytokines mediated mechanisms, metabolic disruption, cell contact dependent mechanisms and cytotoxicity.

In order to study the selection of an allergen-specific subset of Tregs, first PBMC of allergic patients were cultured with the mixture of wild type allergens or with the rPjEDcys hybrid, and were analyzed for the expression of CD25 on CD4⁺ cells. CD25 is expressed by Tregs and also by activated effector T cells, but the density of CD25 expression allows us to discriminate between these two T cell populations.

Our analysis has shown that the CD25 antigen was upregulated on CD4⁺ cells after the *in vitro* stimulation with *Parietaria* major allergens and with the hypoallergenic derivative. Furthermore, hypoallergenic hybrid induced an increase the number of cells with high-density expression of CD25 higher than wild type allergens. Following this line of evidence, we sought to characterize the allergen-specific Treg activated after the *in vitro* stimulation. Nevertheless, the analysis based on the density of CD25 expression allowed us to discriminate between regulatory and activated effector T cells and other markers have been helpful in this discrimination. Moreover, only after

the *in vitro* stimulation with hypoallergenic hybrid, the Mean Fluorescence Intensity (MFI) of CD25 expression in CD4⁺ cells increased compared to an unstimulated culture. These results suggested the selection of a Treg subset in response to rPjEDcys.

Therefore, in addition to high expression of CD4 and CD25, Treg cells were also characterized by low or not expression of CD127. Several papers have identified CD127 as an excellent marker of Treg cells in human peripheral blood. The cell surface marker is expressed at low levels on pTreg cells and distinguishes the CD4⁺ T cells as potential Treg cells. The demonstration of a selective increase of CD4⁺ CD25⁺⁺ T cells that do not express CD127 provides an interesting result. It suggests the induction of a putative Treg subset.

In these experimental settings, we were able to demonstrate that PBMC from *Parietaria* allergic patients stimulated with the wild type allergen and with hypoallergenic hybrid increased the percentage of CD4⁺CD25⁺⁺ CD127⁻ cells. Moreover, the rPjEDcys induced a more significative increase in the percentage of CD4⁺CD25⁺⁺CD127⁻ compared to the mixture of wild type allergens. In order to characterize these CD4⁺CD25⁺⁺CD127⁻ cells, these were isolated by magnetic separation using a two-step procedure. The enriched Treg fractions were purified and total RNA was extracted for a subsequent molecular characterization of enriched CD4⁺ CD25⁺⁺ CD127⁻ cells. We decided to study the level of mRNA expression of FoxP3, IL-10, GARP and TGF-β on the enriched CD4⁺ CD25⁺⁺ CD127⁻ cell fraction. Our analyses on FoxP3 and IL-10 mRNA levels showed that PBMC stimulation with mixture and rPjEDcys induced an increase in mRNA level expression. For the most part, except for one patient, the levels of FoxP3 and IL-10 mRNA were higher in response to rPjEDcys than the mixture. Our analyses on TGF-β did not show significant difference among the various samples. Instead, the RTq-PCR analyses on GARP showed very low levels and high variability within the same patient or between analyzed patients. The latter observation was confirmed by recently data published by Battaglia et al. which suggest the almost undetectable GARP mRNA expression in CD4⁺ T cells, meanwhile the kinetics of GARP expression varies depending on the subset of pTreg cells. Moreover it was suggested a post-transcriptional regulation of GARP expression [35, 128]. In many Treg subsets the level of GARP mRNA increases during the first 6h and decrease at 24h. Moreover, some T helper clones could have high level of GARP mRNA, comparable to that of Tregs. A major limit in the study of Tregs is the absence of specific cell surface markers, but the discovery of potential markers for activated antigen-specific Tregs encouraged the study of these cells looking at the their function.

In line with these data, we decided to study GARP and LAP expression. Our results showed a rapid expression of GARP on CD4⁺ CD25⁺⁺ cell surface 2-6h post-stimulation and a late expression 2 days post-stimulation in response to both antigens. On the other hand, studies on LAP expression showed an heterogeneous response to the mixture and PjEDcys in our population, showing a LAP increase at 2 or 4h followed by a downregulation to resting level 24 hours after stimulation or a second increase at 6 or 24 hours after stimulation (and 3 days after stimulation with hypoallergen). Moreover, using a multiparametric analysis we demonstrate the co-expression of both molecules on CD4⁺ CD25⁺⁺ cell surface after PBMC stimulation with both the w.t. mixture and rPjEDcys. Although the main mechanism by which Tregs mediate immune suppression and promote tolerance is cytokine-mediated, Tregs employ multiple immunoregulatory mechanisms. Therefore we were interested to further characterize the selection of Treg subsets by studying other functional markers of Treg cells. A growing body of evidence supports an important role for the Treg activities mediated by CD39/CD73 adenosine pathway in regulating immune response. CD39 (ENTPD1) and CD73 (ecto-5'-nucleotidase) are two cell surface ecto-enzymes that regulate levels of ATP and adenosine in extracellular microenvironment [132-133]. For these reasons, in order to study the activation of the adenosine pathway by allergen-specific pTreg cells, PBMC of allergic patients were cultured with the mixture of wild type allergens or with the rPjEDcys hybrid, and were analyzed for the expression of CD4 and CD39. Our analyses showed that PBMC *in vitro* stimulation with rPjEDcys induced a more significant increase in percentage of CD4⁺CD39⁺ cells compared to unstimulated sample and the mixture of wild type allergens. Recent data indicated a possible cross-talk between regulatory pathways; these suggested the connection between the TGF- β and the adenosine pathways, in both human and mouse immune system [132-133].

With the aim to investigate if this mechanism is also induced by PBMC stimulation with the mixture and rPjEDcys, we studied the co-expression of CD39 with GARP and LAP on CD4⁺ cells. In summary, our preliminary data demonstrated that rPjEDcys induced a more significant increase in percentage of CD4⁺ cells co-expressing CD39 and GARP or CD39 and LAP than those induced by wild type mixture of allergens.

Finally, we started to study another functional marker of Tregs, as PD1, our data showed the modulation of PD1 in PBMC stimulated with the mixture and rPjEDcys. However, the percentage of CD4⁺ CD25⁺⁺ PD1⁺ cells induced by PBMC stimulation with rPjEDcys is higher than those induced by allergens. Moreover, the MFI of PD1 expression in these cells after the *in vitro* stimulation with the rPjEDcys was significantly higher.

Conclusion

In conclusion, the collective studies performed during my PhD work have analyzed different immunological mechanisms involved in allergic responses to environmental allergens and associated with allergen specific immunotherapy.

Our data suggested that the hypoallergenic hybrid, rPjEDcys expressing disulphide bond variants of Par j 1 and Par j 2, could be an improved tool for a new form of allergy vaccination, as:

- It retains the immunogenicity, such the ability to induce both innate and adaptive immune response;
- It shows a reduced allergenic activity, such reduction of the secretion of two Th2 cytokines that are critical in the development of allergy such as IL-5 and IL-13;
- It appears to induce the selection of regulatory cell subsets which are characterized by the expression of functional markers as GARP, LAP, CD39 and PD1.

Further investigations of these aspects will be useful to elucidate the mechanisms by which rPjEDcys may skew the immune system and consequently reduce the inflammatory allergic response.

The knowledge of the basic mechanisms of allergic response may thus help the development of new therapies. Improvement in understanding and strategies for allergen specific immunotherapy depend on knowledge of immunological mechanisms.

References

1. Abbas AK, Lichtman AH, Pillai S. **Cellular And Molecular Immunology**, Elsevier, Philadelphia. 7th edition, 2011.
2. C.A. Janeway , Jr P. Travers, M. Walport, M.J. Shlomchik **Immunobiology**. 8Th edition. 2011
3. Chaplin DD. **Overview of the immune response**. J Allergy Clin Immunol. 2010 Feb; 125(2 Suppl 2):S3-23.
4. Kawai T, Akira S. **The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors**. Nat Immunol. 2010 May; 373-384 Epub 2010 Apr 20
5. Kollmann T.R., Levy O., Montgomery R.R., Goriely S. **Innate Immune Function by Toll-like Receptors: Distinct Responses in Newborns and the Elderly**. Immunity. 2012 Nov 16; 37(5): 771–783.
6. Aderem A, Ulevitch RJ. **Toll-like receptors in the induction of the innate immune response**. Nature. 2000 Aug 17;406(6797):782-7.
7. Cooper MA, Fehniger TA, Caligiuri MA. **The biology of human natural killer-cell subsets**. Trends Immunol. 2001 Nov;22(11):633-40.

8. Davis CT, Rizzieri D. **Immunotherapeutic applications of NK cells.** *Pharmaceuticals (Basel)*. 2015 May 25;8(2):250-6.
9. Braumüller H, Wieder T, Brenner E, Aßmann S, Hahn M, Alkhaled M, Schilbach K, Essmann F, Kneilling M, Griessinger C, Ranta F, Ullrich S, Mocikat R, Braungart K, Mehra T, Fehrenbacher B, Berdel J, Niessner H, Meier F, van den Broek M, Häring HU, Handgretinger R, Quintanilla-Martinez L, Fend F, Pesic M, Bauer J, Zender L, Schaller M, Schulze-Osthoff K, Röcken M. **T-helper-1-cell cytokines drive cancer into senescence.** *Nature*. 2013 Feb 21;494(7437):361-5.
10. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. **Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.** 1986. *J Immunol*. 2005 Jul 1;175(1):5-14.
11. Zhu J, Paul WE. **CD4 T cells: fates, functions, and faults.** *Blood*. 2008 Sep 1;112(5):1557-69.
12. Luckheeram RV1, Zhou R, Verma AD, Xia B. **CD4⁺T cells: differentiation and functions.** *Clin Dev Immunol*. 2012;2012:925135.
13. Yagi R, Zhu J, Paul WE. **An updated view on transcription factor GATA3-mediated regulation of Th1 and Th2 cell differentiation.** *Int Immunol*. 2011 Jul;23(7):415-20.
14. Yagi R, Junttila IS, Wei G, Urban JF Jr, Zhao K, Paul WE, Zhu J. **The transcription factor GATA3 actively represses RUNX3 protein-regulated production of interferon-gamma.** *Immunity*. 2010 Apr 23;32(4):507-17.
15. Zeng WP. **'All things considered': transcriptional regulation of T helper type 2 cell differentiation from precursor to effector activation.** *Immunology*. 2013 Sep;140(1):31-8.
16. Robinson KM, Manni ML, Biswas PS, Alcorn JF. **Clinical consequences of targeting IL-17 and TH17 in autoimmune and allergic disorders.** *Curr Allergy Asthma Rep*. 2013 Dec;13(6):587-95.
17. Akintunde ME, Rose M, Krakowiak P, Heuer L, Ashwood P, Hansen R, Hertz-Picciotto I, Van de Water J. **Increased production of IL-17 in children with autism spectrum disorders and co-morbid asthma.** *J Neuroimmunol*. 2015 Sep 15;286:33-41.
18. Ueno A, Ghosh A, Hung D, Li J, Jijon H. **Th17 plasticity and its changes associated with inflammatory bowel disease.** *World J Gastroenterol*. 2015 Nov 21;21(43):12283-95.
19. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. **Regulatory T cells and immune tolerance.** *Cell*. 2008 May 30;133(5):775-87.
20. Yang S, Fujikado N, Kolodin D, Benoist C, Mathis D. **Immune tolerance. Regulatory T cells generated early in life play a distinct role in maintaining self-tolerance.** *Science*. 2015 May 1;348(6234):589-94.
21. Eisenstein EM, Williams CB. **The T(reg)/Th17 cell balance: a new paradigm for autoimmunity.** *Pediatr Res*. 2009 May;65(5 Pt 2):26R-31R.
22. Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, Jiang S, Kuchroo VK, Mathis D, Roncarolo MG, Rudensky A, Sakaguchi S, Shevach EM, Vignali DA, Ziegler SF. **Regulatory T cells: recommendations to simplify the nomenclature.** *Nat Immunol*. 2013 Apr;14(4):307-8. doi: 10.1038/ni.2554.
23. Lastovicka J. **The Phenotypic Markers of CD4⁺CD25⁺ T Regulatory Lymphocytes.** *Research in*

Immunology: An International Journal Vol. 2013 (2013)

24. Yu N, Li X, Song W, Li D, Yu D, Zeng X, Li M, Leng X, Li X. **CD4(+)CD25 (+)CD127 (low/-) T cells: a more specific Treg population in human peripheral blood.** *Inflammation*. 2012 Dec;35(6):1773-80.
25. Peterson RA. **Regulatory T-Cells: Diverse Phenotypes Integral to Immune Homeostasis and Suppression.** *Toxicol Pathol*. 2012;40(2):186-204.
26. Hori S. **The FoxP3 interactome: a network perspective of Treg cells.** *Nat Immunol*. 2012 Oct;13(10):943
27. Liu R, Li S, Yang WH, Wang L. **IPEX Syndrome, FOXP3 and Cancer.** *J Syndr*. 2013 Jun;1(1). pii: 7.
28. Ramsdell F, Ziegler SF. **FOXP3 and scurfy: how it all began.** *Nat Rev Immunol*. 2014 May;14(5):343-9.
29. Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. **Genome-wide analysis of FoxP3 target genes in developing and mature regulatory T cells.** *Nature*. 2007 Feb 22;445(7130):936-40.
30. Curotto de Lafaille MA1, Lafaille JJ. **Natural and Adaptive FoxP3+ Regulatory T Cells: More of the Same or a Division of Labor?** *Immunity*. 2009 May;30(5):626-35.
31. Vignali, D. **How many mechanisms do regulatory T cells need?** *Eur. J. Immunol*. 2008 38, 908–911.
32. Raker VK, Domogalla MP, Steinbrink K. **Tolerogenic Dendritic Cells for Regulatory T Cell Induction in Man.** *Front Immunol*. 2015 Nov 9;6:569.
33. Gregori S, Goudy KS, Roncarolo MG. **The cellular and molecular mechanisms of immuno-suppression by human type 1 regulatory T cells.** *Front Immunol*. 2012 Feb 29;3:30.
34. Castro-Sánchez P, Martín-Villa JM. **Gut immune system and oral tolerance.** *Immunol Rev*. 2001 Aug;182:207-14. *Br J Nutr*. 2013 Jan;109 Suppl 2:S3-11.
35. Stockis J, Colau D, Coulie PG, Lucas S. **Membrane protein GARP is a receptor for latent TGF- β on the surface of activated human Treg.** *Eur J Immunol*. 2009 Dec;39(12):3315-22.
36. Tran DQ, Andersson J, Wang R, Ramsey H, Unutmaz D, Shevach EM. **GARP (LRRC32) is essential for the surface expression of latent TGF- β on platelets and activated FOXP3+ regulatory T cells.** *Proc Natl Acad Sci U S A*. 2009 Aug 11;106(32):13445-50.
37. Wang R, Kozhaya L, Mercer F, Khaitan A, Fujii H, Unutmaz D. **Expression of GARP selectively identifies activated human FOXP3+ regulatory T cells.** *Proc Natl Acad Sci U S A*. 2009 Aug 11;106(32):13439-44.
38. Miller MM, Fogle JE, Ross P, Tompkins MB. **Feline Glycoprotein A Repeats Predominant Anchors Transforming Growth Factor Beta on the Surface of Activated CD4 + CD25 + Regulatory T Cells and Mediates AIDS Lentivirus-Induced T Cell Immunodeficiency.** *AIDS Res Hum Retroviruses*. 2013 Apr;29(4):641-51.
39. Dhamne C, Chung Y, Alousi AM, Cooper LJ, Tran DQ. **Peripheral and thymic FoxP3+ regulatory T cells in search of origin, distinction, and function.** *Front Immunol*. 2013 Aug 27;4:253.
40. Muller T, Robaye B, Vieira RP et al. **The purinergic receptor P2Y2 receptor mediates chemotaxis of dendritic cells and eosinophils in allergic lung inflammation.** *Allergy* 2010; 65:1545– 53.
41. Muller T, Vieira RP, Grimm M et al. **A potential role for P2X7R in allergic airway inflammation in mice and humans.** *Am J Respir Cell Mol Biol* 2011; 44:456–64.
42. Manthei DM, Jackson DJ, Evans MD et al. **Protection from asthma in a high-risk birth cohort by attenuated P2X(7) function.** *J Allergy Clin Immunol* 2012; 130:496–502

43. Bono MR, Fernández D, Flores-Santibáñez F, Roseblatt M, Sauma D. **CD73 and CD39 ectonucleotidases in T cell differentiation: Beyond immunosuppression.** FEBS Lett. 2015 Nov 14;589(22):3454-60. doi: 10.1016/j.febslet.2015.07.027. Epub 2015 Jul 29.
44. Haskó G, Linden J, Cronstein B, Pacher P. **Adenosine receptors: therapeutic aspects for inflammatory and immune diseases.** Nat Rev Drug Discov. 2008 Sep;7(9):759-70
45. Bousquet J. **Global Initiative for Asthma (GINA) and its objectives.** Clin Exp Allergy 2000;30(Suppl. 1):2-5.
46. Jin HT, Ahmed R, Okazaki T. **Role of PD-1 in regulating T-cell immunity.** Curr Top Microbiol Immunol. 2011;350:17-37
47. Dolan DE, Gupta S. **PD-1 Pathway Inhibitors: Changing the Landscape of Cancer Immunotherapy .** Cancer Control. 2014 Jul;21(3):231-7.
48. Dai S, Jia R, Zhang X, Fang Q, Huang L. **The PD-1/PD-Ls pathway and autoimmune diseases.** Cell Immunol. 2014 Jul;290(1):72-9.
49. Singh AK, Stock P, Akbari O. **Role of PD-L1 and PD-L2 in allergic diseases and asthma.** Allergy. 2011 Feb;66(2):155-62.
50. Akbari O, Stock P, Singh AK, Lombardi V, Lee WL, Freeman GJ, Sharpe AH, Umetsu DT, Dekruyff RH. **PD-L1 and PD-L2 modulate airway inflammation and iNKT-cell- dependent airway hyperreactivity in opposing directions.** Mucosal Immunol. 2010 Jan;3(1):81-91.
51. Johansson SG, Hourihane JO, Bousquet J, Brujnzeel-Koomen C, Dreborg S, Haahtela T, Kowalski ML, Mygind N, Ring J, van Cauwenberge P, van Hage-Hamsten M, Wüthrich B; **EAACI (the European Academy of Allergology and Clinical Immunology) nomenclature task force. A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force.** Allergy. 2001 Sep;56(9):813-24.
52. AB Ka. **Overview of 'Allergy and allergic diseases: with a view to the future.** Br Med Bull. 2000;56(4):843-64.
53. Galli SJ, Tsai M, Piliponsky AM. **The development of allergic inflammation.** Nature. 2008 Jul 24;454(7203):445-54
54. Taher YA, Henricks PA, van Oosterhout AJ. **Allergen-specific subcutaneous immunotherapy in allergic asthma: immunologic mechanisms and improvement.** Libyan J Med. 2010 Jun 21;5.
55. Kaiser J. **IMMUNOLOGY. How farm life prevents asthma.** Science. 2015 Sep 4;349(6252):1034
56. Liu AH. **Revisiting the hygiene hypothesis for allergy and asthma.** J Allergy Clin Immunol. 2015 Oct;136(4):860-5.
57. Mattes J, Yang M, Mahalingam S, Kuehr J, Webb DC, Simson L, Hogan SP, Koskinen A, McKenzie AN, Dent LA, Rothenberg ME, Matthaei KI, Young IG, Foster PS. **Intrinsic defect in T cell production of interleukin (IL)-13 in the absence of both IL-5 and eotaxin precludes the development of eosinophilia and airways hyperreactivity in experimental asthma.** J Exp Med. 2002 Jun 3;195(11):1433-44.
58. Foster PS, Martinez-Moczygemba M, Huston DP, Corry DB. **Interleukins-4, -5, and -13: emerging therapeutic targets in allergic disease.** Pharmacol Ther. 2002 Jun;94(3):253-64.

59. Skrindo I, Ballke C, Gran E, Johansen FE, Baekkevold ES, Jahnsen FL. **IL-5 production by resident mucosal allergen-specific T cells in an explant model of allergic rhinitis.** Clin Exp Allergy. 2015 Aug;45(8):1296-304
60. Corren J. **Inhibition of interleukin-5 for the treatment of eosinophilic diseases.** Discov Med.2012 Apr;13(71):305-12.
61. Menzella F, Lusuardi M, Galeone C, Taddei S, Zucchi L. **Profile of anti-IL-5 mAb mepolizumab in the treatment of severe refractory asthma and hypereosinophilic diseases** J Asthma Allergy. 2015 Oct 8;8:105-14
62. Gour N, Wills-Karp M. **IL-4 and IL-13 signaling in allergic airway disease..** Cytokine 2015 Sep;75(1):68-78.
63. Pope SM, Brandt EB, Mishra A, Hogan SP, Zimmermann N, Matthaei KI, Foster PS, Rothenberg ME. **IL-13 induces eosinophil recruitment into the lung by an IL-5- and eotaxin-dependent mechanism.** J Allergy Clin Immunol. 2001 Oct;108(4):594-601.
64. Brombacher F. **The role of interleukin-13 in infectious diseases and allergy.** Bioessays. 2000 Jul;22(7):646-56.
65. Zhu Z, Ma B, Zheng T, Homer RJ, Lee CG, Charo IF, Noble P, Elias JA. **IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodeling.** J Immunol. 2002 Mar 15;168(6):2953-62.
66. Corren J **Role of interleukin-13 in asthma.** Curr Allergy Asthma Rep. 2013 Oct;13(5):415-20.
67. Szabo SJ, Sullivan BM, Peng SL, Glimcher LH. **Molecular mechanisms regulating Th1 immune responses.** Annu Rev Immunol. 2003;21:713-58. Epub 2001 Dec 19.
68. Wills-Karp M, Nathan A, Page K, Karp CL. **New insights into innate immune mechanisms underlying allergenicity.** Mucosal Immunol. 2010 Mar;3(2):104- 10. Epub 2009 Dec 23.
69. Aktas E, Akdis M, Bilgic S, Disch R, Falk CS, Blaser K, Akdis C, Deniz G. **Different natural killer (NK) receptor expression and immunoglobulin E (IgE) regulation by NK1 and NK2 cells.** Clin Exp Immunol. 2005 May;140(2):301-9.
70. von Bubnoff D1, Andrès E, Hentges F, Bieber T, Michel T, Zimmer J **Natural killer cells in atopic and autoimmune diseases of the skin.** J Allergy Clin Immunol. 2010 Jan;125(1):60-8.
71. Müller L, Brighton LE, Jaspers I **Mechanisms of immune tolerance to allergens in children..** Am J Physiol Lung Cell Mol Physiol. 2013 Mar 1;304(5):L332-41.
72. Culley FJ. **Natural killer cells in infection and inflammation of the lung.** Immunology. 2009 Oct;128(2):151-63.
73. Carbone T, Nasorri F, Pennino D, Eyerich K, Foerster S, Cifaldi L, Traidl-Hoffman C, Behrendt H, Cavani A. **CD56highCD16-CD62L- NK cells accumulate in allergic contact dermatitis and contribute to the expression of allergic responses.** J Immunol. 2010 Jan 15;184(2):1102-10.
74. Deniz G, van de Veen W, Akdis M. **Natural killer cells in patients with allergic diseases.** J Allergy Clin Immunol. 2013 Sep;132(3):527-35
75. Deniz G, Akdis M, Aktas E, Blaser K, Akdis CA. **Human NK1 and NK2 subsets determined by**

- purification of IFN-gamma-secreting and IFN-gamma-nonsecreting NK cells.** Eur J Immunol. 2002 Mar;32(3):879-84.
76. Romagnani S. **The increased prevalence of allergy and the hygiene hypothesis: missing immune deviation, reduced immune suppression, or both?** Immunology. 2004 Jul;112(3):352-63.
 77. Roncarolo MG, Levings MK. **The role of different subsets of T regulatory cells in controlling autoimmunity.** Curr Opin Immunol. 2000 Dec;12(6):676-83.
 78. O'Garra A, Vieira P. **Regulatory T cells and mechanisms of immune system control.** Nat Med. 2004 Aug;10(8):801-5.
 79. Roncarolo MG, Battaglia M. **Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans.** Nature Reviews Immunol. 2007 Aug;7(8):585-98.
 80. Zhang H, Kong H, Zeng X, Guo L, Sun X, He S. **Subset of regulatory T cells and their roles in allergy.** Journal of Translational Medicine. May 2014, 12:125.
 81. Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis M, Akdis CA. **Role of Treg in immune regulation of allergic diseases.** Eur J Immunol. 2010 May;40(5):1232-40. doi: 10.1002/eji.200940045.
 82. van der Valk JP, de Jong NW, Gerth van Wijk R **Review on immunotherapy in airway allergen sensitised patients.** Neth J Med. 2015 Jul;73(6):263-9.
 83. Schulten V, Tripple V, Aasbjerg K, Backer V, Lund G, Würtzen PA, Sette A, Peters B. **Distinct modulation of allergic T cell responses by subcutaneous versus sublingual allergen-specific immunotherapy.** Clin Exp Allergy. 2015 Oct 5.
 84. Linhart B, Valenta R. **Vaccines for allergy.** Curr Opin Immunol. 2012 Jun;24(3):354-60.
 85. Matsuoka T, Shamji M. H. and Durham S. R. **Allergen Immunotherapy and Tolerance.** Allergology International. 2013;62:403-413.
 86. Larché M, Akdis CA, Valenta R. **Immunological mechanisms of allergen-specific immunotherapy.** Nat Rev Immunol. 2006 Oct;6(10):761-71.
 87. Ferreira F, Wolf M, Wallner M. **Molecular Approach to Allergy Diagnosis and Therapy.** Yonsei Med J. 2014 Jul;55(4):839-52.
 88. Linhart B, Valenta R. **Mechanisms underlying allergy vaccination with recombinant hypoallergenic allergen derivatives Vaccine.** 2012 Jun 19;30(29):4328-35.
 89. Valenta R, Niederberger V. **Recombinant allergens for immunotherapy.** J Allergy Clin Immunol 2007 Apr;119(4):826-30. Epub 2007 Mar 1
 90. Valenta R, Linhart B, Swoboda I, Niederberger V. **Recombinant allergens for allergen-specific immunotherapy: 10 years anniversary of immunotherapy with recombinant allergens.** Allergy. 2011 Jun;66(6):775-83.
 91. Wallner M, Pichler U, Ferreira F. **Recombinant allergens for pollen immunotherapy.** Immunotherapy. 2013 Dec;5(12):1323-38
 92. Valenta R, Niespodziana K, Focke-Tejkl M, Marth K, Huber H, Neubauer A, Niederberger V. et al. **Recombinant allergens: What does the future hold?** J Allergy Clin Immunol 2011;127:860-4
 93. Radulovic S, Jacobson MR Durham SR, Nouri-Aria KT **Grass pollen immunotherapy induces Foxp3-**

- expressing CD4+ CD25+ cells in the nasal mucosa.** *J Allergy Clin Immunol.* 2008 Jun;121(6):1467-72, 1472.
94. Francis JN, Till SJ, Durham SR. **Induction of IL10+CD4+CD25+ T cell by grass pollen immunotherapy.** *Allergy Clin Immunol.* 2003 Jun;111(6):1255-61.
95. Campana R, Vrtala S, Maderegger B, Dall'Antonia Y, Zafred D, Blatt K, Herrmann H, Focke-Tejkl M, Swoboda I, Scheiblhofer S, Gieras A, Neubauer A, Keller W, Valent P, Thalhamer J, Spitzauer S, Valenta R. **Altered IgE epitope presentation: a model for hypoallergenic activity revealed for Bet v 1 trimer.** *Mol Immunol* 2011;48:431–41.
96. Pauli G, Larsen TH, Rak S, Horak F, Pastorello E, Valenta R, Purohit A, Arvidsson M, Kavina A, Schroeder JW, Mothes N, Spitzauer S, Montagut A, Galvain S, Melac M, André C, Poulsen LK, Malling HJ. **Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis;** *J Allergy Clin Immunol.* 2008 Nov;122(5):951-60.
97. Akdis C.A. Blaser K. **Regulation of Specific Immune Responses by Chemical and Structural Modifications of Allergens;** *Int Arch Allergy Immunol* 2000;121:261–269.
98. Valenta R, Ferreira F, Focke-Tejkl M, Linhart B, Niederberger V, Swoboda I, Vrtala S. **From allergen genes to allergy vaccines.** *Annu Rev Immunol* 2010;28:211–41
99. Ferreira F Wallner MBreiteneder H, Hartl A, Thalhamer J, Ebner . **Genetic engineering of allergens: future therapeutic products.** *Int Arch Allergy Immunol.* 2002 Jul;128(3):171-8.
100. Li N, Xu H, Song H, Wang K, Li C. **Analysis of T-cell epitopes of Der f3 in Dermatophagoides farina.** *Int J Clin Exp Pathol.* 2015 Jan 1;8(1):137-45
101. Nouri HR, Varasteh A, Vahedi F, Chamani J, Afsharzadeh D, Sankian M. **Constructing a hybrid molecule with low capacity of IgE binding from Chenopodium album pollen allergens;** *Immunol Lett.* 2012 May 30;144(1-2):67-77.
102. Larché M, Akdis A. C. and Valenta R. **Immunological mechanisms of allergen-specific immunotherapy.** *Nature Reviews Immunology* 6, 761-771 .
103. Till S. **Mechanisms of immunotherapy and surrogate markers..** *Allergy* 2011 Jul;66 Suppl 95:25-7. 70.
104. Gould HJ, Sutton BJ. **IgE in allergy and asthma today.** *Nat Rev Immunol.* 2008 Mar;8(3):205-17.
105. Jacobsen L, Wahn U, Bilo MB. **Allergen-specific immunotherapy provides immediate, long-term and preventive clinical effects in children and adults: the effects of immunotherapy can be categorised by level of benefit the centenary of allergen specific subcutaneous immunotherapy.** *Clin Transl Allergy.* 2012 Apr 13;2:8.
106. Schroder K, Hertzog PJ, Ravasi T, Hume DA. **Interferon-gamma: an overview of signals, mechanisms and functions..** *J Leukoc Biol.* 2004 Feb;75(2):163-89.
107. Shouval DS, Biswas A, Goettel JA, McCann K, Conaway E, Redhu NS, Mascanfroni ID, Al Adham Z, Lavoie S, Ibourk M, Nguyen DD, Samsom JN, Escher JC, Somech R, Weiss B, Beier R, Conklin LS, Ebens CL, Santos FG, Ferreira AR, Sherlock M, Bhan AK, Müller W, Mora JR, Quintana FJ, Klein C, Muise AM, Horwitz BH, Snapper SB. **Interleukin-10 receptor signaling in innate immune cells regulates mucosal immune tolerance and anti-inflammatory macrophage function.** *Immunity.* 2014 May 15;40(5):706-19

108. Akdis CA, Blaser K. **Mechanisms of interleukin-10-mediated immune suppression.** Immunology. 2001 Jun;103(2)
109. Colombo P, Bonura A, Costa M, Izzo V, Passantino R, Locorotondo G, Amoroso S, Geraci D. **The allergens of *Parietaria*.** Int Arch Allergy Immunol. 2003 Mar;130(3):173-9
110. Bonura A, Quarantino S, Gervasi F, Melis MR, Di Sano C, Colombo P. **Innate and adaptive immune responses to the major *Parietaria* allergen Par j 1 in healthy subjects.** Immunobiology. 2013 Jul;218(7):995-1004.
111. Costa MA, Colombo P, Izzo V, Kennedy H, Venturella S, Cocchiara R, Mistrello G, Falagiani P, Geraci D. **cDNA cloning, expression and primary structure of ParjI, a major allergen of *Parietaria judaica* pollen.** FEBS Lett., 1994 Mar 21
112. Costa MA, Colombo P, Izzo V, Kennedy H, Venturella S, Cocchiara R, Mistrello G, Falagiani P, Geraci D. **cDNA cloning, sequence analysis and allergological characterization of Parj 2.0101, a new major allergen of the *Parietaria judaica* pollen.** FEBS Lett, 1996. 399(3): p. 295-8
113. Bonura A, Gulino L, Trapani A, Di Felice G, Tinghino R, Amoroso S, Geraci D, R. Valenta, K. Westritschnig, E. Scala, A. Mari, P. Colombo. **Isolation, expression and immunological characterization of a calcium-binding protein from *Parietaria* pollen.** Molecular Immunology 2008; 45: 2465-2473.
114. J. A. Asturias, I. Ibarrola, J. L. Esevenri, M. Carmen Arilla, R. González, Rioja, A. Martinez. **PCR-based cloning and immunological characterization of *Parietaria judaica* pollen profiling.** J Invest Allergol Clin Immunol 2004; 14(1): 43-48.
115. Colombo P, Duro G, Costa M.A, Izzo V, Mirisola M, Locorotondo G, Cocchiara R, Geraci D. ***Parietaria* pollen allergens.** Allergy 1998 June; 53: 917-921
116. Duro G, Colombo P, Costa M.A, Izzo V, Porcasi R, Di Fiore R, Locorotondo G, Cocchiara R, Geraci D. **Isolation characterization of two cDNA clones coding for isoforms of the *Parietaria judaica* major allergen Par j 1.0101.** Int Arch Allergy Immunol 1997 Apr; 112(4):348-55.
117. Bonura A, Amoroso S, Locorotondo G, Di Felice G, Tinghino R, Geraci D, Colombo P. **Hypoallergenic variants of the *Parietaria judaica* major allergen Parj1: a member of the non-specific lipid transfer protein plant family.** Int Arch Allergy Immunol. 2001 Sep;126(1):32-40.
118. Bonura A, Corinti S, Artale A, Di Felice G, Amoroso S, Melis M, Geraci D, Colombo P. **A hybrid expressing genetically engineered major allergens of the *Parietaria* pollen as a tool for specific allergy vaccination.** Int Arch Allergy Immunol. 2007;142(4):274-84.
119. Minnicozzi M, Sawyer RT, Fenton MJ. **Innate immunity in allergic disease.** Immunol Rev. 2011 Jul;242(1):106-27.
120. Teixeira LK, Fonseca BP, Barboza BA, Viola JP. **The role of interferon- γ on immune and allergic responses.** Mem Inst Oswaldo Cruz. 2005 Mar;100 Suppl 1:137-44. Epub 2005 Jun 14.
121. Ciprandi G, Sormani MP, Filaci G, Fenoglio D. **Carry-over effect on IFN-gamma production induced by allergen-specific immunotherapy.** Int Immunopharmacol. 2008 Dec 10;8(12):1622-5.
122. Maggi E. **T cell responses induced by allergen-specific immunotherapy.** Clin Exp Immunol. Jul 2010;61(1):10-18.

123. Niu N, Qin X. **New insights into IL-7 signaling pathways during early and late T cell development.** *Cell Mol Immunol.* 2013 May;10(3):187-9.
124. Simonetta F, Chiali A, Cordier C, Urrutia A, Girault I, Bloquet S, Tanchot C, Bourgeois C. **Increased CD127 expression on activated FOXP3+CD4+ regulatory T cells;** *Eur J Immunol.* 2010 Sep;40(9):2528-38.
125. Yao Y, Vent-Schmidt J, McGeough MD, Wong M, Hoffman HM, Steiner TS, Levings MK. **Tr1 Cells, but Not Foxp3+ Regulatory T Cells, Suppress NLRP3 Inflammasome Activation via an IL-10-Dependent Mechanism.** *J Immunol.* 2015 Jul 15;195(2):488-97
126. Wang, R., Wan, Q., Kozhaya, L., Fujii, H., and Unutmaz, D., **Identification of a Regulatory T Cell Specific Cell Surface Molecule that Mediates Suppressive Signals and Induces Foxp3 Expression.** *PLoS ONE,* 2008. 3(7): p. e2705.
127. Probst-Kepper M, Geffers R, Kröger A, Viegas N, Erck C, Hecht HJ, Lünsdorf H, Roubin R, Moharreg-Khiabani D, Wagner K, Ocklenburg F, Jeron A, Garritsen H, Arstila TP, Kekäläinen E, Balling R, Hauser H, Buer J, Weiss S. **GARP: a key receptor controlling FOXP3 in human regulatory T cells.** *J Cell Mol Med.* 2009 Sep;13(9B):3343-57.
128. Battaglia M, Roncarolo MG. **The Tregs' world according to GARP.** *Eur J Immunol.* 2009 Dec;39(12):3296-300
129. Gauthy E, Cuende J, Stockis J, Huygens C, Lethé B, Collet JF, Bommer G, Coulie PG, Lucas S. **GARP Is Regulated by miRNAs and Controls Latent TGF- β 1 Production by Human Regulatory T Cells.** *PLoS One.* 2013 Sep 30;8(9):e76186. doi: 10.1371/journal.pone.0076186. eCollection 2013.
130. Nakamura, K., Kitani, A. and Strober, W., **Cell contact-dependent immunosuppression by CD4(1)CD25(1) regulatory T cells is mediated by cell surface-bound transforming growth factor beta.** *J. Exp. Med.* 2001.194: 629–644
131. Nakamura, K., Kitani, A., Fuss, I., Pedersen, A., Harada, N., Nawata, H. and Strober, W. **TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice.** *J. Immunol.* 2004. 172: 834–842.
132. Regateiro FS, Cobbold SP, Waldmann H. **CD73 and adenosine generation in the creation of regulatory microenvironments.** *Clin Exp Immunol* 2013; 171:1–7.
133. Beavis, P.A., Stagg, J., Darcy, P.K. and Smyth, M.J. (2012) **CD73: a potent suppressor of antitumor immune responses.** *Trends Immunol.* 33, 231–237.
134. Antonioli, L., Pacher, P., Vizi, E.S. and Hasko, G. **CD39 and CD73 in immunity and inflammation.** *Trends Mol. Med.* 2013. 19, 355–367.
135. Bastid J, Cottalorda-Regairaz A, Alberici G, Bonnefoy N, Eliaou JF, Bensussan A. **ENTPD1/CD39 is a promising therapeutic target in oncology.** *Oncogene.* 2013 Apr 4;32(14):1743-51. doi: 10.1038/onc.2012.269. Epub 2012 Jul 2.
136. Li P, Gao Y, Cao J, Wang W, Chen Y, Zhang G, Robson SC, Wu Y, Yang JI. **CD39+ regulatory T cells attenuate allergic airway inflammation.** *Clin Exp Allergy.* 2015 Jun;45(6):1126-37. doi: 10.1111/cea.12521.

137. Regateiro FS, Howie D, Nolan KF, Agorogiannis EI, Greaves DR, Cobbold SP, Waldmann H. **Generation of anti-inflammatory adenosine by leukocytes is regulated by TGF- β** . *Eur J Immunol* 2011; 41:2955–65.
138. Mann EH, Chambers ES, Chen YH, Richards DF, Hawrylowicz CM. **1 α ,25-dihydroxyvitamin D3 acts via transforming growth factor- β to up-regulate expression of immunosuppressive CD73 on human CD4+ Foxp3– T cells**. *Immunology*. 2015 Nov;146(3):423-31. doi: 10.1111/imm.12519. Epub 2015 Sep 13.
139. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giotto R, Höpner S, Centonze D, Bernardi G, Dell'Acqua ML, Rossini PM, Battistini L, Röttschke O, Falk K. **Expression of ectonucleotidase CD39 by Foxp3 + Treg cells: hydrolysis of extracellular ATP and immune suppression**. *Blood*. 2007 Aug 15;110(4):1225-32 Epub 2007 Apr 20.
140. Parronchi P, Sampognaro S, Annunziato F, Brugnolo F, Radbruch A, Di Modugno F, Ruffilli A, Romagnani S, Maggi E. **Influence of both TCR repertoire and severity of the atopic status on the cytokine secretion profile of Parietaria officinalis-specific T cells**. *Eur J Immunol*. 1998 Jan;28(1):37-46.
141. Wambre E. **Effect of allergen-specific immunotherapy on CD4+ T cells**. *Curr Opin Allergy Clin Immunol*. 2015 Dec;15(6):581-7.
142. Tomiita M, Campos-Alberto E, Shima M, Namiki M, Sugimoto K, Kojima H, Watanabe H, Sekine K, Nishimuta T, Kohno Y, Shimojo N. **Interleukin-10 and interleukin-5 balance in patients with active asthma, those in remission, and healthy controls**. *Asia Pac Allergy*. 2015 Oct;5(4):210-5.
143. Smarr CB, Bryce PJ, Miller SD. **Antigen-specific tolerance in immunotherapy of Th2-associated allergic diseases**. *Crit Rev Immunol*. 2013;33(5):389-414.
144. Meiler F, Zumkehr J, Klunker S, Rückert B, Akdis CA, Akdis M. In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. *J Exp Med*. 2008 Nov 24;205(12):2887-98.
145. Maggi E, Vultaggio A, Matucci A. T-cell responses during allergen-specific immunotherapy. *Curr Opin Allergy Clin Immunol*. 2012 Feb;12(1):1-6.
146. Schulten V, Tripple V, Sidney J, Greenbaum J, Frazier A, Alam R, Broide D, Peters B, Sette A **Association between specific timothy grass antigens and changes in TH1- and TH2-cell responses following specific immunotherapy**. *J Allergy Clin Immunol*. 2014 Nov;134(5):1076-83

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