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Acknowledgments

I would like thank Constantin Fesel for accepting me in his group, allowing me to use the results of studies that were already running, his availability and patience to explain everything very well. I would also like to thank Nuno Grais for all the help, partnership and availability.

I could not go on without thanking Rúben for all the support, friendship and love, with him on my side everything was much easier. Also my friends that were always supporting this phase, especially Margarida Rocheta, who gave me the necessary stress to finish everything on time. Thank you for your friendship and dedication with me. My family was also very important in this step, my parents support is very important, always. Claudia also help me very much, thank you for helping me, and supporting me. Thanks to all my friends in Lisbon and Coimbra, all the help and availability to me was very important, I didn't realized before how much you cared about me and this phase of my life allowed me to see that, I have no words to describe how much I thank you all.

I would like to thank Madalena Martins that in her busy life always had a little bit to help me and give me some advises. I thank also to Iris Caramalho for her availability, support and tips. I would like to thank to Professor Emilia Duarte, for the availability and comprehension.

I must also thank for the collaborators of this project in Porto that made possible for us to have samples with a great frequency, namely to Oriana and Ana Raquel, as well as the clinicians that were available to indicate the patients to give their blood, and off course, to

all the patients, relatives and healthy controls that offered themselves to be part of this study.

Finally, I would like to thank Dr. Celso for being part of my life, and for giving me a whole new perspective that made my life much easier and responsible.

Abstract

Systemic Lupus Erytematosus (SLE) is an autoimmune disease characterized by the production of antibodies against self, indicating a loss of self recognition and consequently self tolerance. Deficient regulatory T cells (Tregs) have been implicated in this loss of self tolerance with several mechanisms that are thought to play a role in their impairment.

The aim of this study was to characterize Treg phenotypes comparing results from unaffected first degree relatives and SLE patients. Therefore, cytometric analysis was performed in Tregs using peripheral blood from SLE patients and unaffected first degree relatives and also from healthy donors. In parallel SNPs in specific genes were typed, namely *IL2RA*, *IL2RB*, *IL2*, *IL6*, *CTLA4*, *PTPN22* and *PTTG1*. The cytometric Treg phenotypes were analyzed and correlated with the genotypes. Furthermore, cell cultures were performed to induce Foxp3 expressing cells from naïve effector Th cells using anti-CD3 stimulation and TGF-β1.

In agreement with other recent studies, surface CD25, recently proposed as a marker for activation in Tregs, was clearly reduced in relatives and patients. The cytometric results also revealed a higher frequency of $Foxp3^{+}$ cells in patients due to an expansion of the $Foxp3^{low}$ population. In contrast, the frequency of $Foxp3^{+}$ in relatives was lower due to a decrease of the $Foxp3^{high}$ population. This reduction of $CD25^{+}Foxp3^{high}$ cells in relatives was interpreted as a relative exhaustion of these cells due to accelerated activation. Genetic effects in loci encoding *IL-2*, components of the IL2 receptor (IL2R) as well as in *PTTG1* supported this interpretation. Particularly, *IL2RA* genetic variation that in controls was favoring an increase of CD25 was associated with a lower frequency of $Foxp3^{high}$ in relatives. In the patients, genetic effects of an *IL6* SNP appeared to contribute to the

inflammatory condition and possibly influenced the increased frequency of $Foxp3^{low}$. Moreover, in the patients group there was an indication that naïve Th cells had less predisposition to become activated when compared to the relatives and control groups *in vitro*.

In conclusion, this study allowed to characterize some phenotypes in the unaffected relatives, a poorly studied group. The IL2 receptor system seemed to be involved in a compensatory effect of Tregs in this group. Other genetic factors associated with risk for SLE seemed to have a negative effect. The relation of the defective *in vitro* T-cell activation in patients with Treg properties remains to be studied. However, other studies should be performed, especially with the relatives to understand the mechanisms that allow this group to remain unaffected.

Keywords: SLE, autoimmunity, Tregs, Foxp3, CD25, IL2

Resumo

Lupus Eritematoso Sistémico (LES) é uma doença autoimune caracterizada pela produção de anticorpos que reagem contra componentes do próprio organismo, o que indica uma falha no reconhecimento desses componentes e consequentemente pela perda de tolerância imunológica. Disfunções nas células T reguladoras (Treg) têm sido implicadas nesta perda de tolerância com diversos mecanismos sugeridos que estarão na base dessas disfunções.

O objectivo deste estudo foi a caracterização de fenótipos das Tregs comparando os resultados de doentes com LES e familiares em primeiro grau não afectados. Nesse sentido, efectuaram-se análises de citometria em Tregs usando sangue periférico de doentes com LES, familiares em primeiro grau não afectados e dadores de sangue saudáveis. Em paralelo, SNPs de genes específicos foram estudados, nomeadamente *IL2RA*, *IL2RB*, *IL2*, *IL6*, *CTLA4*, *PTPN22* e *PTTG1*. Os fenótipos de citometria das Tregs foram analizados e correlacionados com os genótipos. Além disso, fizeram-se culturas de células para induzir a expressão de Foxp3 em células Th efectoras "naive" usando anti-CD3 para estimulação e TGF-β1.

De acordo com estudos recentes, o CD25 de superfície celular, recentemente proposto como um marcador de activação de Tregs, estava claramente reduzido em familiares e doentes. Os resultados de citometria revelaram também uma maior frequência de células $Foxp3^{+}$ em doentes devido a uma expansão da população $Foxp3^{low}$. Pelo contrário, a frequência de $Foxp3^{+}$ nos familiares era menor devido a uma diminuição da frequência da população $Foxp3^{high}$. Esta redução de células $CD25^{+}Foxp3^{high}$ nos familiares foi interpretada como uma exaustão relativa destas células devido a uma ativação acelerada.

Efeitos genéticos em loci codificantes de *IL2*, de componentes do receptor de IL2 assim como de *PTTG1* suportam a interpretação anterior. Mais especificamente, variações genéticas no *IL2RA* que em controlos favoreciam um aumento de CD25 estavam associadas a uma diminuição da frequência de Foxp3^{high} nos familiares. Nos doentes, os efeitos genéticos de um SNP no locus *IL6* indiciam uma contribuição para a condição inflamatória dos doentes possivelmente influenciando o aumento da população Foxp3^{low}. Além disso, este estudo revelou que células Th efectoras "naive" de doentes tinham uma menor predisposição para activação *in vitro* em comparação com os familiares e controlos.

Em conclusão, este estudo permitiu a caracterização de alguns fenótipos nos familiares não afectados, uma vez que se sabe muito pouco acerca deste grupo. O receptor de IL2 parece estar envolvido num efeito compensatório nas Tregs deste grupo no entanto, outros factores genéticos associados ao risco de LES mostraram ter um efeito negativo. A relação da menor activação das células T dos doentes *in vitro* com propriedades das Tregs ainda está por estudar. No futuro será interessante desenvolver trabalho no sentido de perceber os mecanismos que permitem os familiares de indivíduos que manifestam LES permanecerem não afectados.

Palavras-chave: LES, autoimunidade, Tregs, Foxp3, CD25, IL2

Abbreviations

ADP - Adenosine diphosphate

AMP - Adenosine monophosphate

ATP - Adenosine-5'-triphosphate

BANK1 – B-cell scaffold protein with ankyrin repeats

BLK - B lymphocyte kinase

CD3 – Cluster of differentiation 3

CTLA4 - Cytotoxic T-lymphocyte-associated antigen-4

DNA –Deoxyribonucleic acid

dNTP - Deoxynucleotide Triphosphates

FBS – Fetal Bovine Serum

Foxp3 – Forkhead Box P3

GITR - glucocorticoid-induced TNFR-related protein

IRAK - Interleukin-1 receptor-associated kinase

Jak3- Janus kinase 3

LAG-3 - Lymphocyte-activation gene 3

M - Molar

min – minutes

mg - miligrams

mL – mililiter

mM – milimolar

nL - nanoliter

PCR – Polymerase chain reaction

PI-3 – Phosphatidylinositol 3-kinases

RNA – Ribonucleic acid

rpm – rotation per minute

RPMI - Roswell Park Memorial Institute medium

SAP - Shrimp Alkaline Phosphatase

sec – seconds

SMAD3 - mother against decapentaplegic homologue 3

STAT4 - Signal transducer and activator of transcription 4

STAT5 - Signal transducer and activator of transcription 5

TGF – Transforming growth factor

Th17 – IL-17-producing effector T helper cells

TLR8 – Toll like receptor 8

TREX1 – Three prime repair exonuclease 1

µL – microliter

µM – micromolar

Chapter 1. INTRODUCTION

1. Introduction

1.1. Systemic Lupus Eritematosus (SLE)

Systemic Lupus Erythematosus (SLE) is a multisystemic and complex autoimmune disease. It is one of the most diverse auto-immune diseases, displaying a broad number of clinical and immunological manifestations that may affect any organ (Rahman and Isenberg, 2008). SLE pathogenesis is not well established, the progress in investigation has been giving some clues, however the underlying mechanisms are still to be understood.

The causes of the disease are still a mystery as well as the mechanisms involved in the clinical manifestations. Therefore, the therapy has not improved much in the last few decades (O'Neill and Cervera, 2010; Scheinecker C., 2010). SLE complexity is due to its multifactorial etiology with genetic susceptibility, hormonal and environmental factors:

SLE can develop at any age, ranging from children's with two years old to adults with 80 years old (Danchenko et al., 2006). In the Euro-lupus cohort the mean age of onset was 29 years old while in the American college of Rheumatology (ACR) classifications criteria the mean was 31 years old (Cervera et al., 2009). The incidence of the disease is much higher in females than in males with an estimated ratio of 9:1, however it varies among cohorts (Cervera et al., 2009; Lockshin, 2006). This difference among genders suggests a role for estrogens in the development of SLE and the risk is even increased during pregnancy, suggesting a key role for this hormone (Danchenko et al., 2006; O'Neill and Cervera, 2010).

The concordance for SLE in monozygotic twins is 24% while in dizygotic twins is 2% (Deapen et al., 1992). Furthermore, SLE is more frequent in African-Americans, Hispanics and Asians than in Caucasians which suggests the importance of genetic

predisposition for this auto-immune disorder (Danchenko et al., 2006). There are over than 30 loci confirmed to confer risk for SLE and this demonstrates that genetic predisposition influences the development of the disease through the combination of a large number of genes (Moser et al., 2009; Tsokos and Kammer, 2000) (Fig.1). Therefore, each locus has only a mild contribution and it is postulated that the combination of risk from several alleles increases the risk for SLE (Crispín et al., 2010). These risk loci are involved with nucleic acid sensing or interferon production, others with T cell or B cell signaling pathways. The identification and characterization of candidate genes and alleles may give some understanding about the pathogenesis, their relative contribution for the functional impairment in T and B cells and consequently with the phenotype and severity of the disease (Crispín et al., 2010).

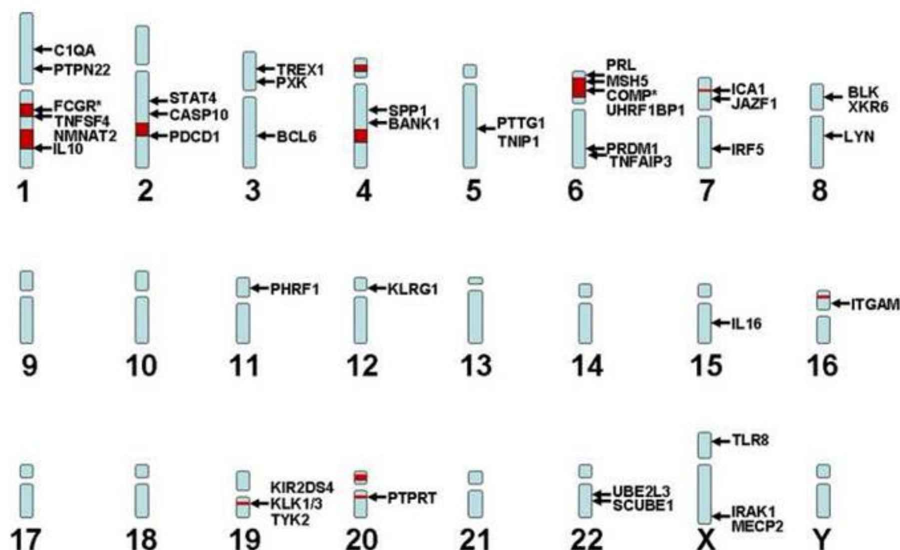

Figure 1 – Multiple genes described to be associated with SLE scattered in the human genome (Crispín et al., 2010).

In this study, we will focus on genes involved in the T cell signaling, which is discussed further in this manuscript.

Several environmental exposures have been associated with SLE pathogenesis in order to explain the higher occurrence in certain communities. However, given the multifactorial nature of SLE, not every person exposed to a certain risk factor develop SLE, decreasing the probability to identify and quantify specific exposures (O'Neill and Cervera, 2010). Some infection agents have been associated with the development of SLE. This is supported by the observation of flares of SLE activity after infection. The strongest relationship between infection and SLE is with Epstein-Barr virus (EBV) (James et al., 2001; James and Robertson, 2012). There are evidences of the presence of antibodies to EBV nuclear antigen-1 epitope prior to the onset of SLE in humans. Furthermore, these antibodies cross-react with Sm antigens (RNA-binding proteins) and Ro epitopes (Ro ribonucleoprotein particle), which in animal models develop a lupus-like auto-immunity (McClain et al., 2005).

1.2. Diagnosis and follow up systems

The disease severity changes over time with alternating episodes of flares and relapses (Luijten et al., 2012). There is the urge to determine a valid and sensitive standard for the clinical practice and research for measuring disease activity, flares and clinical improvement, something that is still a challenge (Wollaston et al., 2004). With the attempt to standardize the studies related to SLE, in 1982 the ACR created a set of classification criteria, based on symptoms and laboratory findings, which was updated in 1997 (Hochberg, 1997; Tan et al., 1982). For clinical studies, it is considered that SLE patients must have 4 of the 11 criteria present in ACR classification. These symptoms could appear apart in time or simultaneously during any period of observations (Table 1) (O'Neill and Cervera, 2010). However, there are still no standardize diagnostic criterion for this disease and the ACR criteria are not used

systematically for this purpose, leading to missed cases that do not follow any treatment (Font and Cervera, 1993).

Table I - Modified ACR Classification criteria for SLE. Table adapted from Wakeland et al 2001 and O'Neill and Cervera 2010.

Category	Symptom
Skin criteria	1. Malar (butterfly) rash (over the cheeks and nose) 2. Discoid rash (scarring rash in sun-exposed areas) 3. Photosensitivity 4. Oral ulcerations
Systemic criteria	5. Arthritis (non-erosive) 6. Serositis: pleuritis or pericarditis 7. Renal disorders: persistent proteinuria or cellular blasts 8. Neurological disorders: seizure or psychosis with no other etiology
Laboratory criteria	9. Hematologic: hemolytic anemia, leukocytopenia, lymphocytopenia or thrombocytopenia 10. Immunologic: anti-dsDNA antibodies, anti-Sm antibodies, antiphospholipid antibodies or false positive serologic test for syphilis. 11. Antinuclear antibodies (ANA)

To evaluate disease activity there are several indices that include an evaluation of disease related features. The mostly used indices are the Physician Global Assessment (PGA), the British Isles Lupus Assessment Group (BILAG), the Systemic Lupus Activity Measure (SLAM), the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and the European Consensus Lupus Activity Measurement (Griffiths et al., 2005). In this study the measurement criteria used by the participant physicians was the SLEDAI indice.

1.3. T lymphocytes in SLE

1.3.1. T cells

In general, T cells can be divided into two main populations according to their functions, namely the effector or conventional T helper cells (Th) and regulatory T cells (Tregs). The molecular differences will be discussed in the Regulatory T cells chapter. T cells have characteristic features like the expression of certain cytokines and the presence of the T cell receptor (TCR). All T cells are generated in the thymus, and T cells recently released to the periphery are possible to distinguish through the expression of CD31, also called PECAM-1. CD31 is a member of the Ig superfamily, characteristic of recent thymic emigrants (CD31+). In the periphery they lose this marker and become central cells (CD31-) (Kimmig et al., 2002; Newman et al., 1990). When T cells are released from the thymus in the naïve state, in the periphery they become memory or mature after stimulation of the TCR and through costimulation. In the naïve state T cells characteristically express CD45RA, and upon activation/stimulation they downregulate CD45RA and start expressing the CD45RO isoform (Sanders et al., 1988; Serra et al., 1988). Besides the thymic emigrants generation, there is another mechanism contributing to the maintenance of T cell frequency. T cells have the ability to proliferate, n the naïve state as well as in the mature state, contributing to the expansion of both compartments in the periphery. Ki67 is a marker that characterizes the proliferation of these cells. The Ki-67 antigen is found in the nuclei of the cells in G1, S and G2 phases and cells in the resting phase G0 have no expression of this protein (Gerdes et al., 1984; Scholzen and Gerdes, 2000).

Tregs and effector Th cells have a common origin as subpopulations of T cells, and they share some extracellular cells markers such as CTLA-4, GITR, LAG-3 and

CD127 (Scheinecker C., 2010). CD127 is the IL-7 receptor α chain, the IL-7 cytokine was described to contribute moderately to Treg homeostasis (Bayer et al., 2008; Liu et al., 2006). Another feature that these two populations share is the T cell receptor (TCR). However Tregs, unlike the effector Th cells, don't proliferate when stimulated through the TCR although they need this type of activation to exert their characteristic suppressive effect (Scheinecker C., 2010).

Effector Th cells and Tregs regulate each other: effector Th cells produce cytokines that are important for Treg maintenance, in contrast Tregs are able to suppress the activity and the pro-inflammatory activity of effector Th cells. This Treg ability is important for self antigen tolerance because, in contrast to effector Th cells, their T cell receptor (TCR) distinguishes self antigens from the foreign ones. There are different mechanisms contributing to the suppressive capacity of Tregs. One example is through the CD39 and CD73 expression in the presence of ATP. It has been described that extracellular ATP has many proinflammatory effects and influence the suppressive capacity and stability of Treg cells (Deaglio et al., 2007). The ectoenzyme CD39 converts ATP and ADP in AMP, which is transformed in extracellular adenosine by the ectoenzyme CD73 (Hasko et al., 2008). This adenosine produced will exert a suppressive effect in the effector Th cells (Deaglio et al., 2007; Fletcher et al., 2009; Liao et al., 2010; Mandapathil et al., 2010; Sitkovsky et al., 2008). One of the most important mechanisms of suppression is through CTLA4. CTLA4 is a surface protein and it was shown to be constitutively expressed in Tregs. Some experiments of blockage of CTLA4 showed an abolishment in the suppressive capacity of these cells. It appears to be involved in Treg homeostasis, however the mechanisms of action are still not well understood (Chistiakov and Turakulov, 2003; Walker and Sansom, 2011). In

turn, effector Th cells express IL-2 that is an important co-stimulatory cytokine for Tregs as it will be discussed above.

1.3.1.1. Cytokines from effector T cells

Cytokines are small peptides or glycoproteins that are produced by a large variety of cells. They play an important role in modulating the immune response against foreign or self-antigens. When the naïve $CD4^+$ T cells are exposed to an antigen, they become active and start to proliferate. The development of an effector phenotype is characterized by the expression of different transcription factors, which in turn, induce the production of specific cytokine profiles. In SLE T cells the cytokine production is impaired, compromising the effector Th cell competences (Crispin et al., 2008a; Yu et al., 2012).

1.3.1.1.1. IL-2

One important cytokine for T cells is IL-2, which plays a central role in the functionality of these cells. Effector Th cells respond to this cytokine and are the main producers at the same time. The production starts with the activation of the naïve Th cells through the TCR, upregulating the expression of its own receptor (IL2R). There is evidence supporting that Tregs depend on IL-2 for their thymic development and to maintain their homeostasis in the periphery, while the deficiency of IL-2 compromise their suppressive capacity (Brandenburg et al., 2008; Setoguchi et al., 2005). Another study revealed that IL-2 is also affecting Th17 cells, IL-17 producers that are described to be involved in many other autoimmune diseases. IL-17 is a proinflammatory cytokine that recruits effector cells to target organs. This cytokine also helps increasing the

survival and proliferation of B cells and consequently their transformation into antibody secreting cells, also called plasma cells (Doreau et al., 2009). Recent studies show that IL-2 limits the production of IL17 *in vivo* and *in vitro* however, in low levels IL-2 favors the occurrence of Th17 cells (Yang et al., 2011). Some interesting experiments with mice demonstrate that mice lacking IL-2 or IL-2 receptor (IL2R) develop severe spontaneous autoimmune disease, with decreased numbers of peripheral Tregs (Malek et al., 2002; Suzuki et al., 1995). Furthermore, IL2/IL2R$^{-/-}$ mice also presented increased levels of serum autoantibody titers, similar to what happens in human SLE, which is known have reduced IL-2 levels (Apostolidis et al., 2011).

IL2R shares structural redundancy with other type I cytokine receptors, sharing a common cytokine receptor γ-chain (γc). The IL2R consists in three different chains, IL-2Rα (CD25), IL-2Rβ and γc (Fig. 2), and has high affinity for this cytokine. IL-2 can also bind to other combinations of receptor chains: with low affinity to IL-2Rα alone and intermediate affinity to IL-2Rβ and γc. The latter two chains are shared with the receptor of IL-15, another type I cytokine (Fig.2) (Rochman et al., 2009). IL-15 is known to promote isotype switching in B cells, and it is involved in the homeostasis and expansion of T cells, natural killer (NK) and NKT cells. It was also described that SLE patients have increased levels of this cytokine in the serum (Apostolidis et al., 2011; Baranda et al., 2005).

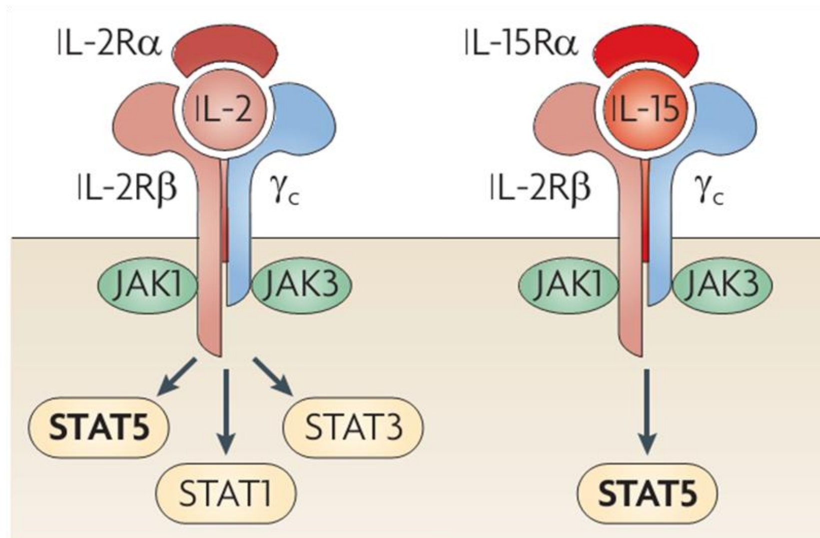

Figure 2 – IL2 and IL15 receptors. IL2Rα is characteristic for the IL2 receptor as well as the IL15Rα is characteristic for IL15 receptor. Both receptors share a common IL-2Rβ and γc chain. They also share STAT5 signaling pathway. Adapted from Rochman et al., 2009.

IL-21 is another type I cytokine thought to be involved in the SLE disorder, which is produced in T cells and its receptor (IL21R) was found in T, NK, NKT and B cells. This cytokine is involved in the activation of B cell and plasma cell differentiation, which is thought to induce the production of autoimmune autoantibodies (Apostolidis et al., 2011).

1.3.1.1.2. Other cytokines

There are many cytokines described to be involved with SLE, but here we focus on the cytokines important for this context. One cytokine of interest is IL-10 that is elevated in the serum of lupus patients and is overproduced by the B cells and monocytes of SLE patients (Hondowicz et al., 2007). It works as a suppressor of activated effector Th cells, however it seems to promote B cell activation as well as the antibody production, which in SLE is thought to contribute to the inflammatory condition (Okamoto et al., 2011; Sabat, 2010). It is produced mainly by

monocytes/macrophages and T cell subsets preventing the production of inflammatory mediators. It also prevents the activation of antigen presenting cells (APCs) and downregulates the expression of co-stimulatory molecules (Okamoto et al., 2011).

Another important cytokine is TGF-β whose functions suppress inflammation. It inhibits the maturation of APCs, as well as the maturation of naïve CD4 T cells into effector Th cells by controlling specific transcription factors (Gorelik and Flavell, 2002). It plays an important role for the development of naturally occurring Tregs, and peripheral Treg induction also depends on this cytokine (Piccirillo et al., 2002; Yamagiwa et al., 2001). TGF-β induces the expression of the transcription factor SMAD3, which in cooperation with NFAT controls the activity of *Foxp*3 intronic enhancer (Gabrysova et al., 2011; Tone et al., 2008; Xu et al., 2010). TGF-β signaling, through the TGF-β receptor, promotes *de novo* expression of *Foxp*3 and is required for the suppressive capacity of Tregs, as well as for their *in vivo* expansion (Huber et al., 2004; Kretschmer et al., 2005). The serum concentration of TGF-β1 is decreased during active SLE. SLE patients with lower numbers of Tregs tend to have lower serum concentrations of this cytokine (Okamoto et al., 2011).

1.3.2. Regulatory T cells

Tregs were initially characterized by a high expression of *IL2RA* ($CD25^{high}$), although it is also expressed in recently activated T cells but in lower levels (Baecher-Allan et al., 2001). This characterization of $CD4^{+}CD25^{high}$ was commonly accepted because some studies supported the suppressive activity of these cells. However, when the Foxp3 marker was discovered there were some discrepancies between the studies using the different markers (Baecher-Allan et al., 2001; La Cava, 2008). Therefore,

Tregs started to be characterized as $Foxp3^+$ cells in general (Fontenot et al., 2005). Afterwards it was found that $CD4^+CD25^{high}Foxp3^+$ could be divided in memory and naïve Tregs by CD45 isoforms (Valmori et al., 2005). Sakagushi's group went further distinguishing the Tregs through the level of *Foxp*3 expression and dividing the total $Foxp3^+$ population in 3 groups: resting Treg cells ($CD45RA^+Foxp3^{low}$), activated Tregs ($CD45RA^-Foxp3^{high}$) and cytokine-secreting non-suppressive T cells ($CD45RA^-Foxp3^{low}$). Therefore $CD45RA^-Foxp3^{low}$ were separated because of their low suppressive activity, while the $CD45RA^-Foxp3^{high}$ subset would be the true active Tregs (Miyara et al., 2009)

Foxp3 (forkhead family transcription factor) is a transcription factor that was found to have stable expression in Tregs. It is important for Treg differentiation, suppressive function, proliferative potential and metabolic fitness (Josefowicz and Rudensky, 2009). Furthermore, the acetylation of several lysines in the forkhead domain of the Foxp3 protein is determinant for optimal Treg function (Tao et al., 2007). There were other T cell subsets found to be functionally suppressive, that also originated in the thymus in the course of the immune response *in vivo*, such as TGF-β-expressing T helper 3 (Th3) cells and IL-10 producing T regulatory 1 (Tr1) cells (Fig.3). However, these subsets didn't express *Foxp*3 (Kretschmer et al., 2005). Furthermore, suppression in Tregs is made through cell-to-cell contact while the Th3 and Tr1 effects depend on the presence of cytokines such as TGF-β and IL-10 respectively (Scheinecker C., 2010).

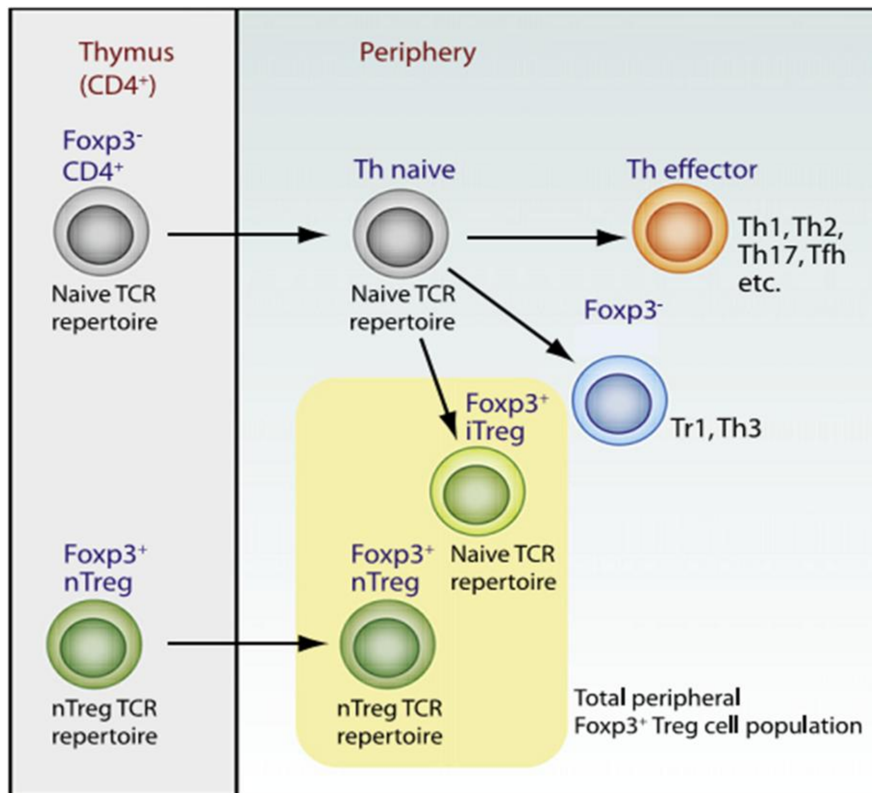

Figure 3 - Thymic and peripheral generation of different T cell subsets. Natural Tregs differentiate in the thymus and migrate to peripheral tissues. Th cells are also generated in the thymus and, once in the periphery, are able to develop different phenotypes. These might become induced Tregs, other regulatory cells like Tr1 or Th3 or even other effector Th cell types such as Th1, Th2, Th17, etc. Adapted from Lafaille and Lafaille, 2009.

1.3.2.1. Natural Tregs versus induced Tregs

In humans, natural Foxp3^{+} Tregs consist only 1-2% of the CD4^{+} cells circulating in the peripheral blood. Their development in the thymus is made in a highly controlled microenvironment (Josefowicz and Rudensky, 2009). Natural Tregs are generated in the thymus through IL-2 mediated activation of Jak3/STAT5 signaling, while in the periphery IL-2 mediates PI-3 kinase activity influencing their homeostasis (Turka and Walsh, 2008). However, in the absence of IL-2 thymic generation still occurs, possibly through IL-15 that is able to generate Tregs using the same signaling pathways. Therefore survival and expansion of Tregs in the periphery may largely depend on IL-2 (Curotto de Lafaille et al., 2004; D'Cruz and Klein, 2005; Fontenot et al., 2003). Natural

Tregs are able to suppress $CD4^+$ T cells, $CD8^+$ T cells, monocytes, B cells and dendritic cells (DC) through cell-to-cell contact and/or through the production of cytotoxic factors such as perforin and granzymes (Grossman et al., 2004).

Adaptive or induced Tregs are generated in the periphery under different conditions (Table II). For example, $CD4^+CD25^-$ effector Th cells are able to become $CD4^+CD25^{high}Foxp3^+$ *in vitro* and *in vivo* through TGF-β and IL-2 cytokines and TCR stimulation (Curotto de Lafaille and Lafaille, 2009; Grossman et al., 2004).

Table II – Different requirements for natural Tregs (nTregs) and induced Tregs (iTregs). Adapted from Laffaille and Laffaille 2009.

		nTreg cells	**iTreg cells**
Generation:	Tissue	Thymus	GALT, spleen, lymph node, inflamed tissue
	Costimulation requirement	CD28	CTLA-4
	Cytokine requirement	IL-2 or IL-15 TGF-β (?)	IL-2 TGF-β
Specificity:		Self (?)	Allergens, commensal microbiota, neoantigens (tumor), alloantigens, self (inflammation)

Adaptive Tregs have a similar phenotype and function as Natural Tregs, and they both have suppressive capacities. The differences are the requirement of cell-to-cell contact in Natural Tregs (Fehervari and Sakaguchi, 2004), and the fact that the adaptive Tregs need the presence of soluble factors like TGF-β for *Foxp*3 induction (Chen et al., 2003; Kang et al., 2005). Studies of the methylation pattern of *Foxp*3 revealed that natural Tregs had complete demethylation within a conserved region upstream of exon 1 known as Treg cell-specific demethylated region (TSDR). In contrast, induced Tregs with high expression of *Foxp*3 had only partial demethylation and these cells generated

in vitro lost *Foxp*3 expression and suppressive capacity after restimulation without TGF-β (Floess et al., 2007; Huehn et al., 2009).

The understanding of how induced Tregs are generated *in vivo* is still not clear. These can be found in lymph nodes during induction of oral tolerance (Coombes et al., 2007), in the lamina propria of the gut in response to microbiota and food antigens (Sun et al., 2007), in chronically inflamed tissues (Curotto de Lafaille et al., 2008) and transplanted tissues (Cobbold et al., 2004). Therefore, the alterations in the tissues and the cytokine pool may influence the balance of the Treg subpopulations (Suen et al., 2009).

1.3.2.2. Tregs and Autoimmunity in SLE

It was described that natural Tregs are low in number or functionally defective in active SLE (Alvarado-Sanchez et al., 2006; Miyara et al., 2005). Treg dysregulation is thought to be one of the major factors contributing to the occurrence of autoimmune diseases like SLE (Suen et al., 2009). As described above, Foxp3 is the marker that is characteristic of the Treg subset. It was described that mutations found in the *Foxp*3 gene resulted in autoimmune manifestations in the Scurfy mouse with immune dysregulations and X-linked syndrome (IPEX) in humans (Gambineri et al., 2003).

Studies in SLE revealed low percentages of $CD4^{+}CD25^{+}$ Treg cells in peripheral blood of SLE patients, however these studies became inconclusive because there was no way to discriminate Tregs from effector T cells without the use of Foxp3 marker. Tregs in humans are represented by the high expression of CD25 but intermediate expression of CD25 may also be observed in effector T cells (La Cava, 2008). It was found that $CD4^{+}CD25^{high}$ Tregs in active SLE had low levels of *Foxp*3 expression. These cells also had reduced capacity to suppress the proliferation and cytokine production of effector

Th cells *in vitro*, while the Tregs from inactive SLE patients showed the same suppressive capacity as healthy controls (Valencia et al., 2007).

Another feature in SLE is the increased frequency of $CD25^{low}Foxp3^{+}$ or even $CD25^{-}Foxp3^{+}$ subpopulations. Some authors suggest that $CD4^{+}CD25^{-}Foxp3^{+}$ T cells might represent adaptive Tregs induced by a systemic autoimmune response while the $CD25^{low}Foxp3^{+}$ subpopulation may include naive natural Tregs and also induced Tregs (Fritzsching et al., 2006; Suen et al., 2009; Valmori et al., 2005). The altered microenvironment in SLE patients in terms of inflammatory mediators is somewhat unbalanced, which might not only influence the function of Tregs but also their phenotypic characteristics. This could be an explanation for the increased $CD4^{+}CD25^{-}$ Foxp3 subpopulation that phenotypically resembles Tregs, however it is not completely functional in terms of suppressive capacity against effector Th cell proliferation and their production of interferon-gamma (Bonelli et al., 2009). Some other groups rather think that this population represents activated T cells rather than defective Tregs, which brings the need to find additional markers for the characterization of Tregs (Scheinecker C., 2010).

As described before, SLE is characterized by a wide spectrum of clinical manifestations but also by the abnormal production of autoantibodies with multiple specificities like nuclear antigens, cell surface molecules and serum proteins (La Cava et al., 2005) resulting in the loss of immune tolerance to self antigens (La Cava, 2008). The increased abundance of autoantibodies promotes the formation of immune-complexes with the antigens that precipitate and accumulate in the tissues, resulting in local damage, chronic inflammation and contributing to the loss of organ function (La Cava et al., 2005). The activation and expansion of T cells in this environment may also contribute to the attack to target cells or tissues, with a production of proinflammatory

cytokines or an increased cell-to-cell adhesion leading to the apoptosis of these cells (Crispin et al., 2008b; Kevil and Bullard, 1999; La Cava, 2008). Another fact that is thought to contribute to the autoreactivity of B cells is the inefficient clearance of apoptotic cell remnants. These may become necrotic and accumulate in the germinal centers leading to survival signals to the autoreactive B cells (Radic et al., 2011). Furthermore, the apoptotic bodies released by blebbing bring DNA microparticles that might represent nucleic acid autoantigens which is compatible with the anti-double strand DNA autoantibodies (anti-dsDNA) and anti-ribonucleic protein autoantibodies (anti-RNP) found in SLE (Cline and Radic, 2004; Reich and Pisetsky, 2009).

Recently it has been proposed that a functional deficiency in regulatory T cells (Tregs) may be at the origin of SLE autoimmunity. B cells and CD4+ Th are contributing to the development and maintenance of autoantibodies (Shlomchik et al., 2001). This could happen by an increase of specific helper T cell activity or by an enhanced B cell activity and both were shown to be regulated by Tregs in healthy subjects (Iikuni et al., 2009; Lim et al., 2005; Lim et al., 2004).

1.4. Genetic Association studies

1.4.1. Allelic association studies

One approach used to analyze genetic information is by doing allele frequency association that relies on differences in the allele frequency. There are two approaches in these studies, one between patients and controls known as the case-control study and another called family based association studies that compare the frequency of alleles that are transmitted from healthy parents to an affected child (Peltonen et al., 1999). The alleles tested are commonly single-nucleotide polymorphisms (SNPs) which, when

found in a higher frequency in SLE patients, can be either a causative variant, directly associated with the disease, or a non-functional variant that might be correlated to a functional variant through linkage-disequilibrium, indirectly associated with the disease. Linkage-disequilibrium is usually associated with short physical distances between SNPs, which restricts the study of a single gene or gene cluster that might have biological significance or has an important position in a linkage region (Rhodes and Vyse, 2008).

1.4.2. Genome-wide association (GWA) studies

In the last 10 years, there was an exponential increase of knowledge about genetics in SLE. With the technological advances and the access of genome-wide associations (GWA) it became possible to genotype hundreds of thousands of SNPs across the whole genome in a single project (Rhodes and Vyse, 2008). This type of studies contributed to the identification of several genes associated with SLE as well as SNPs that are presumed to be involved in the immune response. There are new genes being associated with SLE all the time, with variable functions or even with unknown function (Crispín et al., 2010). Some are involved to nucleic acid sensing and interferon (IFN) production such as *IRF5* (Graham et al., 2007), *STAT4* (Abelson and Delgado-Vega, 2009), *IRAK* (Jacob et al., 2009), *TLR8* (Armstrong et al., 2009), osteopontin (Kariuki et al., 2009) and *TREX1* (Lee-Kirsch et al., 2007). Some are expressed in B cells like *BANK1*, *BLK* (Hom et al., 2008) or *LYN* (Lu et al., 2009), and even others in T cells such as *PTPN22* (Chung and Criswell, 2007), *IL2* (Maiti et al., 2010), *PTTG1* (Lofgren et al., 2012), *IL2RB* (Mirowska-Guzel et al., 2011), *CTLA4* (Benmansour et al., 2010) and many others.

Some genome-wide analysis (GWA) studies have brought to our knowledge a large number of associated SLE genetic factors. However, these associations are discovered in such abundance that our capacity to explain them and assimilate them is overwhelmed (Moser et al., 2009). One problem with these studies relies on the fact that some associations are found in some GWA studies in contrast to other similar studies, raising questions about the reliability of those associations (Suarez-Gestal et al., 2009). Therefore the replication of these studies is mandatory in order to confirm the reproducibility of these associations (Todd, 2006).

Likely, there are more variants that are increasing the susceptibility to SLE that are less frequent, however to detect these variants the cohorts should be much larger than the currently available ones, in order to reach the necessary power. This would be important to identify rare but penetrant alleles in SLE (Rhodes and Vyse, 2008).

Chapter 2. MATERIAL AND METHODS

2. Material and Methods

2.1. Collection of the samples

Peripheral blood samples were collected from 100 unrelated healthy controls and 81 families of SLE patients, one patient per family, and a total of 192 first relatives, after signed and informed consent in different hospitals from Lisbon and Oporto. In this project, I processed approximately 30 families and 60 controls samples, the other samples were processed previously by the group. The disease was clinically diagnosed according to an adequate questionnaire, made for the purpose, and the disease activity score was measured with the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), a classic and validated method. The first degree relatives (parents, siblings and sons older than 18 years old) were clinically ascertained with a proper questionnaire. Most of the SLE patients are women and the age of onset is variable (Table III)

Table III - Gender and age distribution per group.

	Nº	Nº Female	Nº Male	% Female	Median Age	Min Age	Max Age
Patients	81	70	6	92	45	19	81
Relatives	192	113	71	61	48	19	87
Controls	100	58	41	59	44	21	65

2.1.2. Mononuclear cell isolation

Peripheral blood samples were collected in two Cell Preparation Tubes, CPT (BD). The peripheral blood mononuclear cells (PBMC) have low density compared to the other blood cells. CPT tubes use this characteristic to separate the PBMCs and plasma through centrifugation, with a gel that after centrifuging forms a physical barrier separating this fraction from the rest of the cells. The tubes were centrifuged at 3000 rpm for 30 min at 25 ºC. The plasma is mixed up to ressuspend the PBMCs in both

tubes, the plasma is from both tubes is combined and centrifuged at 1300 rpm for 10 min, at 4 ºC. The plasma was removed leaving 1 mL in which the cells were ressuspended and kept on ice. The viable cells were counted in a NeuBauer chamber using 10 µL of 1:100 cell dilution in 0.4% Trypan Blue Stain.

2.2. Flow Cytometry

2.2.1. Cell Staining

The volume equivalent to $1x10^6$ PBMCs of each sample were placed in a 96 well plate, round bottom, and the volume of each well was filled up until 180 µL with FACS buffer. FACS buffer was composed of 2% fetal bovine serum (Sigma) and 1mM sodium azide (NaN_3) in PBS buffer. The cells were centrifuged at 1300 rpm, at 4 ºC, for 3 min and washed through the same procedure. After centrifugation the supernatant was discarded the surface antibodies mix, containing adequately titrated concentrations of antibodies, was added and left for 30 min on the ice. The surface antibodies used were anti-human CD4 Pacific Orange (Invitrogen), anti-CD31 Pacific Blue (EXBIO praha s.a.), anti-CD127 APC/eFluor 780 (eBioscience), anti-CD39 PE/Cy7 (eBioscience), anti-CD45RO PerCP (Invitrogen) and anti-CD25 PE (BD Bioscience). After the incubation period, 150 µL of FACS buffer were added followed by centrifugation at 1300 rpm, at 4 ºC, for 3 min. The cells were washed again with FACS buffer, ressuspended in 180 µL of FixPerm buffer and incubated for 30 min on ice. FixPerm was made with Fixation and Permeabilization concentrate and Fixation and Permeabilization Diluent (eBioscience) according to manufacturer's instructions. The fixation procedure kills the cells, stabilizes the membrane, followed by the permeabilization procedure that creates pores in the membrane of the cells allowing the

intracellular staining antibodies to get inside. The cells were washed 3 times with Perm buffer which was obtained with 10x Permeabilization Buffer (eBioscience) diluted 10 times in MiliQ water. After the washing steps, 20 µL 2% of Rat serum in Perm Buffer solution (Sigma) was added and incubated for 15 min in order to block unspecific binding, followed by the addition of the intracellular antibodies for intracellular proteins: anti-Foxp3 APC (eBioscience) and anti-Ki67 FITC (BD Pharmingen), incubated 30 min on ice. After the incubation the cells were washed 2 times in Perm buffer and ressuspended in 200 µL of FACS buffer. Acquisition of the cytometry data was performed with a Cyan ADP (Beckman Coulter Inc.) and all the data were analyzed in FlowJo Software for Macintosh (Tree Star, Inc., version 9). To analyze the cytometry data, cells were first gated for lymphocytes (FSC, SSC) and then for $CD4^+$ to isolate the CD4 T cells. After this, $CD45RO^+$ was used to distinguish the memory T cell compartment.

2.3. Genotyping Assay

Sequenom's iPLEX® Gold and MassARRAY® technology allows the study of up to 40 markers per sample of genomic DNA or cDNA. It is possible to analyze single-nucleotide polymorphisms (SNPs) or it can be used in the genotyping of insertion/deletion polymorphisms (Ayodo et al., 2007). The method relies on the differences of the masses between alternative alleles that confer differences on the primer extension products using a region-specific template enrichment reaction together with mass spectrometry-based detection (Virginie Orgogozo and Rockman, 2012). The whole experiment is resumed in Figure 4 and each step is described.

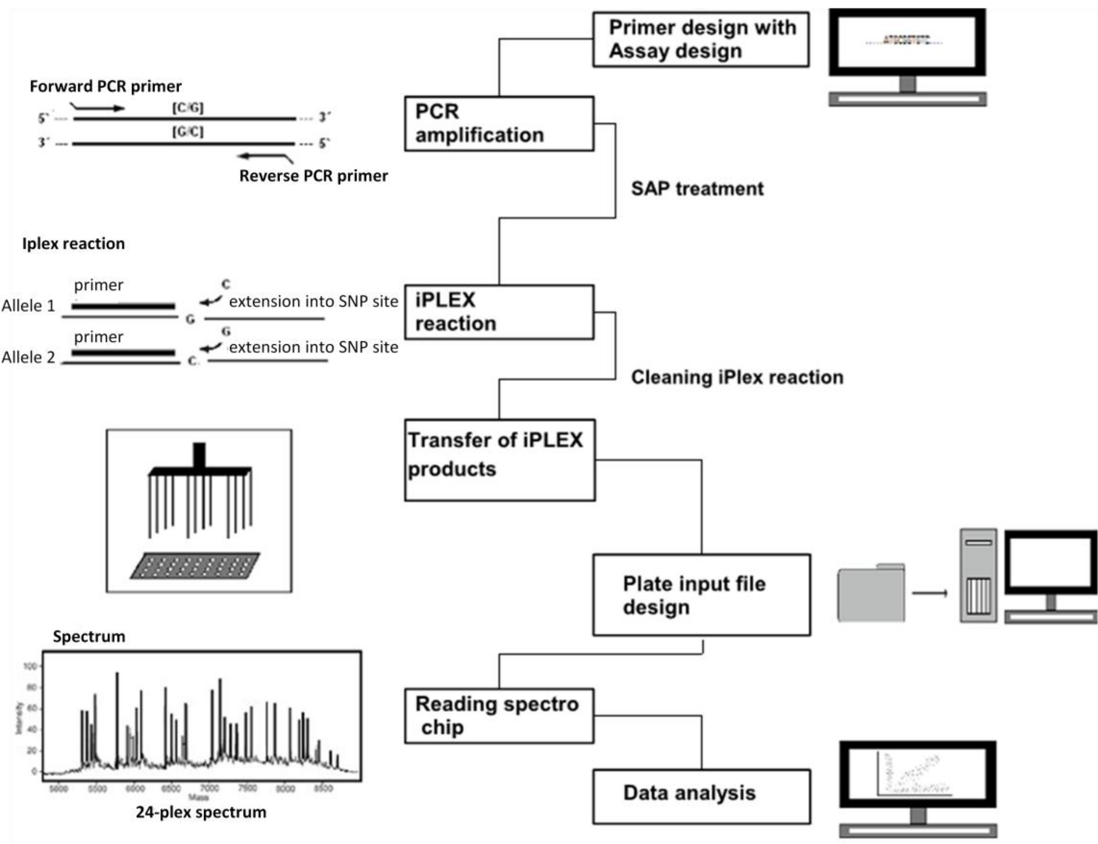

Figure 4: Summary of the Sequenom method (Virginie Orgogozo and Rockman, 2012).

2.3.1. DNA extraction (Salting out method)

The blood samples were collected in EDTA tubes and centrifuged for 5 min at 2000 rpm, 4 ºC. The whole supernatant (plasma and buffy coat) was transferred with some erythrocytes to a 50 mL falcon tube and 30 mL of Red Cell Lysis Buffer (RCLB, 10 mM Tris-HCl, 10 mM NaCl and 5 mM $MgCl_2.6H_2O$) were added and followed by an incubation of 10 min at room temperature. After incubation the cells were centrifuged for 10 min at 2000 rpm and 4 ºC, and the supernatant was discarded, if necessary this step was repeated until no red cell were visible. Then, 3.5 mL of TE (2 mM Tris-HCl, 0.4 mM NaCl and 2 mM EDTA), 200 µL of 10% SDS and 10 µL of 50 mg/mL proteinase K were added to the pellet. The mixture was mixed vigorously in the vortex and left overnight at 42 ºC in slow motion. The solution was transferred into a 10

mL conic tube and 1 mL of 6M NaCl was added and mixed vigorously until it got a milky appearance. Centrifugation was performed for 20-30 min at 3000 rpm, room temperature, and the supernatant was transferred to a new tube, discarding the pellet. DNA was precipitated by the addition of 20 mL of cold (-20 ºC) absolute ethanol. Holding the DNA thread against the tube with a tip, the ethanol was discarded and the DNA was washed with 5 mL of cold ethanol 70%. The tube with the DNA was left open to evaporate the ethanol and then the DNA was ressuspended in an adequate volume of TE (Maniatis et al., 1982).

The DNA quantification was done with a Nanodrop™ 1000 Spectrophotometer (Thermo Scientific) according to the manufacture good practices and quality of the DNA (280/260). DNA samples were diluted into a working concentration of 2.5 ng, in PCR-quality ultrapure PCR-grade water and stored at −20 °C.

2.3.2. Setting up experiment

For SNP selection there was an extensive search for previous genetic associations with SLE and/or Treg function in the literature. SNPs were chosen based on the significance of previous associations, on their role in T cells and/or Tregs, on their frequency in the European population (>20%) and taking into account the linkage disequilibrium analyzed in HapMap (http://hapmap.ncbi.nlm.nih.gov/), with the preference for tag SNPs.

In this group, six multiplex assays (group of SNPs analyzed together) had already been designed previously. In this work, another multiplex assay was constructed with 31 SNPs. However, data analysis described in this work comprises only 39 SNPs from a total of 6 genes related to SLE and Tregs, which are distributed throughout the seven multiplex assays (Table IV).

Table IV – List of genes and the respective SNPs analyzed in this work.

Gene	SNPs	References
***IL2RA* (CD25)**	*rs12359875, rs12244380, rs9663421, rs2076846, rs11256369, rs7072398, rs4749924, rs706781, rs11256497, rs791587, rs791589, rs10905669, rs2256774, rs706779, rs2104286, rs7072793, rs7073236, rs11597367, rs10795791, rs4147359, rs7090530, rs41295061, rs11594656, rs12251307*	(Fesel et al., 2012)
IL2RB	*rs743776, rs2016771, rs3218258, rs228975, rs228979, rs2281094, rs3218292, rs228947*	(Fesel et al., 2012)
CTLA4	*rs231806, rs5742909, rs231775, rs3087243, rs231723*	(Benmansour et al., 2010) and (Kimkong et al., 2011)
IL6	*rs1800795*	(Kimura and Kishimoto, 2010) and (Mirowska-Guzel et al., 2011)
PTTG1	*rs2431099*	(Lofgren et al., 2012)
PTPN22	*rs2488457*	(Chung and Criswell, 2007)

2.3.2.1. Oligonucleotide and Primer Design

Sequenom's MassARRAY Designer software automatically designed PCR and extension primers (probes) for each SNP of interest. The flanking sequences where obtained from the SNP database in the NCBI website.

2.3.3. Running experiment

2.3.3.1. Amplification of the target loci by PCR

To perform an optimal multiplex PCR reaction, amplicons were used as templates for the primer extension reaction. The purpose of this stage was to provide a specific enrichment of template DNA containing the SNPs to be genotyped. A mix of the primers (Metabion) was made with a working concentration of 0.5 mM for each primer (Table V) (Virginie Orgogozo and Rockman, 2012).

Table V: Primer mix preparation for the amplification reaction.

Number of primer pairs in the multiplex assay	31
H_2O	288 µL
Each Primer (100 mM)	2.5 µL
Total volume	443 µL

In each well 4 µL of PCR mix were placed together with 3 µL containing 7.5 ng of DNA. The microplate was sealed and was put in a thermal cycler following PCR conditions described in Table VI.

Table VI: PCR mix preparation for the amplification reaction and PCR cycle conditions.

PCR mix		
Water		1.8 µL
PCR Buffer (10×)		0.5 µL
MgCl2 (25 mM)		0.4 µL
dNTP mix (25 mM each)		0.1 µL
Primer mix (0.5 mM)		1 µL
PCR Enzyme (5 U/mL)		0.2 µL
Total volume		4 µL
PCR cycle conditions		
1 cycle	→	94°C 4 min
45 cycles	→	94°C 20 sec 56°C 30 sec 72°C 60 sec
1 cycle	→	72°C 3 min
Hold	→	6°C

2.3.3.2. Post-PCR with SAP digestion

After the amplification of the target loci a SAP digestion was used to neutralize unincorporated dNTPs from PCR amplification products by cleavage of the 5′phosphate group. In this step 2 µL of SAP solution was added to each well of the 384-well PCR plate (ABgene PCR plates, Thermo Scientific), the plate was sealed and the reaction was performed in a thermal cycler (Table VII).

Table VII - SAP mix solution and PCR cycler conditions.

SAP mix		
Water		1.53 µL
SAP buffer 10×		0.17 µL
SAP		0.3 µL
Total volume		2 µL
PCR cycle conditions		
1 cycle	→	37°C 20 min
1 cycle	→	85°C 5 min
Hold	→	6°C

2.3.3.3. Primer Extension

In MALDI-TOF mass spectrometry the peak intensity and the mass of the analyte are inversely related. In other words, the primer with the higher mass will have the lower peak intensity and vice-versa. In order to compensate this feature, the primer concentration must be adjusted so that the concentration of the primer with lower mass in the reaction mix will have a lower concentration and vice-versa. Therefore the extension primers were divided in four groups (ordered here from the lower to the higher mass) with a final concentration of: 0.93 µM, 1.25 µM, 1.6 µM and 2.1 µM. The iPLEX mix (Table VIII) was distributed in the plate followed by the primer extension mix, the plate was sealed, centrifuged for 1 min at 2000 rpm and placed in a thermal cycler (Virginie Orgogozo and Rockman, 2012).

Table VIII: iPLEX mix solution and PCR cycler conditions.

iPLEX mix		x530 reactions
Water	-	341.9 µL
iPLEX Buffer Plus (10×)	0.2 µL	106 µL
iPLEX Termination mix	0.2 µL	106 µL
Primer mix	-	484.9 µL
iPLEX Enzyme	0.04 µL	21.2 µL
Total volume	-	1,060 µL
PCR cycle conditions		
1 cycle	→	94°C 30 sec
40 cycles	→	94°C 5 sec 52°C 5 sec 80°C 5 sec 52 ºC 5 sec 80 ºC 5 sec 52 ºC 5 sec
1 cycle	→	80° C 5 sec
1 cycle	→	72 ºC 3 min
Hold	→	6°C

After this step, and in order to remove the salts from the previous reactions, a cationic resin was used to avoid background noise in the mass spectrometry analysis.

2.3.3.4. Spotting primer extension products on SpectroCHIPs

In this step, 25 nL of the iPLEX reaction products, from the 384-well plate, were transferred into a SpectroChip, in 384-well format and pre-spotted with a specially formulated MALDI matrix, by the MassARRAY Nanodispenser station with a 384 microtiter. After the sample transfer, the SpectroChip was placed in the MassARRAY mass spectrometer, where the masses of the products resulting from the experimental steps were analyzed (Fig. 5).

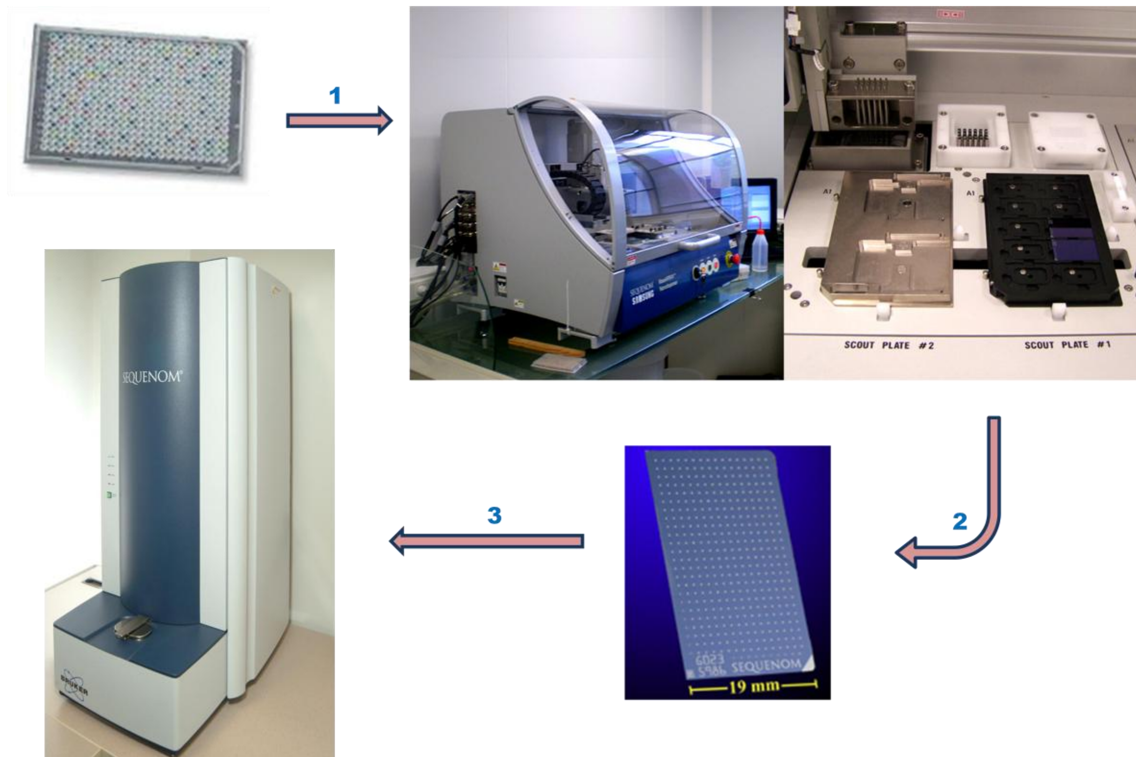

Figure 5 - Schematic representation of spotting primer extension products. Through the MassARRAY Nanodispenser (1), samples are transferred to the SpectroChip (2) and finally the SpectroChip is placed in the MassARRAY mass spectrometer (3) to analyze the PCR products in terms of masses.

2.3.4. Analysis

The resulting spectra of the samples were analyzed in the MassARRAY Typer 4.0 software. This is the first step of quality control where all the calls for each SNP in the experiment can be analyzed and optimized (fig. 6). Other quality controls used were the Hardy-Weinberg equilibrium (HWE) in the healthy controls group that was normal (P>0,01) and a minimum of 90% call rate for each SNP was required. The SNP *rs228979* from *IL2RB* did not meet the quality control criteria and was excluded. All samples had a call rate >75%. All SNPs had a minor allele frequency >1%.

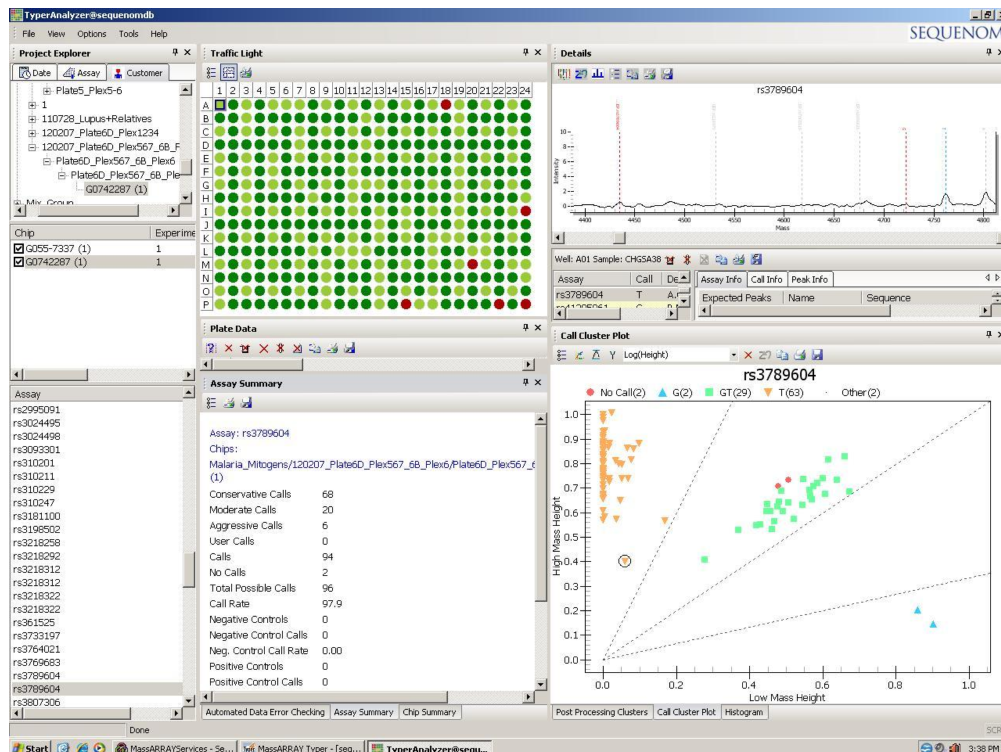

Figure 6 - Main window of the MassARRAY Typer 4.0 software with the plate information and the allelic distribution of the samples for each SNP (http://cancer-seqbase.uchicago.edu/documents/iPLEX SoftwareGuide.pdf).

2.4. Inducing CD25 and *Foxp*3 expression in Naïve Th effector cells

2.4.1. Staining and cell sorting

After counting the cells, 10 to 20x10^6 cells were washed in 150 µL of 1x PBS with 2% FBS for 5 min, 1300 rpm at 4 ºC. Discarding the supernatant, the cells were stained with a mix containing anti-human CD4 PE/Cy5.5 (eBioscience), anti-CD25 PE/Cy7 (BioLegend) and anti-CD45RO FITC (BD Bioscience) in FACS without azide, using 20 µL of mix per 10^6 cells. The cells were incubated for 30 min, at 4 ºC, and washed one time with 1x PBS with 2% FBS. Another wash was made with supplemented medium: RPMI 1640 + GlutaMAX (Gibco) supplemented with 0,05 µM β-Mercaptoethanol (Sigma), 1x Non Essential Amino Acids (Gibco), 1mM Sodium

Pyruvate (Gibco), 1% Penstrep (Gibco), 50 µg/mL Gentamicin (Gibco), 10 mM HEPES (Gibco) and 10% Fetal Bovine Serum (FBS - Sigma).

The cells were sorted in a FacsAria (BD Biosciences) using the following strategy of gating (Fig. 7). The collection tubes were left overnight with supplemented medium to coat the tube walls before collection of sorted cells.

After sorting, the cells were counted to confirm the number of sorted cells given by the sorter. For that, the cells were centrifuged for 5 min, 1300 rpm at 4 ºC and ressuspended with 500 µL of supplemented medium. From the cells suspension, 10 µL were taken and mixed with an equal volume of 0.4% Trypan Blue. 10 µL of this mixture was used for the counting in the NeuBauer chamber.

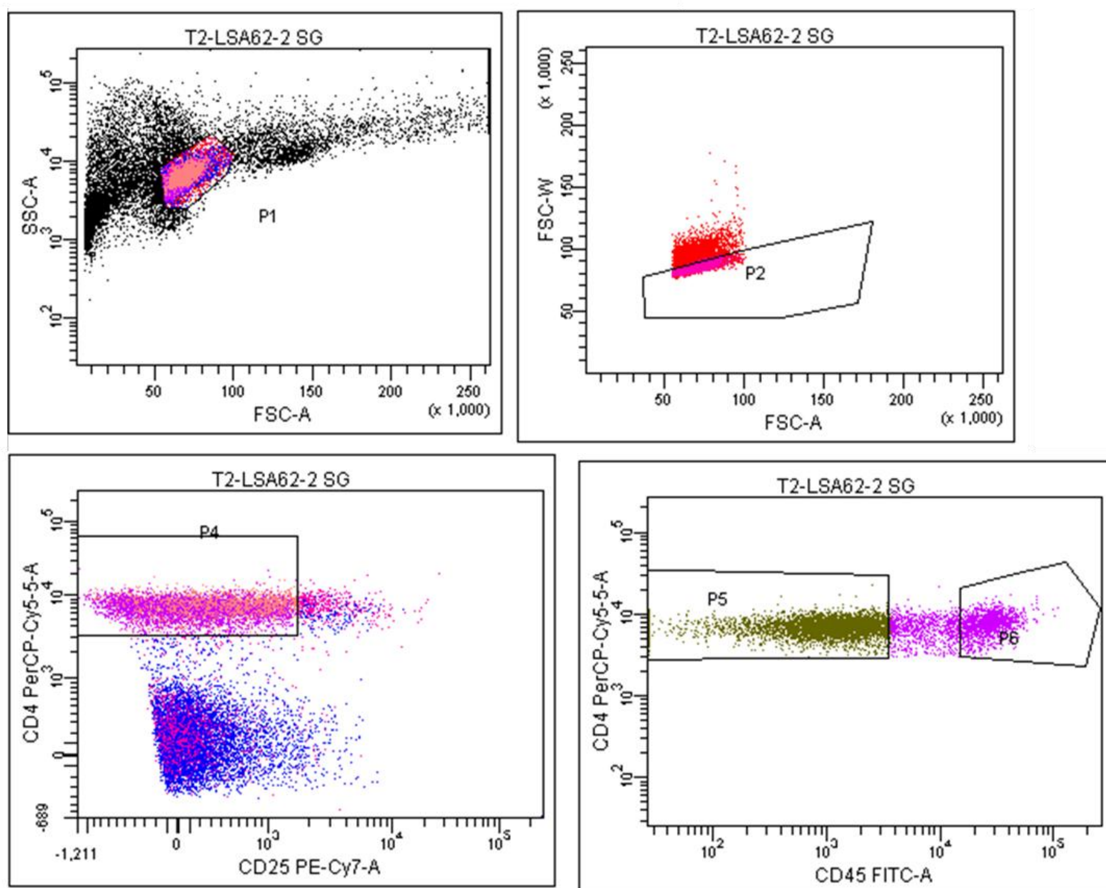

Figure 7 - Gating strategy to sort the naïve effector Th cells. First the lymphocytes were gatted (P1) and within this population the duplets were excluded (P2). Then the $CD4^{+}CD25^{-}$ population inside the P2 is isolated (P4) and afterwards the $CD4^{+}CD25^{-}CD45^{-}$ (population of interest) is isolated from the P4.

2.4.2. Cell Culture

The 96 well culture plates (TPP), flat bottom, were coated with 50 µL per well of 2 µg/mL anti-human CD3 antibody, clone OKT3 (eBioscience), in PBS 1x, overnight at 4 ºC or for at least 4 hours at 37 ºC. After coating, and before placing the cells, the wells were washed three times with 1x PBS and a last time with supplemented medium.

Sorted cells were distributed in the wells in order to range from $5x10^4$ to $10x10^4$ per well, depending on the availability of cells, and PBMCs were cultured with $10x10^4$ cells. After placing the cells, the plate was centrifuged for 3 min, 1300 rpm at 4 ºC and the supernatant discarded to be replaced with the stimulatory culture medium.

Different combinations of cytokines and concentrations were used to test the capacity of naïve effector Th cells to express CD25 and Foxp3 (Table IX). The cytokines used were anti-human CD28, clone CD28.2 (eBioscience), TGF-β1 (eBioscience) and IL-2. The cytokines were diluted in supplemented medium. The amount of medium used in each well was proportional to the number of cells in order to put them all at the same density, for example if there were $5x10^4$ cells 100 µL of medium and if there were $10x10^4$ cells 200 µL were used. Cells were then incubated for 5 days at 37 ºC, 5% CO_2.

Different conditions were tested to explore the activation capacity of anti-CD3 antibody together with anti-CD28 and the influence of TGF-β1 on the expression of *Foxp*3 (Table IX). To exclude the presence of TGF-β1 in the serum or the influence of possible production of this cytokine by the cells, the condition 0 was done with an anti-TGF-β1 antibody, clone 1D11 antibody (produced in house).

Table IX - Set of cytokines used to create different conditions and to perceive how they influence the rate of activation in Foxp3 expressing cells.

	0	1	2
Anti-CD3	2 µg/mL		
Anti-CD28	1µg/mL		
TGF-β1	-	-	2ng/mL
1D11	50µg/mL	-	-
IL-2	50 U/mL		

Depending on the number of cells available from each group they were distributed throughout the conditions established (Table X).

Table X – Number of cell culture conditions performed per group.

Conditions	0	1	2
Patients	-	4	6
Relatives	2	10	12
Controls	2	17	24

2.4.2.1. Staining of the cultured cells

The volume from each well of the culture plate was transferred into a 96 well plate, round bottom, and centrifuged for 3 min, 1300 rpm, at 4 ºC. The cells were washed with 1x PBS and after centrifugation the supernatant was discarded and 20 µL of surface antibodies mix were added and left for 30 min on ice. The surface antibodies used were anti-human CD4 Pacific Orange (Invitrogen), anti-CD127 APC/eFluor 780 (eBioscience), anti-CD39 PE/Cy7 (eBioscience), anti-CD45RO PerCP (Invitrogen) and anti-CD25 PE (BD Bioscience). A Fixable Viability Dye eFluor 450 (eBioscience) was also used in the surface mix, with a dilution factor of 1/40 µL in 1x PBS. After incubation of 30 min at 4 ºC, 150 µL of FACS buffer was added followed by

centrifugations at 1300 rpm, at 4 ºC, for 3 min. From this step on the protocol was the same as in the normal staining.

2.5. Data Analysis

Data analysis and graphic displays were performed using IgorPro v6.1 (Wavemetrics), programmed for the purpose. Statistical methods were made with distribution independent methods using Mann-Whitney test for group comparisons and Spearman rank correlation for quantitative relations. P-values below 0.05 were considered significant.

Considering the data from the genotyping analysis, the allele information from the genotyping data were converted into a numeric score (example: AA was 1, AG was 2 and GG was 3), to analyze it in quantitative terms. SNP associations were corrected for multiple testing by the Bonferroni method. Bonferroni correction is the most conservative method to correct for multiple testing and it consists in multiplying the P-value of a certain SNP with the number of SNPs tested for the same gene.

Genetic associations in the relatives group were assessed by Spearman rank correlation between all first degree relatives, irrespectively of family relations. Since in most of the families more than one relative was included, it may be that intrafamiliar correlation of phenotypes influences the results so that they have to be considered as approximate. Definitive analysis of the data will adopt a statistical test that considers the family structures explicitly.

Chapter 3. RESULTS

3. Results

3.1. Flow Cytometry Data

3.1.1. Characterizing the Treg population

In this work we followed the Sakagushi characterization mentioned before, where resting Treg were characterized as CD45RA^{+}Foxp3^{low}, activated Tregs as CD45RA^{-}Foxp3^{high} and cytokine secreting CD45RA^{-}Foxp3^{low} as non-suppressive T cells (Miyara et al., 2009). Here CD45RO^{+} was used instead of CD45RA^{-} and the analysis is based on the CD45RO^{+}Foxp3^{low} and CD45RO^{+}Foxp3^{high} that will be referred to in this study as Foxp3^{low} and active Tregs respectively. All Foxp3 expressing cells are referred to as Foxp3^{+} cells.

Analyzing the frequencies of Foxp3^{+} and active Tregs in the CD4^{+} compartment, it is already possible to visualize differences between the groups: patients, relatives and controls (Fig. 8).

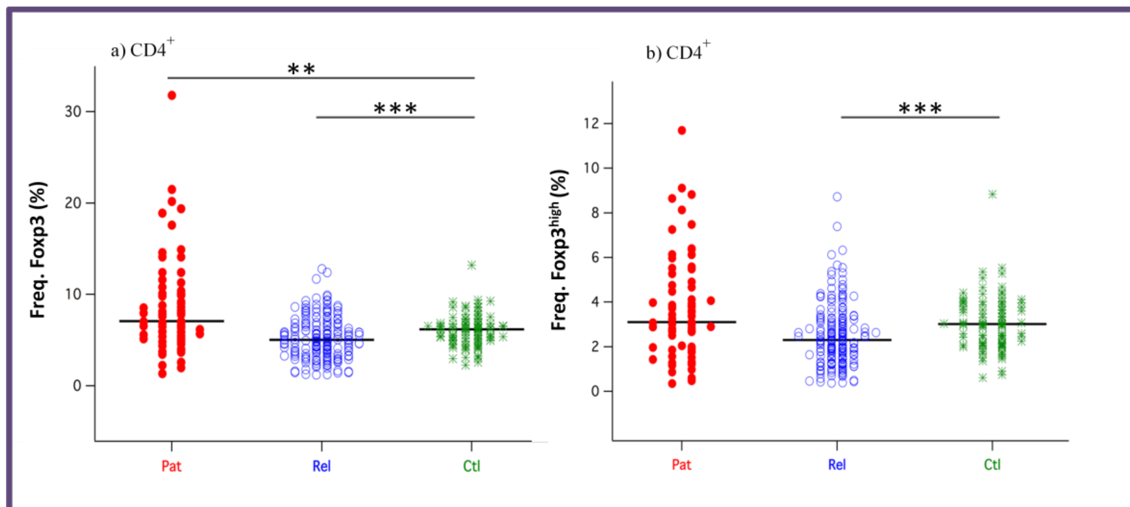

Figure 8 - Frequency of Foxp3 cells in the CD4^{+} compartment. a) all Foxp3-expressing cells (Foxp3^{+}) and b) the active Tregs (Foxp3^{high}). Each dot represents the cytometry result of one individual: the patients are represented in red, in blue are the relatives and in green the healthy controls. The horizontal lines indicate the median and the stars indicate the p-values calculated with the group-wise Mann-Whitney test (**$P<0.01$; ***$P<0.001$).

The patients had higher frequency of Foxp3 expressing cells in $CD4^+$ (P=0.001) but the frequency of $Foxp3^{high}$ in $CD4^+$ cells was not different compared to controls (Fig. 8 a and b). This means that another Foxp3 population was influencing the $Foxp3^+$ frequency in $CD4^+$. Therefore, the frequency of $Foxp3^{low}$ was determined and the results demonstrate that the frequency of Foxp3 expressing cells was indeed higher in the patients because the $Foxp3^{low}$ compartment was expanded ($P=1.8x10^{-7}$) (fig. 9). The origin of these cells is controversial and their frequency is usually low as it was observed in the relatives and controls (Fig. 9).

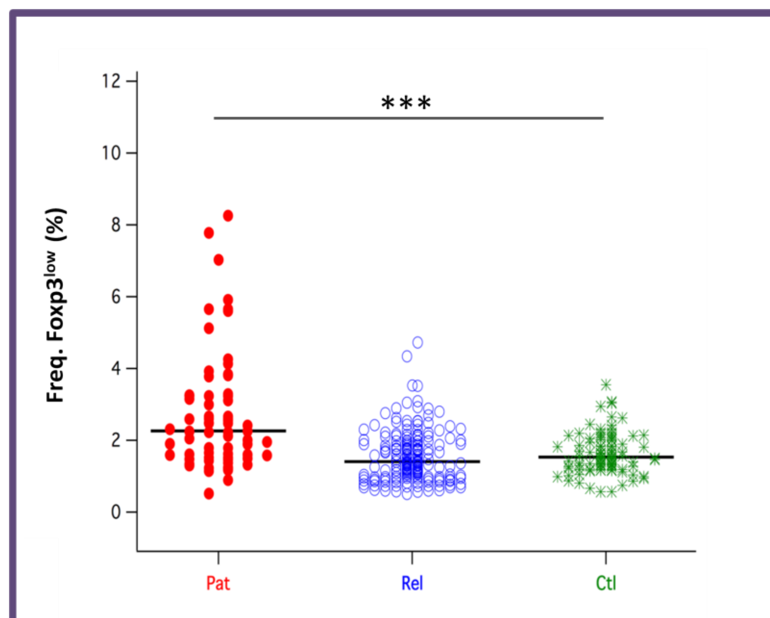

Figure 9 - Frequency of $Foxp3^{low}$ cells in the $CD4^+$ compartment. Each dot represents the cytometry result of one individual: red represents patients, in blue are the relatives and in green the healthy controls. The horizontal lines indicate the median and the p-values where calculated with the group-wise Mann-Whitney test. (*** P<0.001)

In the relatives both the frequency of $Foxp3^+$ (P=0.0008) and the frequency of active Tregs (P=0.0002) were significantly lower compared to the frequency of the same compartments in controls (Fig. 8) while the frequency of $Foxp3^{low}$ cells there was not different compared to controls (Fig. 9).

The next step was to characterize Foxp3^{+} cells in terms of CD25 expression, which was measured in terms of the median fluorescence intensity (MFI). The MFI is an indication of the amount of IL2 receptor α chain (CD25) present on the surface of the cells.

The patients group had the lowest CD25 median fluorescence intensity (MFI) inside the active Treg compartment ($P=1.7x10^{-12}$) (Fig. 10b) while the frequency of active Tregs in patients was not significantly different compared to controls (Fig. 10a).

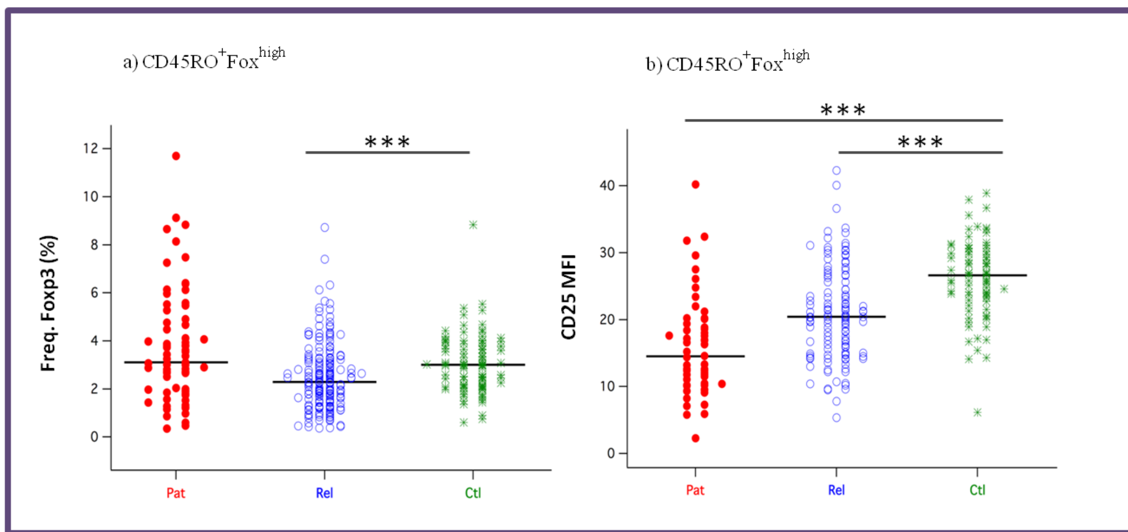

Figure 10 - Frequency of Foxp3 and CD25 MFI within CD4^{+}CD45RO^{+}Foxp3^{high} subset (active Tregs). In this figure, there is a) the frequency Foxp3 and b) the CD25 MFI, both in Foxp3^{high}. Each dot represents the cytometry result of one individual: in red the patients are represented, in blue are the relatives and in green the healthy controls. The horizontal lines indicate the median and the p-values where calculated with the Mann-Whitney test. (*** $P<0.001$)

This shows that the Tregs in patients had a downregulation of CD25 which was likely a reflection of their reduced activity. This is also an indication that the characterization of Tregs as CD4^{+}Foxp3^{high} is not sufficient for regulatory function activity (Bonelli et al., 2008; Wang et al., 2010). This “dissociation” effect between CD25 and Foxp3 expression is consistent with what is described for SLE. (Bonelli et al., 2008; Wang et al., 2010)

Therefore, it was confirmed that the presence of CD25 is also important to define Treg activity. In the case of the relatives the frequency of Foxp3 and CD25 MFI were both significantly reduced in Tregs (P=0.0001 and P=5.7x10^{-8} respectively).

The relation between the frequency of Foxp3 and CD25 MFI for each group demonstrates that in all the groups the CD25 MFI was positively correlated with the frequency of Foxp3^{high}, however it was clear that the patients and the relatives had lower levels of CD25 MFI in Foxp3^{+} compared to controls (Fig. 11).

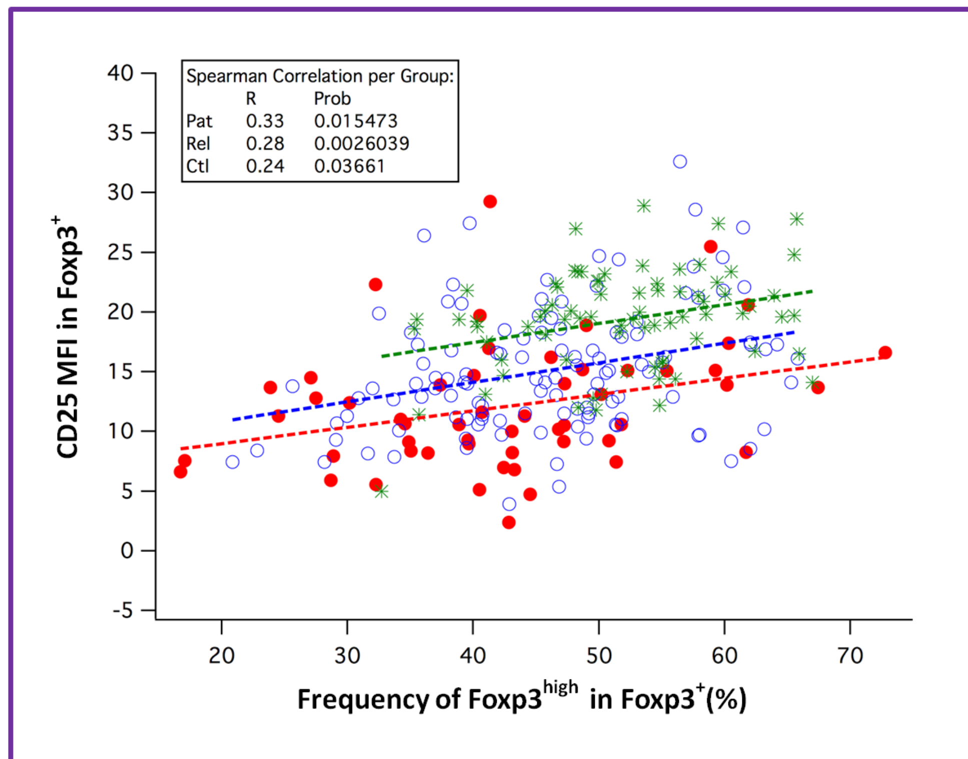

Figure 11 - Correlation between CD25 MFI with the frequency of active Tregs (Foxp3^{high}), both within Foxp3^{+}. Each dot represents the correlation result of one individual: red represents the patients, in blue are the relatives and in green the healthy controls. The lines indicate the linear regression for the respective group color. The box indicates the R Spearman correlation and the respective P-value per group.

These results demonstrate that the patients had no impaired frequency of Tregs but rather a deficit of CD25 expression in the active Tregs, however the influence of the Foxp3^{low} cells in the Treg compartment is not clear. In this study, it is rather shown that

the frequency of Foxp3^{low} in CD4^{+} was correlated with the CD25 MFI either in Foxp3 expressing cells (Foxp3^{+}) or in the active Tregs, both only in patients.

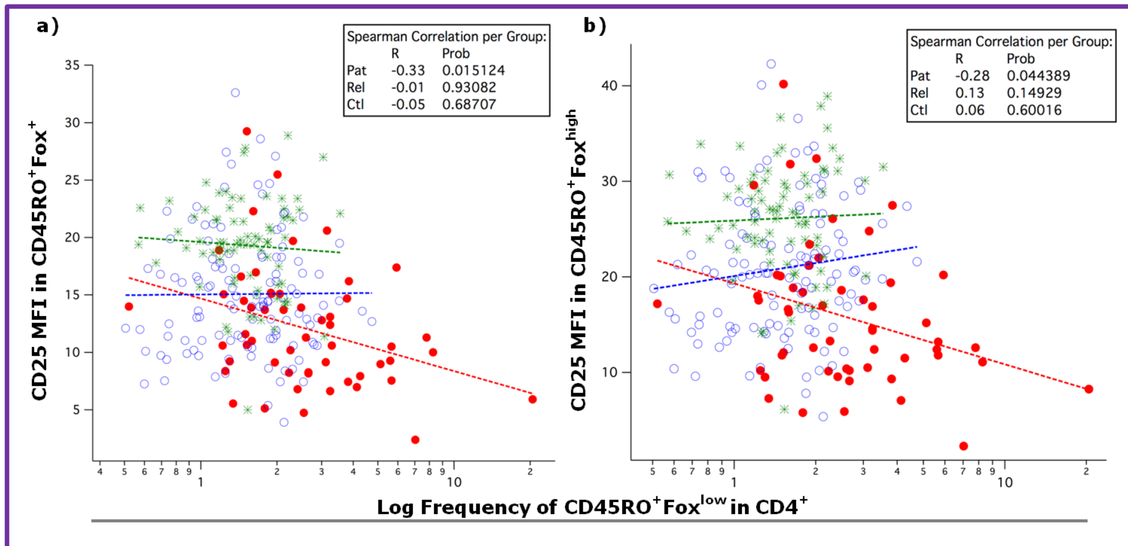

Figure 12 - Correlation of Foxp3^{low} with the CD25 MFI in Foxp3 compartments. a) CD25 MFI in Foxp3^{+} and b) CD25 MFI in active Tregs (Foxp3^{high}). Each dot represents the correlation result of one individual: red represents the patients, in blue are the relatives and in green the healthy controls. The lines indicate the linear regression for the respective group color. The boxes indicate the R Spearman correlation and the respective P-value per group.

This demonstrates that the frequency of Foxp3^{low} also had an effect on the expression of CD25 in the active Tregs. Whether this is a direct or indirect effect, there is an indication that the expansion of this compartment was accompanied by a downregulation of CD25 also in active Tregs (Fig. 12)

The proliferation marker Ki67 and the thymic emigrant marker CD31 were also analyzed. However, cytometry did not give statistically significant differences between the groups for these two markers.

Considering the marker CD39 in Foxp3^{high}, patients and relatives did not have significant differences compared to controls. The cytometry results of the marker CD127 were excluded from the analysis because the MFI values were not consistent and varied too much over time.

3.2. Correlations Genotype-Phenotype

Genetic studies usually have the main goal of finding risk factors of a phenotype such as a human disease. However, in this study the objective was rather to characterize genetic influences on phenotypes that indicate differences in T cell regulation between the patients, unaffected relatives and controls.

The genes selected for this study were described to have a direct or indirect effect in the Tregs. Only the genes whose SNPs were associated with the phenotypes after Bonferroni correction were considered, therefore the tables presented here are simplified, the complete ones are in annex I.

3.2.1. Locus-wise associations

SNP associations were corrected for multiple testing by the Bonferroni method, which allows a locus-wise analysis where the genetic influence on Treg phenotypes can be studied. Some genes had more than one SNP significant after Bonferroni considering only the one with the highest significance. In this work, the interest was to analyze the active Tregs, therefore the associations with the phenotype Foxp3^{high} in Foxp3^{+} were analyzed (Table XII).

Table XII - Associations of SNPs with the cytometric frequency of active Tregs (CD45RO^{+}Foxp3^{high}) inside the Foxp3^{+}. The associations of the SNPs in the respective locus, were tested using Spearman Correlation within the groups of study: patients, relatives and controls. Nominal P-values (P) were corrected for multiple testing over all SNPs typed is the respective loci by Bonferroni correction (Bonf).

Gene	SNP	CD45RO^{+}Foxp3^{high} in Foxp3^{+}								
		Patients			Relatives			Controls		
		R	P	Bonf	R	P	Bonf	R	P	Bonf
IL2RA* (CD25)	*rs9663421*	-0.264	0.055	-	-0.290	**3E-4**	**0.008**	-0.017	0.876	-
IL2RB	*rs743776*	0.229	0.102	-	0.230	**0.004**	**0.035**	0.056	0.614	-
IL6	*rs1800795*	0.303	**0.028**	**0.028**	0.102	0.205	-	-0.176	0.097	-
CTLA4	*rs231775*	-0.024	0.864	-	0.034	0.677	-	-0.347	**0.001**	**0.007**
PTTG1	*rs2431099*	0.141	0.313	-	-0.302	**2E-4**	**2E-4**	0.169	0.117	-

However the role of CD25 seems important to determine the activity of the Tregs and so the associations for the CD25 MFI in Foxp3^{high} were also analyzed (Table XIII).

Table XIII - Associations between the SNPs with the cytometry data of the CD25 MFI in active Tregs (CD45RO^{+}Foxp3^{high}). The associations of the SNPs in the respective locus, were tested using Spearman Correlation within the groups of study: patients, relatives and controls. Nominal P-values (P) were corrected for multiple testing over all SNPs typed is the respective loci by Bonferroni correction (Bonf).

Gene	SNP	CD25 MFI in CD45RO^{+}Foxp3^{high}								
		Patients			Relatives			Controls		
		R	P	Bonf	R	P	Bonf	R	P	Bonf
IL2-IL21	*rs1479924*	0.029	0.862	-	0.305	0.003	**0.045**	-0.064	0.584	-
	rs6849238	-0.061	0.704	-	-0.306	0.003	**0.040**	0.070	0.553	-
IL2RA	*rs706779*	-0.203	0.200	-	-0.014	0.886	-	-0.410	**0.001**	**0.013**
CTLA4	*rs5742909*	-0.053	0.742	-	-0.288	**0.005**	**0.026**	-0.096	0.411	-
	rs3087243	0.101	0.523	-	-0.254	**0.011**	-	-0.341	**0.003**	**0.017**
PTPN22	*rs2488457*	0.012	0.942	-	0.330	**0.001**	**0.005**	0.004	0.972	-

IL2RA was associated with CD25 MFI in the active Tregs in controls (Table c). This indicates a direct effect of genetic variation in the *IL2RA* locus on CD25 expression, which is probably contributing to the activation of these cells, as it was previously described in the results and introduction. However, in the relatives this locus was not associated with CD25 MFI but rather with the frequency of Foxp3^{high} in Foxp3^{+}. To see if both effects, in controls and in the relatives, were due to the same genetic variants, the Spearman R values for both phenotypes were correlated (Fig. 13). The graphic demonstrates that there was a strong negative correlation between the effects of the individual *IL2RA* SNPs, suggesting that the genetic nature of these effects was identical on both phenotypes. In other words, the same effects that made the expression of CD25 higher in controls was driving down the frequency of active Tregs (Foxp3^{high}) in the relatives.

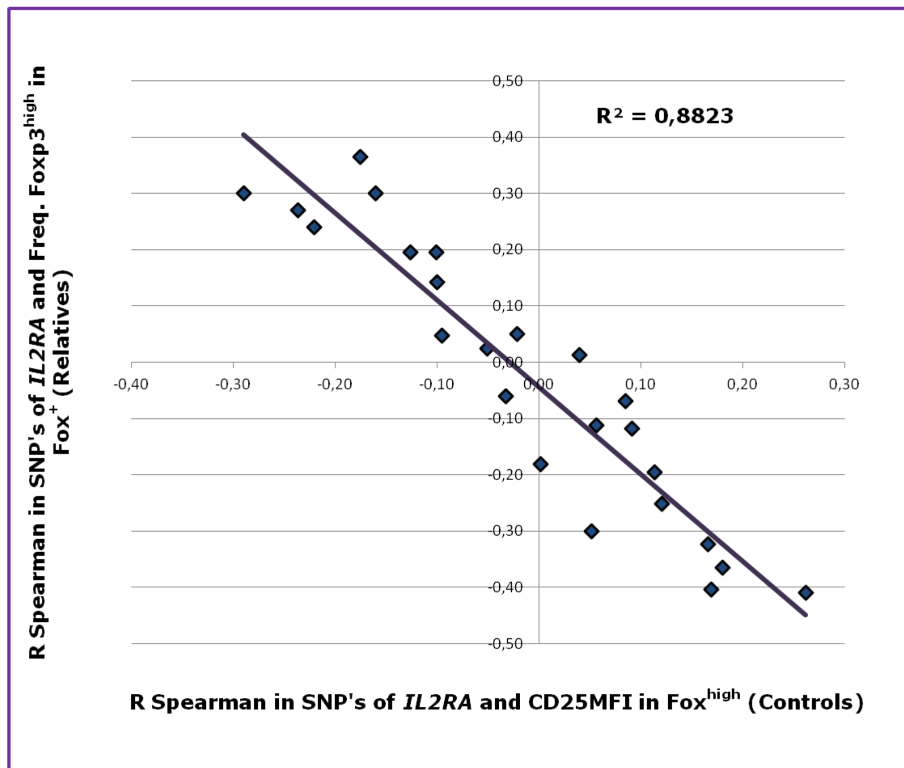

Figure 13: Correlation of the effect of *IL2RA* SNPs on CD25 MFI in Foxp3^{high}, in controls, with the effect of the same SNPs on Foxp3^{high} in Foxp3^{+}, in relatives. Each dot represents the same SNP in both phenotypes.

Individual SNPs may not have a direct influence on the associated phenotype but can also be associated due to a linkage effect. Therefore, linkage disequilibrium (LD) maps give some clues about these linkage structures.

To study the genetic effect of *IL2RA* gene (CD25), tag SNPs were used to cover the entire locus. The results demonstrate that the SNPs *rs9663421* and *rs706779* were associated with the frequency of active Tregs in Foxp3^{+} in relatives. In this group, the SNP *rs9663421* is located in the 3' region of the locus while the SNP *rs706779* was rather in the 5' region of the locus (Fig. 14).

In controls all the Bonferroni corrected SNPs were associated with CD25 MFI in active Tregs (Annex I) and are indicated in Figure 14. However, for the controls the associated SNPs are rather located in the middle, SNPs *rs2256774* and rs*7067799*, and 5' region of the locus (Fig. 14).

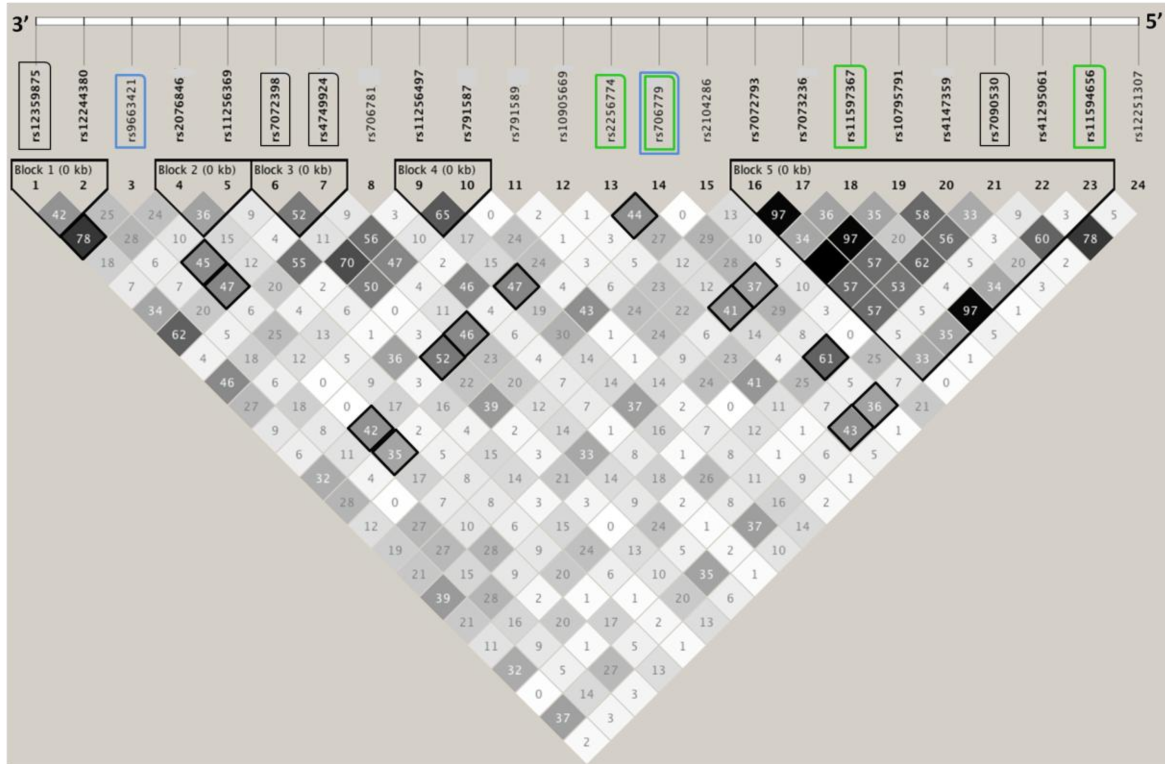

Fig. l4 - Linkage disequilibrium map of *IL2RA* SNPs typed in this study. The squares inside the pyramid represent the linkage percentage between the SNPs, the darker are the squares the higher is the linkage between the SNPs. The SNPs strongly associated with the selected phenotypes are marked in blue for the relatives and in green for the controls. These SNPs are in linkage with other SNPs inside in the grey boxes.

There are no studies available about the influence of this locus on SLE. However, Lowe et al. 2007 found that *IL2RA* was associated with Type 1 Diabetes (T1D) but the associated SNPs were mainly in the 5' region of the locus (Lowe et al., 2007). All the SNPs that were associated with the Treg phenotypes are in linkage with this 5' region described by Lowe et al (2007), except for *rs9663421* whose effects cannot be explained by the effects suggested for T1D SNPs, because it is not in linkage with this 5' region of the locus. In fact, the SNP *rs9663421* had the strongest association in this analysis and its effect was not yet described by other groups. It appears that the linkage structure is more complicated than what was described by the other group implicating the SNPs *rs41295061* and *rs11594656* as candidate genes for the effect on CD25 in T1D, therefore more studies about this locus are required.

IL2RB is another subunit of the IL-2 receptor, and the main receptor for IL-15 (Rochman et al., 2009). In this study, the SNP *rs743776* was associated with the frequency of $Foxp3^{high}$ in $Foxp3^{+}$ cells in the relatives. The SNP *rs743776* does not have described associations but is tagged by the SNP *rs743777* (Malek et al., 2002; Mirowska-Guzel et al., 2011). The *IL2RB* SNP *rs743777* is associated to autoimmune diseases such as rheumatoid arthritis (RA). However there is no evidence for association of this SNP with SLE (Jin et al., 2010).

Another region of interest was the linked loci *IL2-IL21* because IL2 is a very important cytokine for Treg activation as a co-stimulator. *IL2* is very close to *IL21* and they form a big linkage block (van Heel et al., 2007). In this study, the SNPs *rs1479924* and *rs6849238* are localized in *IL-2* and *IL21* respectively and both were associated with the CD25 MFI in $Foxp3^{high}$ in the relatives. The IL2-IL21 region was described to be highly associated with many autoimmune diseases: Celiac disease, T1D, RA, ulcerative colitis, juvenile idiopathic arthritis (JIA), psoriatic arthritis, psoriasis, and SLE (Maiti et al., 2010).

3.2.2. Alleles associated with autoimmune phenotypes

The other typed SNPs had alleles described to be associated with specific phenotypes related to autoimmunity or even with SLE. The risk alleles described in the literature for these SNPs, that were associated with the Treg phenotypes in our study, are shown in Table XIV.

Table XIV - Risk alleles for each SNP associated with the Treg phenotypes.The associations of the SNPs, of the respective gene, were tested using Spearman Correlation within the groups of study: patients, relatives and controls. Nominal P-values (P) were corrected for multiple testing over all SNPs typed is the respective loci by Bonferroni correction (Bonf) and the R Spearman values (R).

Gene	SNP	Risk Allele	Patients		Relatives		Controls		Associated Phenotype
			R	P	R	P	R	P	
IL6	*rs1800795*	G	0.30	**0.028**	0.10	0.205	-0.18	0.097	$Foxp3^{high}$
PTPN22	*rs2488457*	C	-0.01	0.942	-0.33	**0.001**	-0.00	0.972	CD25 MFI
PTTG1	*rs2431099*	C	0.14	0.313	0.30	**2E-4**	-0.17	0.117	$Foxp3^{high}$
CTLA4	*rs5742909*	T	-0.05	0.742	-0.29	**0.005**	-0.10	0.411	CD25 MFI
	rs3087243	G	0.10	0.523	-0.25	**0.011**	-0.34	**0.003**	CD25 MFI
	rs231775	G	-0.02	0.864	0.03	0.677	-0.35	**0.001**	$Foxp3^{high}$

CTLA4 is a transmembrane glycoprotein that suppresses T cell activation through the TCR signaling while in Tregs it has a positive effect in their homeostasis. Its downregulation, or loss of activity, was associated with a higher risk for autoimmunity (Benmansour et al., 2010; Kimkong et al., 2011). *CTLA4* polymorphisms were found to be associated with SLE and other autoimmune diseases such as Graves' disease, autoimmune hypothyroidism, celiac disease, T1D (Benmansour et al., 2010). In this study, the risk allele G of the SNP *rs3087243* was associated with reduced CD25 MFI in $Foxp3^{high}$ in both controls and relatives. The G risk allele in *rs231775* was associated with diminished $Foxp3^{high}$ frequency in controls. The last *CTLA4* SNP *rs5742909* was associated with reduced CD25 MFI in active Tregs, in relatives. For some SNPs, mechanisms for direct molecular effects were described before. For example, the SNP *rs231775* risk allele causes an amino acid change while the SNP *rs3087243* is described to be important for efficient splicing and its G risk allele was associated with a downregulation of CTLA4 (Chistiakov and Turakulov, 2003; Kimkong et al., 2011; Repnik and Potocnik, 2010). However, the risk allele G in SNP *rs5742909* was controversial in terms of association with autoimmune diseases (Benmansour et al., 2010).

PTPN22 (Protein tyrosine phosphatase nonreceptor type 22) encodes the lymphoid specific phosphatase (Lyp), an intracellular protein tyrosine phosphatase that binds to Csk kinase. This binding is important for the Csk ability to suppress T cell activation. Genetic variations in *PTPN22* were associated with a risk for autoimmunity in several diseases including SLE (Bottini et al., 2004). In this study *PTPN22* SNP *rs2488457* was associated with reduced CD25 MFI in Foxp3^{high} in the relatives. This SNP is located in the promoter of the gene and its risk allele C is described to be associated with acute onset of T1D in Japanese subjects (Chung and Criswell, 2007).

In the case of *PTTG1*, it was shown that the SNPs associated with SLE within this locus were not influencing *PTTG1* expression but rather the microRNA146a (*miR146*), which is encoded in the same locus. The *miR146* was shown to be crucial for the suppressive activity in Tregs (Lu et al., 2010). The C risk allele in the SNP studied here, *rs2431099*, was associated with the increased frequency of Foxp3^{high} in Foxp3^{+}. This SNP was not described to be associated with a particular phenotype, however it is in LD with the SNP *rs2431697* that was directly associated with the expression of *miR146a* (Lofgren et al., 2012).

Finally, IL-6 is a pro-inflammatory cytokine involved in regulating the balance between IL-17 producing Th17 cells and Tregs, and polymorphisms in its gene were associated with multiple sclerosis (Mirowska-Guzel et al., 2011). In this study, the SNP *rs1800795* G risk allele was associated with increased frequency of active Tregs in Foxp3^{+} in patients. This SNP *rs1800795* risk allele was associated with an increase of *IL-6* expression (Kimura and Kishimoto, 2010; Mirowska-Guzel et al., 2011).

3.3. Cell Cultures

Since Tregs are described to be less active in SLE, it is a matter to study if the peripherally induced Tregs would be also impaired. Therefore, the naïve effector Th cells were sorted ($CD4^{+}CD25^{-}CD45RO^{-}$) and stimulated through the TCR using anti-CD3 antibody and the co-stimulation was made with anti-CD28, IL2, and the cytokine TGF-β1, known to influence *Foxp*3 expression. The main objective was to see if the T cells belonging to different groups would respond differently to the same stimulus. However, the influence of TGF-β1 in the activation of the cells and consequently in their induction to Foxp3 expressing cells was also studied. This analysis involved two steps, the first was the activation of the cells, measured through the frequency of $CD25^{+}$, and the second was if after activation cells would express *Foxp*3. Another important consideration was the fact that Tregs suppressor capacity depends on the amount of *Foxp3* expressed, here the same logic was followed, analyzing if the cells had low expression ($Foxp3^{low}$) or high expression ($Foxp3^{high}$).

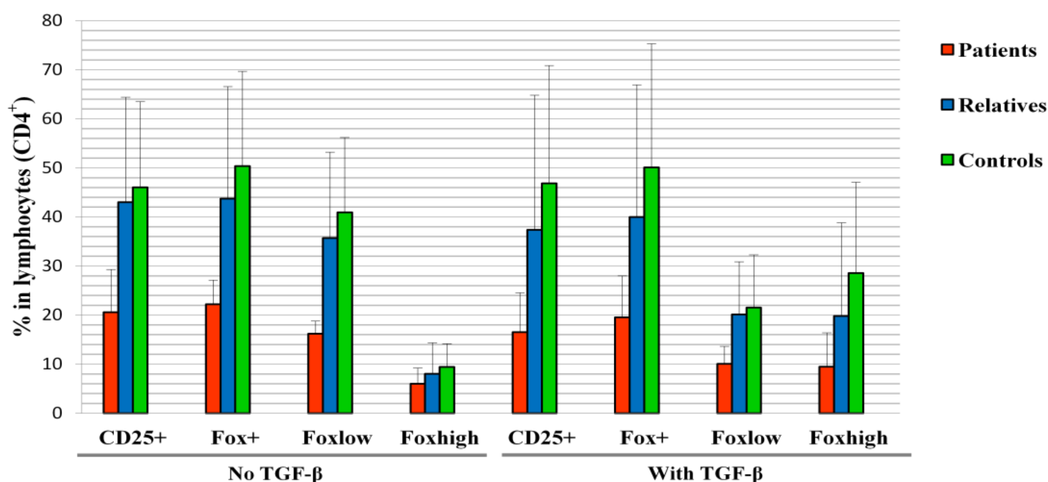

Figure 15 - TGF-β1 influence in the activation and *Foxp*3 induction capacity. This graphic was made with cytometry results after 5 days of culture and the bars represent the average percentage of the indicated phenotypes in the lymphocyte/$CD4^{+}$ compartment. The lines on the bars indicate the standard deviation.

The results showed that in terms of activation (frequency of $CD25^+$) patients had lower frequencies compared to relatives and controls. It also revealed that the activation was not different with and without TGF-β1, showing that this cytokine did not influence the activation of these cells (Fig. 15). Analyzing the frequency of Foxp3, it was also observed that there were differences between the groups. However these differences in $Foxp3^+$ were probably due to the frequency of activation because they had similar frequencies inside $CD4^+$ cells. In fact, the *Foxp*3 expression was close to 100% inside the activated cells, which means that in practically all activated cells *Foxp*3 expression was induced (Fig. 16). An interesting feature was that with and without TGF-β1 the frequencies of $Foxp3^+$ were similar. However, the difference was in the amount of *Foxp*3 expressed with TGF-β1 contributing to a higher frequency of $Foxp3^{high}$ cells (Fig. 16). Moreover, to exclude the influence of any other sources of TGF-β1, that could be present in the FBS or produced by the cells in culture, TGF-β1 was blocked with an anti-TGF-β1 (1D11). The results demonstrate that, in terms of *Foxp*3 expression, there was no difference between the cultures without TGF-β1 and the cultures with 1D11. Therefore, the induction of *Foxp*3 expression was not influenced by this cytokine but rather though the TCR stimulation (anti-CD3) and co-stimulation (anti-CD28 and IL2), observed by the higher frequency of $Foxp3^{low}$ (Fig. 16).

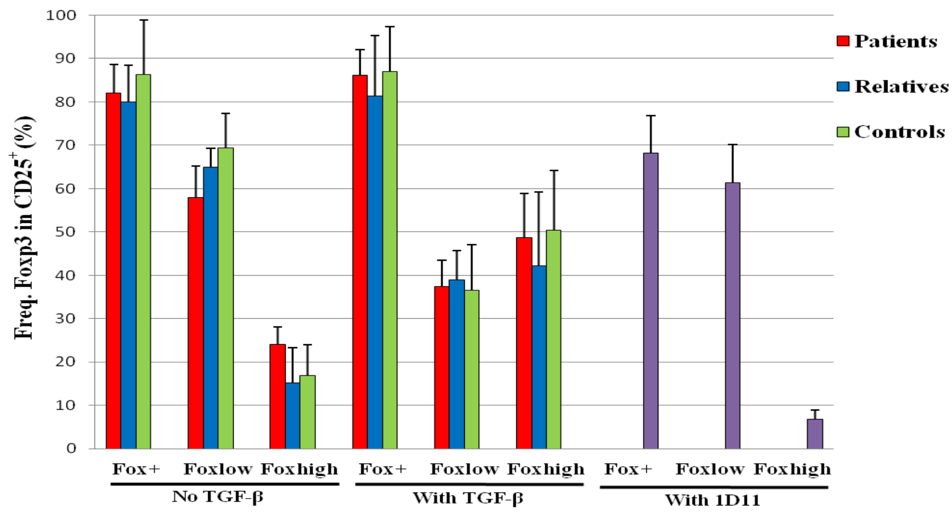

Figure 16 - Frequency of Foxp3 expressing cells within the activated cells ($CD25^+$). This graphic was made with cytometry results after 5 days of culture, the bars represent the average percentage of Foxp3 (Fox) frequency inside activated cells from different conditions of culture (with or without TGF-β and with 1D11 in purple). The lines on the bars indicate the standard deviation.

Chapter 4. DISCUSSION

4. Discussion

One particularity of this study was the presence of the unaffected relatives since they share genetic information with the patients. It was interesting to address how the genetic background influences the physiological aspects in the Tregs of patients compared with the relatives. However, the genetic information might not reflect all the mechanisms behind the Treg phenotypes, that is why the combination of genetic information and cellular phenotypic parameters was necessary. Therefore, the genotypes were correlated with the cytometry data (phenotypes) to analyze what is the genetic influence in the studied groups.

The balance of the frequency in the T cell subpopulations was described to be crucial for the disease process. It was suggested that in SLE patients the reduced numbers of $CD4^{+}C25^{high}$ could be an effect of the higher sensitivity to apoptosis (Miyara et al., 2005). Later, by the same group, $Foxp3^{high}$ populations were described as the active Tregs (Miyara et al., 2009). In this study what was found was not a reduced frequency of active Tregs but rather an increase of the $CD4^{+}Foxp3^{+}$ cells in the patients, which was consistent with the findings of Bonelli et al, 2010 (Fig. 10). Tregs from SLE patients were also described to be less functional, in terms of reduced capacity to suppress the proliferation of the effector Th cells (Valencia et al., 2007). In this study, there was a downregulation of CD25 in the Tregs from patients, which is thought to be an indication of reduced activity within this population (Bonelli et al., 2008; Wang et al., 2010). An interesting finding was that reduced CD25 MFI was correlated with the frequency of $CD4^{+}Foxp3^{low}$ cells (Fig. 12). This population was expanded in the $Foxp3^{+}$ population in SLE patients compared to the other groups (Fig. 8a) which was also found by other groups (Fritzsching et al., 2006; Suen et al., 2009; Valmori et al., 2005). These cells were described as non-suppressive and cytokine producers of mainly IL-17,

contributing to the inflammatory condition (Miyara et al., 2009). In fact, as it was mentioned before, the patients have a deficit of IL-2 which was described to favour the occurrence of Th17 cells (Yang et al., 2011). It was shown here that SLE patients and unaffected relatives had a downregulation in CD25, which indicates that this downregulation might be due to a genetic influence and possibly be one characteristic that, together with many other factors, is preceding the disease onset. One hypothesis is that in patients the inactivation of the active Tregs starts with a downregulation of CD25 followed by a downregulation of Foxp3, and so the $CD4^{+}Foxp3^{low}$ could be inactive Tregs. However, the controversy about the origin of the $Foxp3^{low}$ cells continues. Another finding was the *IL-6* SNP *rs1800795* risk allele G being correlated with an increased frequency of $Foxp3^{high}$ in $Foxp3^{+}$. As mentioned before, IL-6 is a pro-inflammatory cytokine and could be promoting the increased frequency of active Tregs since these are the cells that control the pro-inflammatory cytokine producer cells, avoiding the severe damages of an uncontrolled inflammatory process. There is evidence that IL-6 is increased in several autoimmune diseases and the risk allele G in *rs1800795* was associated with an IL-6 upregulation (Kimura and Kishimoto, 2010; Mirowska-Guzel et al., 2011). It was also described that when IL6 was increased, besides blocking the *Foxp*3 expression, it promoted the differentiation of Th17 cells, which could be related with the increased frequency of $Foxp3^{low}$ cells. Since Tregs are becoming inactive in some extent, this might trigger a compensation effect for the production of these cells. There were associations of IL-6 with other autoimmune diseases such as rheumatoid arthritis, multiple sclerosis or T1D. However, there is still no evidence of association with SLE.

In contrast to these findings in patients, the relatives had a lower frequency of the total $Foxp3^{+}$ cells as well as a lower frequency of Fox^{high} cells (Fig. 8). It was

described that the relatives share autoantibody production with the SLE patients, although they don't suffer from an autoimmune condition and so there is no reason to believe that Tregs are functionally impaired in this group. Since Tregs contribute to self antigen tolerance, probably together with other mechanisms, it might even be that this group develops mechanisms to avoid the disease (Fesel et al., 2012). However, this is a poorly studied group and there are no studies about IL-2 production or Treg suppressive capacity. In this work, the genetic influence of *IL2RA* variants on the Treg phenotypes was studied. It was observed that in control subjects the effect of *IL2RA* SNPs favored CD25 MFI while in relatives it was rather favoring a lower frequency of $Foxp3^{high}$ cells (Fig. 13). As mentioned before, CD25 is since recently considered as an important marker for Tregs activity (Wang et al., 2010). Therefore, if the *IL2RA* gene would be upregulated, the Treg cells would become more active ($CD25^{high}$). The observed reduction of $Foxp3^{high}$ cells with the influence of genotypes that otherwise favored CD25 expression, support the hypothesis that the frequency of $Foxp3^{high}$ cells was lower in relatives because their active Tregs were being rapidly consumed.

Another genetic effect observed in this study was the association of *IL2RB* SNPs, also in the relatives with the same phenotype, the frequency of $Foxp3^{high}$ (Table XII). This is consistent with the *IL2RA* effects, because IL2Rα would need the presence of IL2Rβ in the same proportion to combine and form the high affinity receptor for IL2 (Rochman et al., 2009), allowing the capture of IL2 even if this cytokine would be limiting to some extent. It is known that SLE patients have a deficit of IL-2 and if this is explained by a genetic influence it can be expected that the relatives would also have an intermediate deficit of IL-2. In fact, a genetic effect of *IL-2* SNPs was observed in these results. SNPs in the *IL2* locus were influencing only the CD25 MFI in $Foxp3^{high}$ and only in the unaffected relatives (Table XIII). This also supports that these cells might be

more rapidly consumed, once there is less IL2 the activated Tregs would rapidly exhausted. Alltogether, these genetic effects show that certainly, this IL2 receptor system is doing something different compared to the patients and also to the controls, which could represent a compensatory effect to maintain tolerance.

There is also another evidence supporting the exhaustion of the Tregs. The *PTTG1* SNP *rs2431099*, in this study, had a strong association with the frequency of Foxp3^{high} in Foxp3^{+} cells. Genetic association of SNPs in the PTTG1-miR146a region was found associated with SLE in Europeans due to lower expression of *miR146a* (Lofgren et al., 2012), and miR146a was found to be crucial for Treg activity since their suppressive capacity was impaired in miR146a-deficient mice (Lu et al., 2010). These findings suggest that the molecular mechanisms of Tregs in the relatives might reflect their activity leading to a higher exhaustion of these cells.

The induced Tregs might be contributing to peripheral self tolerance mechanisms. Induced Tregs are thought to differentiate from naïve precursors and to be specific for antigens that are not presented in the thymus, with the ability to suppress effector Th cells through the release of cytokines (Buckner and Ziegler, 2004; Groux, 2001). It is well described that Tregs can be induced from conventional T cells (CD4^{+}CD25^{-}) (Ben Ahmed et al., 2009; Walker et al., 2003). These cells are thought to help controlling inflammatory processes (Apostolou et al., 2008; Suen et al., 2009), and it was interesting to see that these cells were differently converted into induced Tregs in this study. In fact, the naïve effector Th cells showed different capacity of activation between the groups (Fig. 14), although the total Foxp3^{+} was not different within the activated fraction (Fig. 15). This still indicates that in the different groups the cells had different predispositions to become CD25^{+} expressing cells *in vitro*. Furthermore, these results indicate that TGF-β1 was rather increasing the *Foxp*3 expression instead of

inducing its expression *de novo*. In agreement with this, it was described that TGF-β1 acts like an enhancer of *Foxp*3 contributing to a higher expression and maintenance (Gabrysova et al., 2011; Xu et al., 2010). In SLE this is the first time where this experiment was done and the only study of cell culture induction in SLE was with isolated Tregs from patients where it was argued that these cells recovered their function after stimulation (Valencia et al., 2007). It is possible that Tregs are behaving differently, but more studies are needed to support these findings.

At last, there were other genes where SNPs were associated with SLE, some with direct effects on their expression which also affected the phenotypes studied here. The CTLA4 protein is known to play an important role in the suppression of effector Th cell proliferation, and when it is downregulated or loses activity there is a higher risk for autoimmunity (Benmansour et al., 2010; Kimkong et al., 2011). An interesting fact is that all the risk alleles for the SNPs studied in this work were negatively correlated with the CD25 MFI and with the frequency of Foxp3^{high} cells. If risk alleles in these SNPs are contributing to the downregulation of CTLA4, as it is the case for *rs3087243* mentioned before, this could not only affect their activated state but also the expression of CD25.

In this study, the *PTPN22* risk allele in SNP *rs2488457* was also negatively influencing the expression of CD25. Genetic variation in this gene was associated with several autoimmune diseases such as SLE, RA, T1D, and autoimmune thyroid disease. Particularly, one SNP in this locus was found to change the amino acid sequence from arginine to tryptophan at codon 620 (R620W), conferring a gain of function in the encoded protein Lyp. The protein became more active interfering with the TCR signaling and consequently with the activation of T cells. Moreover, this mechanism is thought to inhibit the regulatory function of Tregs resulting in immune responses

against autoantigens (Chung and Criswell, 2007). The locus-wise significant association found in this gene strongly supports that *PTPN22* was playing a role in the downregulation of CD25 and consequently in the activated state of the Tregs.

Altogether, it was interesting to see that in this genetic study, these SNPs were related to Tregs mainly in relatives and not in the patients. In the relatives this might be due to the shared genetic background, while in the patients a multiplicity of effects as well as the disease itself might be obscuring genetic effects. In this sense, phenotypic alterations in consequence of inflammatory processes could be mediated by the disease condition rather than a genetic effect. An explanation that supports this idea is that interferon α (IFNα) production, described to be involved with SLE, contributes to the inflammatory process through the induction of a specific pattern of genes with pro-inflammatory capacity. In this way the observed genetic influence wouldn't be the direct cause but rather converge in an IFNα gene expression pattern (Golding et al., 2011). Another feature of this study that could be influencing the results was the different sampling numbers for each group, with the relatives being the group with more collected samples. Furthermore, the presence of more than one relative per family could result in inflated p-values.

The analysis made using a gene-phenotype correltaion was a valid method described before by other group (Dendrou et al., 2009). This was shown to be a valuable tool to investigate genetic influence in specific subphenotypes secondary to the disease condition.

Chapter 5. CONCLUSIONS

4. Conclusions

This study might be interesting to understand how first degree relatives from SLE patients remain unaffected although they share a genetic background that contains risk factors for SLE. This was the first time that the unaffected relatives group was studied in such extent. It was found that the IL2 receptor system might be an important mechanism used by the Tregs in the relatives to maintain self tolerance. On the other hand, in SLE patients, apparently the mechanisms around the Treg function were not so dependent on the genetic influence and so, there may be external influences that contribute to impair the function of these cells. The method of correlating genotypes with cytometry phenotypes, although it is uncommon, was principally a valid method to infer the genetic influence on specific phenotypes. Other statistical analysis will be followed to exclude family genetic influence, and more samples collected to diminish the sampling bias. There is still a long journey to understand the mechanisms underlying SLE but all the steps are important to get us closer to the truth, and Tregs are very likely to play an important role.

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Annex I.

Table XV – List of all SNPs typed for each gene and the association with the frequency of active Tregs (Foxp3^{high} in Foxp3^{+}). The associations of the SNPs in the respective locus, were tested using Spearman Correlation within the groups of study: patients, relatives and controls. Nominal P-values (P) were corrected for multiple testing over all SNPs typed is the respective loci by Bonferroni correction (Bonf).

Gene	SNP	Ref. Allele	Foxp3^{high} in Foxp3^{+}								
			Patients			Relatives			Controls		
			R	P	Bonf	R	P	Bonf	R	P	Bonf
IL2RA	*rs12359875*	G	-0,132	0,336	8,057	-0,237	**0,003**	0,079	-0,060	0,570	13,678
	rs12244380	T	-0,079	0,570	13,682	0,085	0,291	6,982	0,114	0,281	6,733
	rs9663421	G	-0,264	0,055	1,315	-0,290	**0,000**	**0,008**	-0,017	0,876	21,015
	rs2076846	G	0,210	0,130	3,115	0,091	0,261	6,253	0,065	0,540	12,971
	rs11256369	T	0,209	0,129	3,085	0,121	0,135	3,229	-0,062	0,556	13,338
	rs7072398	G	0,310	**0,024**	0,572	0,166	**0,039**	0,942	0,170	0,110	2,634
	rs4749924	G	-0,096	0,484	11,616	-0,221	**0,006**	0,148	-0,076	0,471	11,303
	rs706781	T	-0,029	0,835	20,038	-0,022	0,786	18,873	0,121	0,252	6,049
	rs11256497	T	0,231	0,117	2,814	0,052	0,536	12,874	0,093	0,382	9,176
	rs791587	G	0,186	0,177	4,241	0,056	0,484	11,622	0,063	0,555	13,312
	rs791589	T	-0,192	0,178	4,268	0,002	0,982	23,577	0,132	0,223	5,342
	rs10905669	T	-0,094	0,496	11,898	-0,032	0,688	16,515	-0,056	0,598	14,362
	rs2256774	C	0,324	**0,018**	0,438	0,169	**0,040**	0,949	0,012	0,913	21,912
	rs706779	G	0,292	**0,034**	0,810	0,262	**0,001**	**0,028**	-0,080	0,461	11,074
	rs2104286	C	-0,237	0,088	2,111	-0,100	0,217	5,216	-0,140	0,186	4,458
	rs7072793	T	-0,008	0,952	22,858	-0,127	0,127	3,054	0,026	0,803	19,283
	rs7073236	T	-0,001	0,995	23,871	-0,101	0,210	5,038	0,026	0,803	19,283
	rs11597367	G	-0,138	0,316	7,594	-0,176	**0,029**	0,701	-0,038	0,720	17,285
	rs10795791	T	0,010	0,939	22,542	0,114	0,158	3,801	-0,026	0,803	19,283
	rs4147359	T	0,018	0,894	21,459	-0,096	0,235	5,645	0,142	0,180	4,331
	rs7090530	C	-0,113	0,414	9,937	-0,161	**0,046**	1,110	-0,025	0,810	19,441
	rs41295061	G	-0,085	0,539	12,940	0,039	0,626	15,026	-0,115	0,279	6,695
	rs11594656	T	0,134	0,332	7,976	0,180	**0,025**	0,607	0,038	0,720	17,285
	rs12251307	T	0,085	0,549	13,171	-0,051	0,527	12,640	0,100	0,346	8,316
IL2RB	*rs743776*	T	0,229	0,102	0,816	0,230	**0,004**	**0,035**	0,056	0,614	4,911
	rs2016771	T	0,230	0,098	0,781	0,157	0,051	0,408	0,014	0,899	7,190
	rs228979	T	-0,012	0,940	7,524	0,113	0,234	1,869	-0,133	0,235	1,876
	rs3218258	G	-0,194	0,158	1,265	-0,196	**0,015**	0,120	0,012	0,912	7,293
	rs228975	C	-0,109	0,431	3,450	0,061	0,451	3,610	0,036	0,734	5,875
	rs2281094	T	-0,081	0,560	4,484	-0,028	0,726	5,806	-0,001	0,991	7,926
	rs3218292	G	0,032	0,831	6,650	0,231	**0,008**	0,060	-0,160	0,132	1,058
	rs228947	T	0,017	0,900	7,198	0,102	0,206	1,645	-0,063	0,552	4,417
IL6	*rs1800795*	G	0,303	**0,028**	**0,028**	0,102	0,205	0,205	-0,176	0,097	0,097
CTLA4	*rs231806*	G	0,145	0,335	1,677	0,023	0,790	3,948	-0,100	0,571	2,853
	rs5742909	T	0,107	0,450	2,249	-0,034	0,678	3,391	0,130	0,222	1,109
	rs231775	G	-0,024	0,864	4,322	0,034	0,677	3,386	-0,347	**0,001**	**0,007**
	rs3087243	G	0,159	0,246	1,231	0,041	0,609	3,043	-0,196	0,065	0,325
	rs231723	G	-0,043	0,752	3,759	0,010	0,898	4,492	-0,310	**0,003**	**0,017**
PTTG1	*rs2431099*	T	-0,141	0,313	0,313	-0,302	**0,000**	**0,000**	0,169	0,117	0,117

Table XV – List of all SNPs typed for each gene and the association with CD25 MFI in active Tregs (Foxp3^{high} in Foxp3^{+}). The associations of the SNPs in the respective locus, were tested using Spearman Correlation within the groups of study: patients, relatives and controls. Nominal P-values (P) were corrected for multiple testing over all SNPs typed is the respective loci by Bonferroni correction (Bonf).

Gene	SNP	Ref. Allele	CD25 MFI in Foxp3^{high}								
			Patients			Relatives			Controls		
			R	P	Bonf	R	P	Bonf	R	P	Bonf
IL2-IL21	*rs1479924*	G	0,029	0,862	12,924	0,305	**0,003**	**0,045**	-0,064	0,584	8,763
	rs2069762	G	0,029	0,856	12,841	-0,077	0,443	6,647	-0,128	0,270	4,043
	rs2069763	C	0,056	0,723	10,839	0,201	**0,046**	0,683	-0,163	0,161	2,409
	rs2069778	C	0,172	0,278	4,164	-0,077	0,445	6,682	0,088	0,447	6,702
	rs2069770	ID	0	1	15,000	0,213	**0,034**	0,513	0,036	0,755	11,320
	rs2069772	T	0,063	0,689	10,333	0,176	0,080	1,207	-0,107	0,358	5,368
	rs11575812	G	0,030	0,851	12,771	0,292	**0,004**	0,055	-0,078	0,504	7,560
	rs6534347	G	0,141	0,374	5,603	0,155	0,124	1,862	-0,199	0,088	1,314
	rs1398553	G	0,050	0,757	11,359	0,037	0,728	10,915	-0,055	0,639	9,584
	rs17005931	G	0,105	0,512	7,677	-0,062	0,540	8,095	-0,149	0,200	2,996
	rs907715	T	-0,069	0,665	9,973	-0,246	**0,018**	0,272	-0,013	0,914	13,710
	rs6822844	T	-0,154	0,329	4,932	0,067	0,511	7,667	-0,048	0,681	10,212
	rs12642902	G	-0,017	0,917	13,749	-0,214	**0,033**	0,502	0,024	0,839	12,580
	rs6852535	G	-0,042	0,792	11,883	0,077	0,443	6,647	0,128	0,270	4,043
	rs6849238	G	-0,061	0,704	10,566	-0,306	**0,003**	**0,040**	0,070	0,553	8,298
IL2RA	*rs12359875*	G	0,080	0,613	14,718	-0,080	0,426	6,383	0,271	**0,020**	0,476
	rs12244380	T	-0,172	0,283	6,793	0,025	0,803	12,043	-0,069	0,550	13,205
	rs9663421	G	-0,059	0,708	17,000	-0,072	0,471	7,066	0,300	**0,010**	0,236
	rs2076846	G	0,093	0,563	13,509	0,084	0,403	6,043	-0,118	0,315	7,565
	rs11256369	T	0,035	0,825	19,811	-0,102	0,312	4,676	-0,251	**0,031**	0,744
	rs7072398	G	0,059	0,708	16,992	0,154	0,126	1,889	-0,323	**0,005**	0,130
	rs4749924	G	0,011	0,944	22,655	-0,173	0,084	1,266	0,240	**0,039**	0,939
	rs706781	T	0,193	0,223	5,358	0,024	0,811	12,170	0,051	0,660	15,849
	rs11256497	T	-0,135	0,432	10,360	0,182	0,081	1,217	-0,300	**0,010**	0,249
	rs791587	G	-0,074	0,641	15,393	0,129	0,200	3,000	-0,112	0,335	8,035
	rs791589	T	-0,097	0,563	13,501	0,146	0,158	2,367	-0,182	0,126	3,022
	rs10905669	T	-0,169	0,284	6,815	0,034	0,736	11,037	-0,060	0,609	14,609
	rs2256774	C	-0,048	0,762	18,299	0,128	0,214	3,204	-0,404	**0,001**	**0,014**
	rs706779	G	-0,203	0,200	4,805	-0,014	0,886	13,293	-0,410	**0,001**	**0,013**
	rs2104286	C	-0,204	0,203	4,877	-0,108	0,287	4,300	0,142	0,222	5,322
	rs7072793	T	0,361	**0,026**	0,630	0,114	0,278	4,163	0,195	0,093	2,231
	rs7073236	T	0,352	**0,026**	0,625	0,088	0,381	5,709	0,195	0,093	2,231
	rs11597367	G	0,276	0,081	1,945	0,055	0,585	8,772	0,365	**0,002**	**0,041**
	rs10795791	T	-0,349	**0,027**	0,659	-0,076	0,448	6,726	-0,195	0,093	2,231
	rs4147359	T	0,232	0,148	3,555	0,065	0,519	7,781	0,048	0,681	16,340
	rs7090530	C	0,216	0,177	4,251	0,034	0,738	11,073	0,301	**0,010**	0,232
	rs41295061	G	0,024	0,879	21,097	0,034	0,733	10,997	0,013	0,909	21,816
	rs11594656	T	-0,279	0,082	1,964	-0,037	0,710	10,644	-0,365	**0,002**	**0,041**
	rs12251307	T	-0,154	0,343	8,223	-0,051	0,617	9,248	0,025	0,831	19,944
CTLA4	*rs231806*	G	0,110	0,541	2,707	-0,314	**0,005**	**0,026**	-0,167	0,434	2,168
	rs5742909	T	-0,053	0,742	3,712	-0,288	**0,005**	**0,026**	-0,096	0,411	2,054
	rs231775	G	0,139	0,384	1,920	0,064	0,533	2,665	-0,181	0,128	0,641

	rs3087243	G	0,101	0,523	2,617	-0,254	**0,011**	0,057	-0,341	**0,003**	**0,017**
	rs231723	G	0,158	0,317	1,585	0,045	0,657	3,284	-0,184	0,113	0,563
PTPN22	*rs6679677*	C	0,135	0,404	2,019	-0,095	0,357	1,785	0,145	0,211	3,166
	rs3789604	T	0,020	0,904	4,522	0,108	0,307	1,533	0,226	0,055	0,832
	rs1310182	G	0,042	0,790	3,952	-0,151	0,134	0,669	0,190	0,102	1,528
	rs2476601	G	0,188	0,252	1,259	-0,142	0,159	0,793	0,145	0,211	3,166
	rs2488457	G	0,012	0,942	4,708	0,330	**0,001**	**0,005**	0,004	0,972	14,585