Haplotype 4 of the multiple sclerosis-associated interleukin-7 receptor alpha gene influences the frequency of recent thymic emigrants.

Non Peer-reviewed author version

BROUX, Bieke; HELLINGS, Niels; VENKEN, Koen; RUMMENS, Jean-Luc; HENSEN, Karen; VAN WIJMEERSCH, Bart & STINISSEN, Piet (2010) Haplotype 4 of the multiple sclerosis-associated interleukin-7 receptor alpha gene influences the frequency of recent thymic emigrants.. In: GENES AND IMMUNITY, 11(4). p. 323-336.

DOI: 10.1038/gene.2009.106
Handle: http://hdl.handle.net/1942/10710

Haplotype 4 of the multiple sclerosis associated interleukin-7 receptor alpha gene influences the frequency of recent thymic emigrants

Bieke Broux[1]; Niels Hellings, PhD[1]; Koen Venken, PhD[2]; Jean-Luc Rummens, MD[3]; Karen Hensen, PhD[3]; Bart Van Wijmeersch, MD, PhD[1,4]; Piet Stinissen, PhD[1]

[1]Hasselt University, Biomedical Research Institute and transnationale Universiteit Limburg, School of Life Sciences, Diepenbeek, Belgium

[2]Ghent University Hospital, Department of Rheumatology, Laboratory for Molecular Immunology and Inflammation, Gent, Belgium

[3]Virga Jesse Hospital, Clinical Laboratory of Experimental Hematology, Hasselt, Belgium

[4]Mariaziekenhuis Noord-Limburg and Revalidatie & MS-Centrum, Overpelt, Belgium

Corresponding author:

Prof. Dr. P. Stinissen[1]

Biomedical Research Institute

Agoralaan, building C

3590 Diepenbeek, Belgium

e-mail: piet.stinissen@uhasselt.be

Phone number: +3211269204

Fax number: +3211269299

Running title: Functional analysis of IL-7Rα haplotypes

Abstract

The receptor for the homeostatic T cell cytokine interleukin-7 (IL-7Rα) has recently shown genetic association to multiple sclerosis (MS). To investigate the functional contribution of IL-7Rα polymorphisms to the pathogenesis of MS, we correlated the IL-7Rα haplotypes with different T cell parameters in a group of MS patients and healthy controls. We demonstrate that carriers of one of the four IL-7Rα haplotypes (Hap4) show a higher expression of IL-7Rα (CD127) on their $CD4^+$ T cells, compared to non-carriers (p=0.04). Moreover, Hap4 carriers possess higher frequencies of recent thymic emigrants (RTEs, $CD31^+$) in both the regulatory T cell (Treg; p=0.007) and conventional T cell (Tconv) population (p=0.0001). This effect is most pronounced within the MS population (Treg: p=0.0077; Tconv: p=0.0007), while in healthy controls significance was only reached for Tconv (p=0.043; Treg: p=0.11). Since previous studies demonstrated a decreased RTE-Treg frequency in MS patients compared to healthy subjects, we here conclude that this decrease is localized within the MS population of non-Hap4 carriers. In conclusion, our findings suggest that IL-7Rα polymorphisms can influence T cell development and homeostasis, and thereby contribute to the altered immune regulation which is associated with disease development in patients with MS.

Keywords: interleukin-7 receptor, polymorphism, recent thymic emigrants, multiple sclerosis

Introduction

Multiple sclerosis (MS) is the most common disorder of the central nervous system (CNS) in young adults. Although the pathogenesis of the disease has not yet been fully elucidated, there is increasing evidence that MS occurs in genetically predisposed persons. **Many studies have already described that the Human Leukocyte Antigen (HLA) class II genes, located on chromosome 6, represent the strongest risk for MS, accounting for 14%-50% of the genetic susceptibility[1,2]. Since this locus is in strong linkage disequilibrium, the identification of the true susceptibility allele is a complex matter. Chao et al. recently showed that the once-leading candidate, HLA-DRB1*1501, is not the susceptibility allele itself, but is probably part of a susceptibility haplotype.[3] This haplotype includes at least HLA-DQA1*0102 and HLA-DQB1*0602, which interact with HLA-DRB1*1501 through epistatic mechanisms.[4] In addition, it appears that vitamin D, through a vitamin D response element in the promoter region of HLA-DRB1*1501, interacts with this locus to influence its expression.[5]**

Recently, other non-HLA genes have been shown to be associated with MS. One of these genes is the interleukin-7 receptor α chain (IL-7Rα) gene[6-9]. Its product forms a heterodimer with the common IL-2R γ-chain to bind IL-7, or with the Thymic Stromal Lymphopoietin Receptor (TSLPR) to bind TSLP. IL-7 is an essential cytokine in the development, survival, proliferation and differentiation of T and B lymphocytes. TSLP, an epithelial cell derived cytokine, facilitates the dendritic cell mediated differentiation of $Foxp3^+$ regulatory T cells (Tregs) in the thymus[10], and promotes T helper (Th) 2 differentiation in the periphery[11]. Therefore, polymorphisms within the gene that encodes the receptor of these two cytokines could contribute to an altered T cell homeostasis.

Four haplotypes have been described within the IL-7Rα gene, which all include multiple single nucleotide polymorphisms (SNPs) in this gene region[6]. The SNP that shows the strongest linkage to MS, is a nonsynonymous coding SNP in exon 6 (rs6897932)[7-9]. Functionally, when a C allele is present in this position, a twofold increase of the skipping of exon 6 occurs, which leads to the formation of the soluble form of the IL-7R[7]. This changes the ratio of membrane bound versus soluble receptor, which could cause a reduction of CD127 (IL-7Rα) expression on T cells.

Recent reports which show that CD127 expression is low on Tregs in comparison with activated conventional T cells (Tconv)[12,13], have triggered the discussion that polymorphisms in the IL-7Rα gene might influence Treg function. We and others have recently shown that an altered Treg homeostasis occurs in MS patients. Venken et al. demonstrated that natural naive Treg development and function are disturbed in MS patients, but that the memory Treg pool recovers in the chronic phase of the disease[14]. Also, two independent studies of Venken et al. and Haas et al. showed that CD31 (PECAM-1) expressing naive Tregs are significantly reduced in the peripheral blood of MS patients[14,15]. CD31 is a 130 kDa transmembrane glycoprotein expressed by endothelial cells, platelets, monocytes, neutrophils and T cell subsets. It was further shown that this adhesion molecule is a marker for recent thymic emigrants (RTE), which are characterized by a high number of T cell receptor excision circles (TREC) and a low number of cell divisions[16,17].

To investigate the possible contribution of IL-7Rα polymorphisms to the pathogenesis of MS, we sought for a possible correlation between the IL-7Rα genotypes and different T cell parameters in a group of MS patients and healthy controls. We found that the haplotype which is tagged by the MS associated SNP rs6897932 does not influence the frequency of $CD4^{+}CD25^{hi}CD127^{lo}$Tregs, nor affects the frequency of $CD31^{+}$ T cells or expression levels of CD127 for both Tconv and Tregs in MS patients and healthy controls. Analysing the

haplotype which has been called “protective” by the group of Stewart (Hap4, tagged by the promoter SNP rs11567685), a significantly higher expression of CD127 on total $CD4^+$ T cells for $Hap4^+$ MS patients was found (p=0.041). Furthermore, for the first time we show that the frequency of $CD31^+$ naive ($CD45RA^+$) Tconv and Tregs was significantly reduced in MS patients who did not express Hap4 (Tconv, p=0.0007; Tregs, p=0.0077). In healthy controls, we could only find a statistically significant difference in the Tconv subset (p=0.043), but a trend could also be seen for Tregs (p=0.11). These results point towards a possible contribution of haplotype 4 to alterations in T cell development and homeostasis.

Materials and methods

Study subjects

Peripheral blood samples were collected from a total of 33 healthy controls and 65 patients with clinically definite MS (RR-MS, n=39; SP-MS, n=15; PP-MS, n=11). Thirty-two of these patients were untreated in that they had not received corticosteroids or immunomodulatory drugs within at least 3 months of blood collection. The other 33 patients were treated with IFN-β (n=25), glatiramer acetate (GA, n=7) or both (n=1) at time of blood sampling. Within our population of MS patients, a diverse range of clinical subtypes was included. The mean age of MS patients is 44.7±11.2 years (range 20-68 years), the average disease duration is 10.3±7.8 years (range of 3 mo-28 years), and Expanded Disability Status Scale (EDSS) scores range from 1 to 5.5 (median of 2±1.5). The mean age of the healthy controls is 33.2±10.7 years (range 21-55 years). This study was approved by the local Medical Ethical committee of Hasselt University and informed consent was obtained from all study subjects.

IL-7Rα genotyping

MS patients and healthy controls were genotyped for the IL-7Rα locus using a restriction fragment length polymorphism (RFLP) procedure, as previously described[6]. Briefly, a fragment of the IL-7Rα gene promoter region, including three tagging SNPs (rs7718919, rs11567685 and rs11567686), was PCR amplified from genomic DNA isolated from PBMC of MS patients and healthy controls. This fragment was then cut by the restriction enzymes HphI (Fermentas, Burlington, Ontario, Canada) and PstI (New England Biolabs, Ipswich, MA, USA) to obtain banding patterns specific for each haplotype. To verify our genotyping assay, selected samples were sequenced using an ABI automated DNA sequencer (PerkinElmer, Waltham, MA, USA) to confirm the genotype.

Flow cytometric analysis

PBMC were isolated from whole blood by Ficoll density gradient centrifugation (Histopaque, Sigma-Aldrich, St. Louis, MO, USA). Cells were quadruple stained with the following mAbs: anti-human CD4, CD25 (both from BD Biosciences, Franklin Lakes, NJ, USA), CD45RA, CD31 (both from ImmunoTools, Friesoythe, Germany) or CD127 (eBioscience, San Diego, CA, USA), and subsequently analyzed by flow cytometry using a FACSCanto II (BD Biosciences). Statistical analysis was performed using FACSDiva software (BD Biosciences). Based on four of these markers, namely CD4, CD25, CD45RA and CD127, four populations of T cells can be distinguished (see Figure 1)[13,14]: naive Tregs (nTregs; $CD4^+CD25^{hi}CD127^{lo}CD45RA^{hi}$), memory Tregs (mTregs; $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$), naive Tconv (nTconv; $CD4^+CD25^-CD127^+CD45RA^{hi}$) and memory Tconv (mTconv; $CD4^+CD25^-CD127^+CD45RA^-$). CD31 staining was performed together with CD4, CD25 and CD45RA, to determine its expression in nTregs ($CD4^+CD25^{hi}CD45RA^{hi}$), mTregs ($CD4^+CD25^{hi}CD45RA^-$), nTconv ($CD4^+CD25^-CD45RA^{hi}$) and mTconv ($CD4^+CD25^-CD45RA^-$).

Cell culture

For the analysis of the effect of IL-7 on CD31 kinetics *in vitro*, PBMC were cultured in the presence or absence of recombinant human IL-7 (10 ng/ml; R&D Systems, Minneapolis, MN, USA), additionally stimulated with monoclonal anti-CD3 antibody (αCD3; 2 µg/ml; BIOMED, Diepenbeek, Belgium). On days 0 and 5, flow cytometric analysis was performed with the following antibodies: CD4-PERCP (BD Biosciences) and CD31-FITC (ImmunoTools). Flow cytometric analyses were done using a FACSCalibur (BD Biosciences). Statistical analysis was performed using Cellquest software (BD Biosciences).

Because of high inter-donor variation, values are represented as relative to day 0 (*ex vivo*) values (set at 100%).

Statistical analysis

Differences of genotype or allele frequencies between MS patients and HC were analysed using the χ^2 test. For comparison of T cell parameters between carriers and non-carriers of certain alleles, a Mann Whitney test was performed using Prism software version 4.0 (GraphPad Software). Results are expressed as mean values ± SEM. Differences were considered significant when $p<0.05$.

Results

Genotyping IL-7Rα polymorphisms in a population of Belgian MS patients and healthy controls

Three tagging SNPs in the promoter region of the IL-7Rα gene (rs7718919, rs11567685 and rs11567686) were analysed by means of a RFLP procedure to determine the genotypes of our Belgian population of MS patients (n=65) and healthy controls (n=33). The four haplotypes tagged by these three promoter SNPs (according to Teutsch et al[6]) are summarized in Table 1. Allele and genotype frequencies in our study groups were similar to those previously reported[6-9] (Table 2). In addition, the allele and genotype frequencies for the MS associated exon 6 SNP (rs6897932) were determined (Table 3). Our results confirm the published overexpression of the C allele in MS patients[7-9]. However, no statistical significancy was reached (χ^2, p=0.780), as a result of the low sample size.

Treg frequencies in MS patients and healthy controls are not influenced by the IL-7Rα polymorphisms

In this part, we determined the effect of IL-7Rα polymorphisms on Treg frequencies in a group of untreated MS patients and healthy controls. Since several groups reported that $Foxp3^+$ Tregs have a low expression of the IL-7Rα chain ($CD127^{lo}$)[12,13], polymorphisms within this gene may affect Treg homeostasis. We focussed on two specific SNPs within the IL-7Rα locus, known to influence the CD127 expression on T cells. First, the MS associated exon 6 SNP (rs6897932) is thought to influence the ratio of membrane bound versus soluble receptor isoform, because of increased skipping of exon 6 in C allele carriers[7,9]. Study groups were subdivided based on the presence of haplotype 2 (Hap2), which is tagged by the exon 6 SNP (Hap2 carriers express the T allele; Table 1). Second, the group of Stewart reported that

the promoter SNP rs11567685 has an effect on the CD127 expression on T cells in PP-MS patients[18]. Moreover, they stated that the haplotype including the C allele at this site (Hap4; see table 1) is protective in these patients[19]. Correlation analyses were performed by stratifying our study groups based on the presence of Hap4. All analyses were performed on untreated MS patients, since it was reported that treatment strongly influences Treg function[14].

Frequencies of total Tregs ($CD4^+CD25^{hi}CD127^{lo}$), nTregs and mTregs (see Figure 1) were determined in our population of untreated MS patients (n=32) and healthy controls (n=27). No significant differences in frequencies of the mentioned Treg populations were found between Hap2 and non-Hap2 carriers within the total population (totTreg: p=0.31; mTreg: p=0.45; nTreg: p=0.98), nor within the MS or HC population (Figure 2A). Moreover, Hap4 and non-Hap4 carriers did not differ in their Treg frequencies in the total population (totTreg: p=0.80; mTreg: p=0.73; nTreg: p=0.70) nor within the MS or HC population (Figure 2B).

Haplotype 4 carriers have a significantly higher CD127 expression on total $CD4^+$ T cells

In light of the functional role of the exon 6 SNP rs6897932, we analysed the expression of CD127 on total $CD4^+$ T cells, Tconv ($CD4^+CD25^-CD127^+$) and Treg ($CD4^+CD25^{hi}CD127^{lo}$). The presence or absence of Hap2 had no effect ($p>0.05$) on the mean fluorescence intensity (MFI) of CD127 on all three T cell populations in the total study group (Figure 3, upper panel). Also, no significant difference was found when analysing MS patients (total $CD4^+$: p=0.81; Tconv: p=0.78; Treg: p=0.29) and controls (total $CD4^+$: p=0.74; Tconv: p=0.83; Treg: p=0.37) separately.

When these data were analysed based on the presence or absence of Hap4, a statistically significant difference was found on total $CD4^+$ T cells (p=0.041) in the total study population (Figure 3, lower panel). More specifically, carriers of Hap4 showed a higher CD127

expression on total $CD4^+$ T cells as compared to non-Hap4 carriers. For Tconv, a trend could also be observed (p=0.082), but for Tregs no difference was found (p=0.80). When individual groups were analysed, only trends were found in the MS group (total $CD4^+$: p=0.11; Tconv: p=0.14; Treg: p=1.00), but not in healthy controls (total $CD4^+$: p=0.23; Tconv: p=0.46; Treg: p=0.54).

The frequency of $CD31^+$ naive T cells is higher in MS patients carrying haplotype 4

IL-7 plays a major role in lymphocyte development, as has been shown by the paucity of T and B cells in IL-7- and IL-7Rα-deficient mice[20,21]. To investigate the effect of IL-7Rα polymorphisms on T cell development, we examined the amount of RTEs present in the peripheral blood of MS patients and healthy controls. For this purpose, CD31 was used as a marker for these RTEs[16]. Two independent studies of Venken et al.[14] and Haas et al.[15] previously showed that RTE-Treg ($CD4^+CD25^{hi}CD127^{lo}CD31^+$) are significantly reduced in MS patients, in comparison to healthy controls. In this study, we reconfirmed these results (HC: 65.30±9.65%; MS: 58.93±11.35%; p=0.035). To determine the contribution of IL-7Rα polymorphisms to this difference, four different T cell subsets were analysed in this experiment: nTconv, mTconv, nTreg and mTreg (see Figure 1). The memory T cell pool intrinsically possesses a low percentage of CD31 expressing cells, and this amount was not significantly different between any of the studied haplotypes (data not shown). When analyzing our data based on carriers or non-carriers of Hap2, no correlation with the amount of CD31-expressing T cells was found in the total study population (nTconv: p=0.16; nTreg: p=0.28), nor within the MS patient and healthy control group (Figure 4, upper panel).

In contrast, the presence of Hap4 did have an effect on the frequency of CD31 expressing naive T cells (both nTconv and nTreg) in the total population (nTconv: p=0.0001; nTreg: p=0.005). More specifically, non-Hap4 carriers have a lower percentage of $CD31^+$ nTregs

(p=0.007) and nTconv (p=0.0001). The mean age of Hap4 carriers and non-carriers was not significantly different (39.4 ± 10.9 versus 40.1 ± 12.7; p=0.86), excluding the effect of age. When MS patients and controls were analysed separately, we found that the MS groups contributed most to the significant difference found in the total study population (nTreg: p=0.0077; nTconv: p=0.0007). In healthy controls, a significant difference was only reached in the nTconv subset (p=0.043), while a trend could also be seen for nTregs (p=0.11; figure 4). Within non-Hap4 carriers, there was a significant reduction of $CD31^+$ nTregs (p=0.041), but not of $CD31^+$ nTconv (p=0.37) in the MS population compared to healthy controls. Within the Hap4 carriers, no significant differences were found (nTregs: p=0.44; nTconv: p=0.62). Therefore, we show for the first time that the overall reduction of the RTE-Treg frequency in MS patients compared to controls is localized within the non-Hap4 carriers.

Analysis of CD31 expression after TCR stimulation in the presence or absence of IL-7

To gain more insight into the mechanism behind the effect of Hap4 on the frequency of CD31 expressing naive T cells, an *in vitro* experiment was set up using healthy Hap4 carriers (n=4) and non-Hap4 carriers (n=4). PBMC from these donors were cultured in the presence of anti-CD3, to induce TCR stimulation, known to induce a loss of CD31. In parallel, the effect of IL-7 on CD31 expression was determined, by culturing the cells in the presence or absence of IL-7. On days 0 and 5, flow cytometric analysis was performed. TCR stimulation of PBMC clearly resulted in a decrease of the percentage of $CD4^+CD31^+$ T cells at day 5 of culture (Hap4: 67.81±8.25%; non-Hap4: 75.24±2.28%; relative to percentage directly *ex vivo*). The addition of IL-7 during TCR stimulation induced an increased percentage of $CD4^+CD31^+$ T cells at day 5 of culture (Hap4: 81.79±18.07%; non-Hap4: 82.27±8.82%; relative to percentage directly *ex vivo*), although this increase was not significant (Figure 5). Moreover, the effect of IL-7 is more pronounced in Hap4 carriers compared to non-Hap4 carriers. Three

out of 4 Hap4 carriers showed a significant stabilization of CD31 expression by IL-7, while this was only the case in 1 out of 4 non-Hap4 carriers. This observation may provide a link between the observed differences found for the CD127 and CD31 expression in Hap4 versus non-Hap4 carriers.

Discussion

While the association between IL-7Rα polymorphisms and MS has been demonstrated earlier by several groups[6-9,22], the functional relevance of this association is not clear yet. In this study, we provide evidence that polymorphisms in the IL-7R gene can have an influence on the development of naive T cells. To our knowledge, we are the first to show that carriers of the previously reported protective Haplotype 4 have a higher percentage of RTEs in both naive Treg and Tconv subsets, compared to non-Hap4 carriers. In addition, Hap4 carriers express higher levels of IL-7Rα on their $CD4^+$ T cells. In contrast, the MS associated exon 6 SNP did not affect Treg frequency or CD127 expression on T cells.

Recently, investigators of our[14] and another group[15] compared the frequency of RTE-Treg in MS patients and healthy controls. Both studies provide evidence that this subset of naive Tregs was significantly reduced in MS patients as compared to HC. In addition, Haas et al. showed that CD31 expressing Tregs are mainly responsible for the functional properties of the entire Treg population. Therefore, a decrease in this subpopulation of Tregs might explain the dysfunctional immune regulation seen in MS patients[15]. CD31 is an adhesion molecule that is expressed on T cells which have recently emigrated out of the thymus and entered the peripheral circulation. These RTEs are characterized by a high TREC content and hardly any recent cell divisions[16]. In the present study, we confirm that RTE-Tregs are decreased in MS patients compared to healthy controls, and we provide a link between the frequency of RTEs and IL-7Rα polymorphisms. More specifically, the frequency of RTE-Tregs was decreased in MS patients versus healthy controls in non-Hap4 carriers, but not in the Hap4 carrier population. These results suggest that the decrease in RTE-Tregs in MS patients is a consequence of a lower frequency in non-Hap4 carriers. The observation that RTEs are reduced for both the Treg as well as the Tconv subsets in non-Hap4 individuals, may indicate

that Hap4 expression has an overall effect on thymic T cell development. Early experiments with IL-7 and IL-7R knockout animals showed that IL-7 signalling is crucial for thymic T cell development[20,21]. Recently, Mazzucchelli et al. also discovered that Treg development is dependent on either IL-7 or TSLP. These two cytokines both bind to the IL-7Rα, and their combined function leads to the normal development of Tregs[23]. Taken together, these results link IL-7Rα polymorphisms to a possible defect in thymic T cell development.

Furthermore, we performed a functional assay to determine the contribution of these polymorphisms to CD31 kinetics *in vitro*. We found that, after TCR stimulation, the addition of IL-7 stabilized the CD31 phenotype in $CD4^+$ T cells. This phenomenon was most pronounced in Hap4 carriers, thus pointing towards a possible role of IL-7Rα polymorphisms in peripheral T cell homeostasis. More importantly, this may provide us with a link between the expression levels of CD127 and CD31, which were also different between Hap4 carriers and non-carriers. A very recent study of Azevedo et al. determined the role of IL-7 in the homeostasis of RTEs. They found that IL-7 driven proliferation does not result in a loss of CD31 expression on naive $CD4^+$ T cells. This suggests that IL-7 preferentially maintains RTEs during adult life [24]. Our results now provide a link between the genetic variation within the IL-7Rα gene and the stabilization of the CD31 phenotype in anti-CD3 stimulated $CD4^+$ T cells. This mechanism might play an important role in the peripheral T cell homeostasis in MS patients, since the differences in CD31 expression levels were more evident in MS patients compared to healthy controls. Based on this, we can conclude that the observed alterations in RTE frequency are not only a result of genetic influences, but also of ongoing inflammatory processes, which are present in MS patients but not in healthy controls. Taken together, these data suggest that IL-7Rα polymorphisms not only affect thymic T cell development, but could also play a role in peripheral RTE homeostasis.

Because we found an effect of IL-7Rα polymorphisms on the development or homeostasis of T cells, we hypothesized that this might reflect a difference on the molecular level (e.g. the expression of CD127). Indeed, we observed an increased CD127 expression on total $CD4^+$ T cells in Hap4 carriers compared to non-carriers in the total study population. This is in line with the reportings of McKay et al. who found that the CD127 expression on $CD4^+$ T cells (both Treg and Tconv) was higher in Hap4 carrying compared to non-Hap4 carrying primary progressive (PP) MS patients. Therefore they proposed that this haplotype is protective in PP-MS[19]. However, our total study group consisted of healthy controls, as well as RR-MS and SP-MS patients. This may indicate that the protective effect of Hap4 is not restricted to PP-MS patients alone.

When subjects were subdivided in Hap2 carriers and non Hap2 carriers, no significant differences were found for CD127 expression. This is again in line with the findings of McKay et al.[18] However, a study of Gregory et al., which analysed the mRNA expression of the membrane bound versus the soluble form of the receptor, reported that carriers of the T allele at the exon 6 SNP rs6897932 (which tags Hap2) show a higher mRNA expression of the membrane-bound IL-7Rα protein, compared to carriers of the C allele[7]. Because our study cannot confirm this result on the protein level, two explanations can be given: 1) posttranslational modifications, which cannot be seen by real-time PCR, bias these results, or 2) flow cytometry cannot pick up subtle differences in the expression levels of CD127. To address this issue, other and more sensitive detection techniques should be used in the near future.

The observed molecular effects in our study can have consequences on the cellular level and even on the organism in total. A deficiency in Treg frequencies (especially in RTE-Tregs), can for example be detrimental in patients with autoimmune diseases such as MS. Also, a decrease in the output of Tconv might have disadvantages. That is, a decrease in thymic

output of Tconv might lead to homeostatic proliferation, which on its turn causes dividing cells to age. Aging of the immune system, also called immunosenescence, has been found to be accelerated in patients with a diverse range of autoimmune diseases[25,26].

Given the relatively low odds ratio (1.18)[8] of the IL-7Rα gene regarding its association to MS, a small effect on the MS risk could be expected. However, the results described here clearly show a significant effect of Hap4 on the frequency of RTEs. Since MS is a complex disease, with many genetic factors probably contributing to the predisposing genotype, the effect of Hap4 on T cell parameters might be the result of genetic interactions with other alleles influencing the immune system (e.g. two alleles within the IL-2R[8]). The low odds ratio of the IL-7Rα gene also in part explains why some Hap4 carriers still develop MS. As has been stated, the strongest genetic risk is represented by the HLA class II genes, but still other, non-genetic factors contribute to the disease, such as viral load and exposure to sunlight, all of which may affect MS susceptibility.

To conclude, our results provide the first evidence that IL-7Rα polymorphisms influence the frequency of RTE in the peripheral blood of MS patients, either on the level of T cell development, and/or in the periphery. These results might therefore contribute to a better understanding of the biological role of IL-7Rα polymorphisms in complex diseases such as MS.

Acknowledgements

The authors thank patients and healthy controls for their blood donations, Tom Broekmans and Anne Bogaers for assistance in collecting the blood samples. We also thank Hanne Jongen of the hematology unit of the Virga Jesse hospital Hasselt for performing flow cytometric analyses on the FACSCanto.

Conflict of Interest statement

The authors declare no conflict of interest.

Reference list

1 Hafler DA, Slavik JM, Anderson DE, O'Connor KC, De Jager P, Baecher-Allan C. Multiple sclerosis. *Immunol Rev* 2005; **204:** 208-231.

2 Ebers GC, Kukay K, Bulman DE, Sadovnick AD, Rice G, Anderson C et al. A full genome search in multiple sclerosis. *Nat Genet* 1996; **13:** 472-476.

3 Chao MJ, Barnardo MC, Lincoln MR, Ramagopalan SV, Herrera BM, Dyment DA et al. HLA class I alleles tag HLA-DRB1*1501 haplotypes for differential risk in multiple sclerosis susceptibility. *Proc Natl Acad Sci U S A* 2008; **105:** 13069-13074.

4 Lincoln MR, Ramagopalan SV, Chao MJ, Herrera BM, Deluca GC, Orton SM et al. Epistasis among HLA-DRB1, HLA-DQA1, and HLA-DQB1 loci determines multiple sclerosis susceptibility. *Proc Natl Acad Sci U S A* 2009; **106:** 7542-7547.

5 Ramagopalan SV, Maugeri NJ, Handunnetthi L, Lincoln MR, Orton SM, Dyment DA et al. Expression of the multiple sclerosis-associated MHC class II Allele HLA-DRB1*1501 is regulated by vitamin D. *PLoS Genet* 2009; **5:** e1000369.

6 Teutsch SM, Booth DR, Bennetts BH, Heard RN, Stewart GJ. Identification of 11 novel and common single nucleotide polymorphisms in the interleukin-7 receptor-alpha gene and their associations with multiple sclerosis. *Eur J Hum Genet* 2003; **11:** 509-515.

7 Gregory SG, Schmidt S, Seth P, Oksenberg JR, Hart J, Prokop A et al. Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. *Nat Genet* 2007; **39:** 1083-1091.

8 Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, De Jager PL et al. Risk alleles for multiple sclerosis identified by a genomewide study. *N Engl J Med* 2007; **357:** 851-862.

9 Lundmark F, Duvefelt K, Iacobaeus E, Kockum I, Wallstrom E, Khademi M et al. Variation in interleukin 7 receptor alpha chain (IL7R) influences risk of multiple sclerosis. *Nat Genet* 2007; **39:** 1108-1113.

10 Watanabe N, Wang YH, Lee HK, Ito T, Wang YH, Cao W et al. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. *Nature* 2005; **436:** 1181-1185.

11 Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N et al. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med* 2005; **202:** 1213-1223.

12 Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 2006; **203:** 1701-1711.

13 Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 2006; **203:** 1693-1700.

14 Venken K, Hellings N, Broekmans T, Hensen K, Rummens JL, Stinissen P. Natural naive CD4+CD25+CD127low regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression. *J Immunol* 2008; **180:** 6411-6420.

15 Haas J, Fritzsching B, Trubswetter P, Korporal M, Milkova L, Fritz B et al. Prevalence of newly generated naive regulatory T cells (Treg) is critical for Treg suppressive function and determines Treg dysfunction in multiple sclerosis. *J Immunol* 2007; **179:** 1322-1330.

16 Kimmig S, Przybylski GK, Schmidt CA, Laurisch K, Mowes B, Radbruch A et al. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. *J Exp Med* 2002; **195:** 789-794.

17 Kohler S, Wagner U, Pierer M, Kimmig S, Oppmann B, Mowes B et al. Post-thymic in vivo proliferation of naive CD4+ T cells constrains the TCR repertoire in healthy human adults. *Eur J Immunol* 2005; **35:** 1987-1994.

18 McKay FC, Swain LI, Schibeci SD, Rubio JP, Kilpatrick TJ, Heard RN et al. Haplotypes of the interleukin 7 receptor alpha gene are correlated with altered expression in whole blood cells in multiple sclerosis. *Genes Immun* 2008; **9:** 1-6.

19 McKay FC, Swain LI, Schibeci SD, Rubio JP, Kilpatrick TJ, Heard RN et al. CD127 immunophenotyping suggests altered CD4+ T cell regulation in primary progressive multiple sclerosis. *J Autoimmun* 2008; **31:** 52-58.

20 Freeden-Jeffry U, Vieira P, Lucian LA, McNeil T, Burdach SE, Murray R. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* 1995; **181:** 1519-1526.

21 Peschon JJ, Morrissey PJ, Grabstein KH, Ramsdell FJ, Maraskovsky E, Gliniak BC et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* 1994; **180:** 1955-1960.

22 Booth DR, Arthur AT, Teutsch SM, Bye C, Rubio J, Armati PJ et al. Gene expression and genotyping studies implicate the interleukin 7 receptor in the pathogenesis of primary progressive multiple sclerosis. *J Mol Med* 2005; **83:** 822-830.

23 Mazzucchelli R, Hixon JA, Spolski R, Chen X, Li WQ, Hall VL et al. Development of regulatory T cells requires IL-7Ralpha stimulation by IL-7 or TSLP. *Blood* 2008; **112:** 3283-3292.

24 Azevedo RI, Soares MV, Barata JT, Tendeiro R, Serra-Caetano A, Victorino RM et al. IL-7 sustains CD31 expression in human naive CD4+ T cells and preferentially expands the CD31+ subset in a PI3K-dependent manner. *Blood* 2008.

25 Thewissen M, Somers V, Venken K, Linsen L, van Paassen P, Geusens P et al. Analyses of immunosenescent markers in patients with autoimmune disease. *Clin Immunol* 2007; **123:** 209-218.

26 Thewissen M, Somers V, Hellings N, Fraussen J, Damoiseaux J, Stinissen P. CD4+CD28null T Cells in Autoimmune Disease: Pathogenic Features and Decreased Susceptibility to Immunoregulation. *J Immunol* 2007; **179:** 6514-6523.

Figure legends:

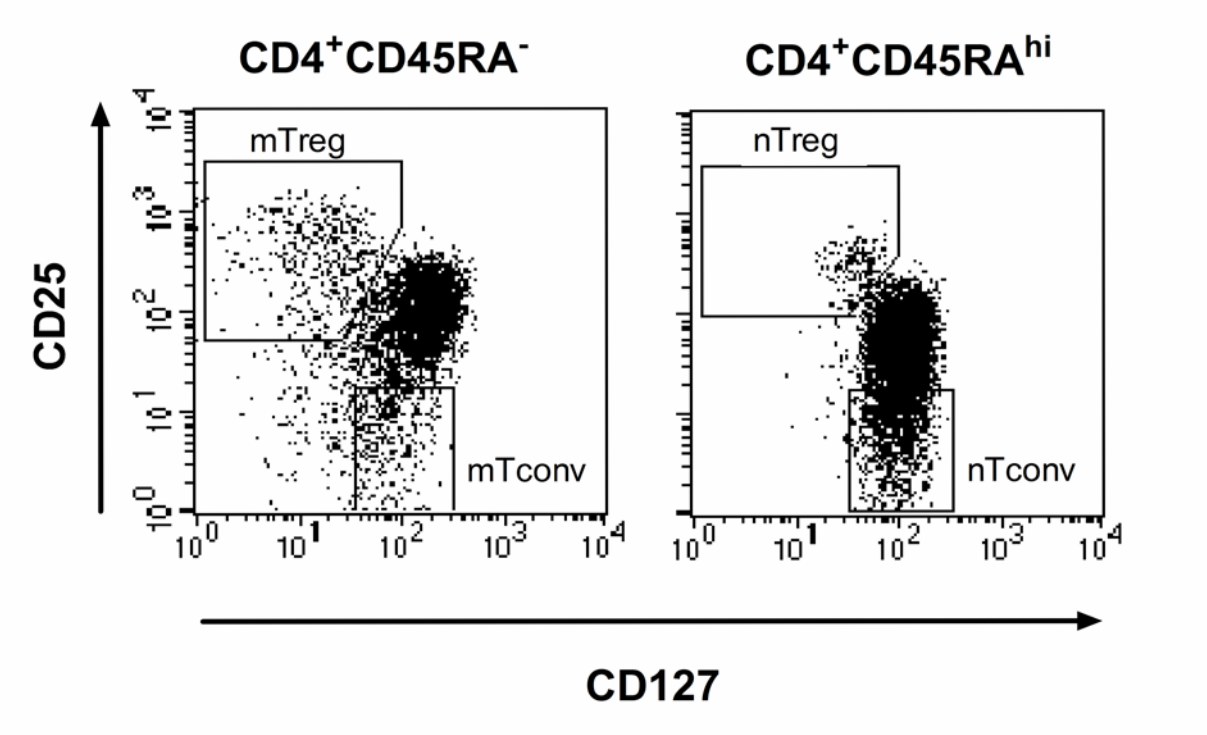
FIGURE 1. Four different populations of T cells can be distinguished in peripheral blood by means of CD4, CD25, CD127 and CD45RA expression. The plot on the left is gated on $CD4^{+}CD45RA^{-}$ memory T cells; the plot on the right is gated on $CD4^{+}CD45RA^{hi}$ naive T cells. The markers CD25 and CD127 can then discriminate between Tconv ($CD25^{-}CD127^{+}$) and Tregs ($CD25^{hi}CD127^{lo}$).

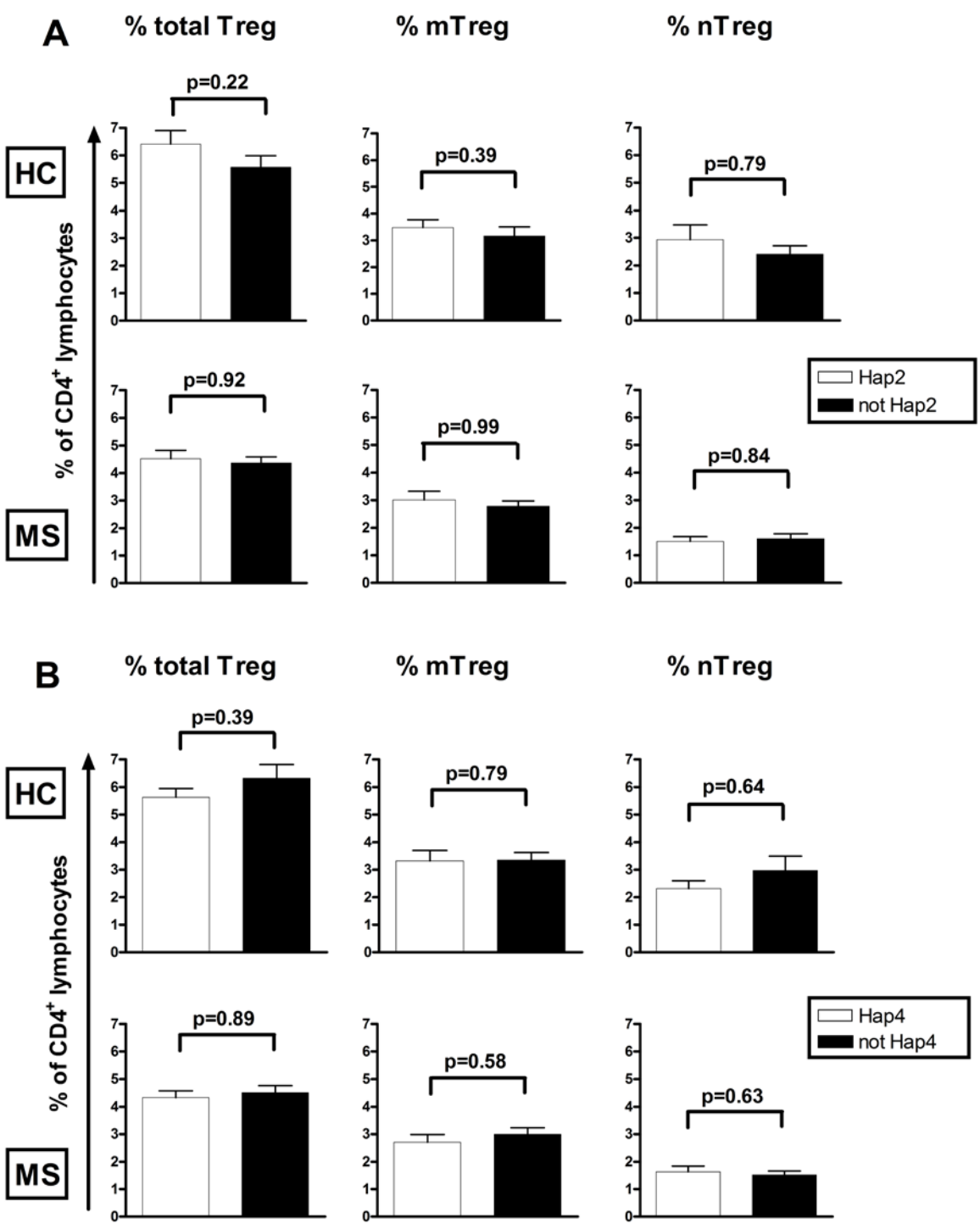
FIGURE 2. Treg frequencies within the $CD4^{+}$ lymphocytes of MS patients and healthy controls. A: Hap2 carriers versus non-Hap2 carriers; B: Hap4 carriers versus non-Hap4 carriers. Total Treg: $CD4^{+}CD25^{hi}CD127^{lo}$; mTreg: $CD4^{+}CD25^{hi}CD127^{lo}CD45RA^{-}$; nTreg: $CD4^{+}CD25^{hi}CD127^{lo}CD45RA^{hi}$. Differences were not significant in any of the populations ($p>0.05$).

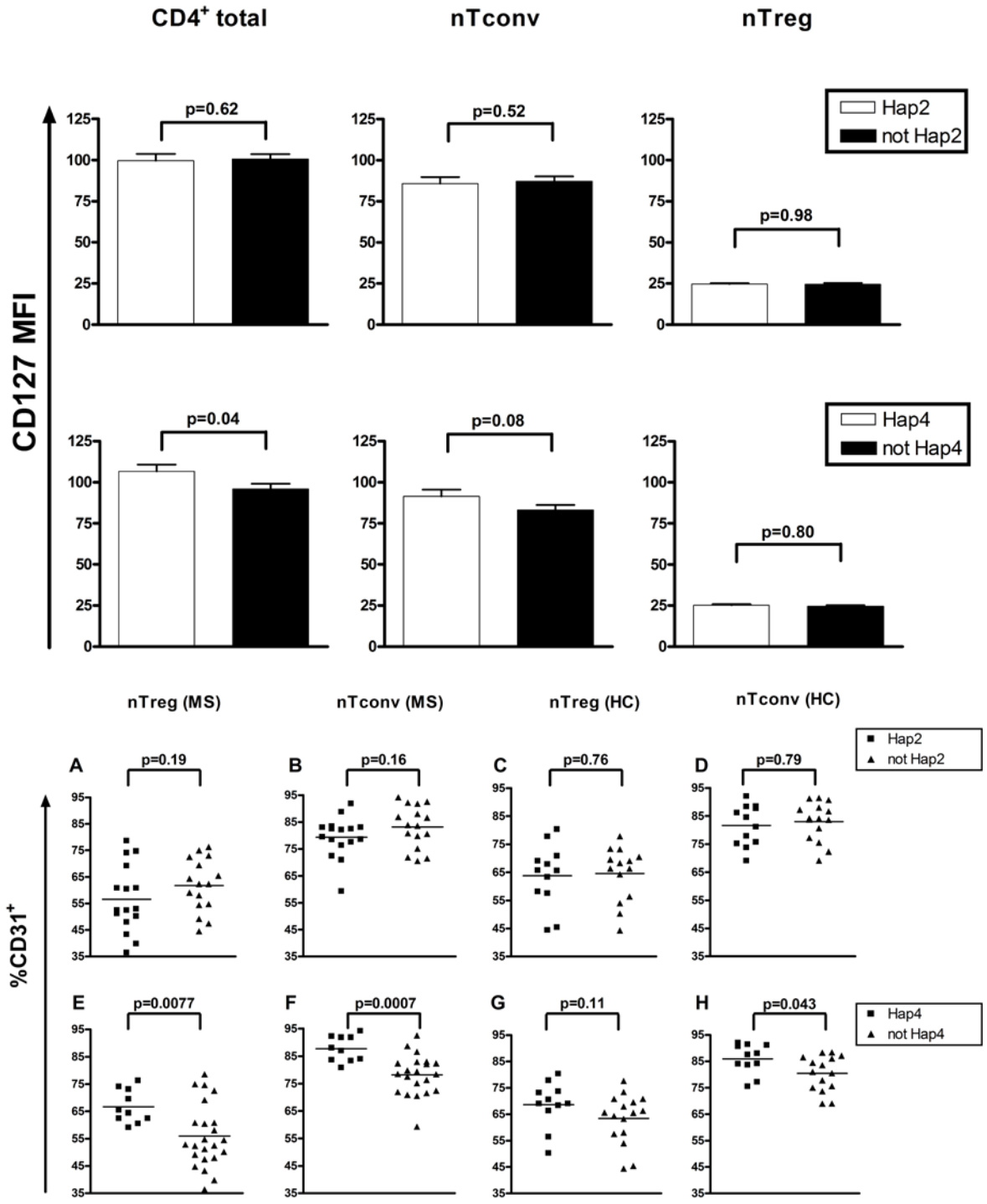
FIGURE 3. Mean fluorescence intensity of CD127 on total $CD4^{+}$ T cells ($CD4^{+}$ total) , $CD4^{+}CD25^{-}CD127^{+}$ T cells (nTconv) and $CD4^{+}CD25^{hi}CD127^{lo}$ Tregs (nTreg) of pooled MS patients and HC. The MFI is plotted against presence or absence of Hap2 (upper panel) and the presence or absence of Hap 4 (lower panel).

FIGURE 4. The frequency of CD31-expressing T cells in different T cell populations in Hap2 carriers versus non-Hap2 carriers (A-D) and Hap4 versus non-Hap4 carriers (E-H). nTreg: $CD4^{+}CD25^{hi}CD127^{lo}CD45RA^{hi}$; nTconv: $CD4^{+}CD25^{-}CD127^{+}CD45RA^{hi}$. Results are considered significant when $p<0.05$.

FIGURE 5. PBMC from four Hap4 and four non-Hap4 healthy donors were stimulated with αCD3, with or without additional IL-7. The frequency of $CD4^{+}CD31^{+}$ cells was measured on days 0 and 5. Values for every donor on day 5 of culture are shown (relative to the *ex vivo* measurements on day 0).


CD4+CD45RA-
CD4+CD45RAhi
mTreg
mTconv
nTreg
nTconv
CD25
CD127


A
% total Treg
% mTreg
% nTreg
p=0.22
p=0.39
p=0.79
p=0.92
p=0.99
p=0.84
HC
MS
% of CD4+ lymphocytes
Hap2
not Hap2
B
% total Treg
% mTreg
% nTreg
p=0.39
p=0.79
p=0.64
p=0.89
p=0.58
p=0.63
HC
MS
% of CD4+ lymphocytes
Hap4
not Hap4


CD4+ total
nTconv
nTreg
CD127 MFI
p=0.62
p=0.52
p=0.98
Hap2
not Hap2
p=0.04
p=0.08
p=0.80
Hap4
not Hap4
nTreg (MS)
nTconv (MS)
nTreg (HC)
nTconv (HC)
%CD31+
A
p=0.19
B
p=0.16
C
p=0.76
D
p=0.79
E
p=0.0077
F
p=0.0007
G
p=0.11
H
p=0.043

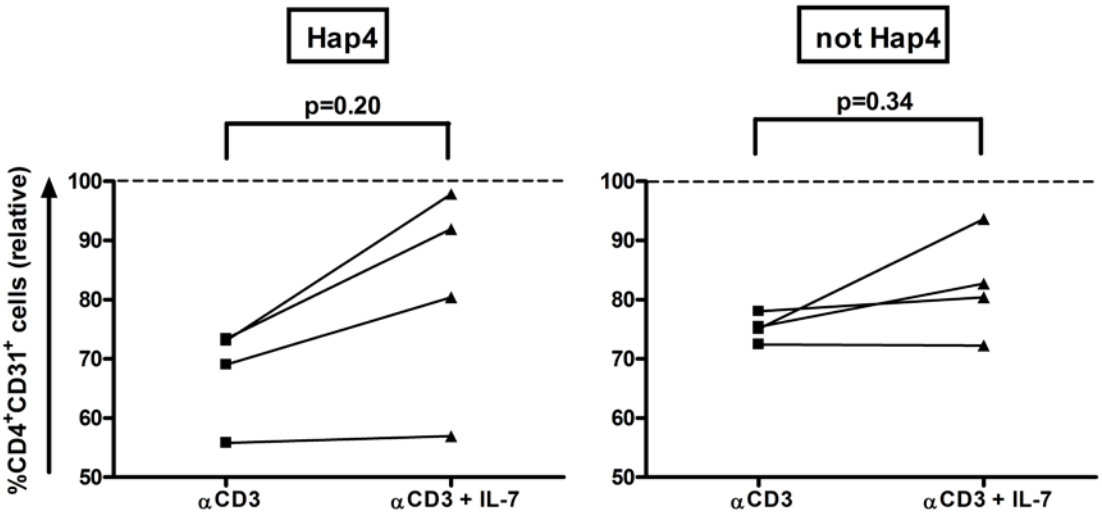

Hap4
not Hap4
p=0.20
p=0.34
%CD4+CD31+ cells (relative)
100
90
80
70
60
50
αCD3
αCD3 + IL-7

TABLE 1. The four different haplotypes of the IL-7Rα gene. The tagging SNPs (rs7718919, rs11567685 and rs11567686) are represented in bold; the MS associated exon 6 SNP (rs6897932) is added in the last column

	rs7718919	**rs11567685**	**rs11567686**	rs6897932
Hap 1	**G**	**T**	**G**	C
Hap 2	**G**	**T**	**A**	T
Hap 3	**T**	**T**	**A**	C
Hap 4	**G**	**C**	**A**	C

TABLE 2. IL7Ra haplotype frequencies in MS patients and healthy controls.

Haplotypes	*1*	*2*	*3*	*4*
Controls (n=33)	0.227	0.273	0.182	0.318
MS patients (n=65)	0.346	0.254	0.085	0.315

TABLE 3. Allele and genotype frequencies of the exon 6 SNP (rs6897932) in MS patients and healthy controls.

rs6897932	*C*	*T*	*CC*	*CT*	*TT*
Controls (n=33)	0.727	0.273	0.485	0.485	0.030
MS patients (n=65)	0.746	0.254	0.538	0.415	0.046