

ORIGINAL ARTICLE

IL7RA haplotype-associated alterations in cellular immune function and gene expression patterns in multiple sclerosis

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Interleukin-7 receptor alpha (*IL7RA*) is among the top listed candidate genes influencing the risk to develop multiple sclerosis (MS), an inflammatory demyelinating disease of the central nervous system. Soluble IL-7RA (sIL-7RA) protein and mRNA levels vary among the four common *IL7RA* haplotypes. Here we show and confirm that protective haplotype carriers have three times lower sIL-7RA serum levels than the other three haplotypes. High sIL-7RA concentrations significantly decrease IL-7-mediated STAT5 phosphorylation in CD4⁺ T cells. Transcriptome analysis of unstimulated and stimulated CD4⁺ T cells of MS patients carrying the different *IL7RA* haplotypes revealed complex and overlapping patterns in genes participating in cytokine signaling networks, apoptosis, cell cycle progression and cell differentiation. Our findings indicate that genetic variants of *IL7RA* result in haplotype-associated differential responsiveness to immunological stimuli that influence MS susceptibility not exclusively by varying levels of sIL-7RA.

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INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system. It affects mainly adults between 20 and 40 years of age.¹ The etiology of MS is still incompletely understood, but both a complex genetic trait and environmental factors contribute to disease expression.² At the level of individual genes, the most important contribution to genetic risk stems from alleles in the human leukocyte antigen class two region, namely the DR15 haplotype.³ Genome-wide association studies have discovered until today more than 50 common genetic variants, single-nucleotide polymorphisms (SNPs), that contribute to MS susceptibility,^{4,5} and it is expected that additional ones will be discovered in the near future. SNPs in the genomic region coding for the interleukin-7 receptor alpha (IL-7RA) were the second set of confirmed MS risk alleles.^{6–8}

Among the *IL7RA* SNPs, the nonsynonymous SNP rs6897932 located in exon 6 of the *IL7RA* gene shows the strongest association with MS.^{7,8} Several functional studies demonstrated that the risk 'C' allele leads to an increased skipping of exon 6 resulting in a decreased ratio of full-length- to soluble IL-7RA (sIL-7RA)-encoding mRNA and an increased amount of sIL-7RA (H6).^{7,9,10} Although this was repeatedly shown, it remains an open question why only one out of three haplotypes comprising rs6897932 is positively associated with MS. Four SNPs, rs1494555, rs6897932, rs987107 and rs987106, are sufficient to tag the four main haplotypes that arise from the *IL7RA* gene locus (Figure 1).⁷ Haplotype 1 is also referred to as the 'risk' haplotype because it is more prominent in MS patients compared with healthy donors (HDs), whereas the opposite effect has been observed for haplotype 3, which has therefore been termed 'protective' haplotype.

Regarding the functional effects of *IL7RA* haplotypes beyond altered sIL-7RA levels, previous studies have shown differences between haplotype 2 and non-haplotype 2 carriers with respect to the frequency of recent thymic emigrants¹¹ and response of CD4⁺ lymphocytes to interferon beta.¹² The functional effects on T-cell stimulation of haplotype 2, which has not been found to be related to MS risk, is thought to arise from differences in the genomic sequences within the promoter region of the *IL7RA* locus, which are also tagged by the SNPs used to define haplotypes.

In the context of MS and autoimmune diseases, IL-7-mediated signaling likely acts through its involvement in lymphocyte development and/or homeostasis, but this may not be the only mechanism. IL-7 signaling through the IL-7R is indispensable for the development of human T lymphocytes¹³ as demonstrated by the X-linked severe combined immunodeficiency phenotype of patients carrying a defect in the *IL7RA*. Given the central role of T cells in MS and other autoimmune diseases, it is therefore reasonable to assume that IL-7/IL-7R interactions are functionally involved in their pathogenesis, and it is interesting that SNPs not only in *IL7RA* but also in the cytokine IL-7 are associated with MS.¹⁴ The IL-7RA is mainly expressed by cells of the lymphoid lineage, but also by dendritic cells and monocytes.^{15,16} IL-7 is produced by non-hematopoietic stromal and epithelial cells but to lesser amounts also by dendritic cells.¹⁷ Binding of IL-7 leads to dimerization of the IL-7RA chain and the so-called common γ -chain, which then form the high affinity IL-7R.¹⁸ IL-7R shares its α -chain with the thymic stromal lymphopoietin receptor and the common γ -chain with the receptors for IL-2, IL-4, IL-9, IL-15 and IL-21.¹⁹ IL-7R-mediated signaling activates the JAK/STAT and

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PI3K/AKT signaling pathways.^{15,16} The homeostasis of naive T lymphocytes is regulated by T-cell receptor and self-peptide major histocompatibility complex contact and the availability of pro-survival cytokines such as IL-7.^{16,18} Upon ligand engagement, activated- as well as IL-7-stimulated T lymphocytes downregulate the IL-7R, and as IL-7 is produced at relatively stable, but low levels by the abovementioned stromal cells, more IL-7 is then available for naive and memory T lymphocytes with the result that diverse repertoires of naive and antigen-experienced memory T-cell populations are maintained.¹⁵

IL-7 also has a role in other autoimmune diseases such as rheumatoid arthritis where it has been shown that IL-7 serum and synovial fluid concentrations are elevated in comparison with HDs and in addition are correlated with disease activity.²⁰ Hartgring *et al.*²¹ were able to demonstrate in an *in vitro* model that recombinant sIL-7RA reduces IL-7-mediated proliferation and production of interferon gamma of peripheral blood mononuclear cells (PBMCs). Interestingly, anti-IL-7RA antibody treatment of either mice with collagen-induced arthritis or experimental autoimmune encephalomyelitis led to reduced disease severity.^{22–24} The above data indicate that dysregulation of the IL-7/IL-7R system may contribute to disease pathogenesis and pathological immune activation during autoimmune diseases. Its physiological roles include not only the functions in T-lymphocyte generation and -homeostasis but also coping with chronic viral infections.²⁵

Despite robust confirmation of the associations of *IL7RA* SNPs with MS, the understanding of the possible functional contributions of *IL7RA* risk-conferring alleles to MS pathogenesis is still incomplete. The goals of the present study were to characterize

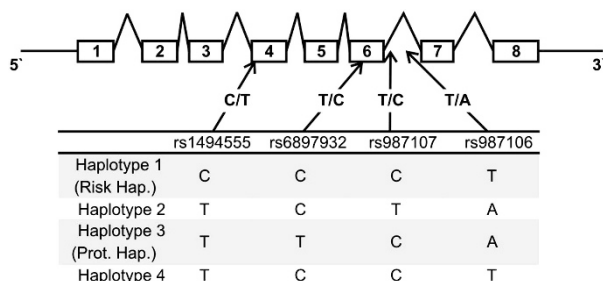


Figure 1. Schematic diagram of the *IL7RA* sequence showing the four SNPs used to stratify the Hamburg cohort into four common haplotypes.

immune cells of MS patients carrying risk or protective *IL7RA* alleles with respect to membrane and sIL-7RA expression, to examine how the increased sIL-7RA serum concentrations in risk allele carriers have an impact on IL-7/IL-7R functions, and whether transcriptional profiling in individuals carrying the various *IL7RA* haplotypes leads to additional insight into their potential functional contribution to MS.

RESULTS

Association of SNP rs6897932 with MS

First, we analyzed the *IL7RA* allele, genotype and haplotype distribution in a cohort of Northern German, Caucasian MS patients ($n=484$) and HDs ($n=310$). Four tagging SNPs (rs1494555, rs6897932, rs987107 and rs987106) were used to identify the four common *IL7RA* haplotypes. Owing to the small sample size, we did not expect to reach statistical significance. Surprisingly, however, this was the case for SNP rs6897932 (Table 1). The dominant model fit best to our data and confirmed an increased MS susceptibility for homozygous carriers of the 'C' allele of the SNP rs6897932 ($P=0.03$; odds ratio = 1.38 (95% confidence interval: 1.04–1.84)). No significant association with disease could be detected for the other three SNPs. However, the genotype frequencies in the Hamburg cohort were similar to the previously published reports.^{7,26} As expected for a small cohort, no statistical significance was reached for the haplotype distribution (Table 2).

mRNA and protein expression correlate with *IL7RA* haplotype

Previous studies^{9,10} demonstrated an influence of the *IL7RA* haplotype on the splicing resulting in an increased serum concentration of sIL-7RA. To determine whether or not we can confirm these effects, we analyzed the IL-7RA mRNA as well as protein expression in haplotype 1–4 carrying homozygous MS patients. The relative expression of the full-length (H20), the soluble isoform missing exon 6 (H6) and all common IL-7RA isoforms was determined using reverse transcriptase PCR. Carriers of the protective haplotype had an increased ratio in the expression of membrane bound to sIL-7RA compared with carriers of the other haplotypes (Figure 2a). In contrast and consistent with previous results, risk allele carriers showed a significantly reduced ratio, that is, a relative increase of the soluble isoform and a slight reduction of the full-length isoform (Supplementary Material, Supplementary Figure S1).

We then examined the IL-7RA serum concentration of homozygous MS patients of the four common *IL7RA* haplotypes using

Table 1. Allele and genotype analysis of the four haplotype-tagging SNPs rs1494555, rs6897932, rs987107 and rs987106 in 484 MS patients and 310 HDs from Hamburg

SNP	Allele	Frequency in individuals with MS	Frequency in controls	Genotype	Frequency in individuals with MS	Frequency in controls	P-value (dominant coding)	Odds ratio (95% confidence interval)
rs1494555	C	0.35	0.34	C/C	0.12	0.14	0.38	0.88 (0.66–1.17)
	T	0.65	0.66	C/T	0.45	0.40		
				T/T	0.43	0.46		
rs6897932	T	0.24	0.27	T/T	0.07	0.05	0.03	1.38 (1.04–1.84)
	C	0.76	0.73	C/T	0.33	0.43		
				C/C	0.60	0.52		
rs987107	T	0.30	0.26	T/T	0.10	0.06	0.28	0.85 (0.64–1.13)
	C	0.70	0.74	C/T	0.40	0.39		
				C/C	0.50	0.55		
rs987106	T	0.46	0.48	T/T	0.24	0.23	0.30	1.19 (0.87–1.63)
	A	0.54	0.52	A/T	0.45	0.50		
				A/A	0.31	0.27		

Abbreviations: HDs, healthy donors; MS, multiple sclerosis; SNPs, single-nucleotide polymorphisms.

enzyme-linked immunosorbent assay. Analysis of sIL-7RA protein expression is in accordance with the gene expression analysis. Patients carrying the protective haplotype had three times lower sIL-7RA serum concentrations compared with carriers of the other three haplotypes (Figure 2b). MS patients heterozygous for the SNP rs6897932 had intermediate sIL-7RA serum levels (Figure 2b). Therefore, consistent with the reports of Hoe et al.,⁹ we demonstrate an allele dosage influence of SNP rs6897932 on the sIL-7RA serum concentration.

Altered IL-7RA surface expression on NK cells in relation to the IL7RA haplotype

Next, we addressed whether carriers of the risk versus protective IL7RA haplotypes show differences in membrane expression of the receptors on different immune cells including CD4⁺ and CD8⁺ bulk T cells, the naive and memory populations of these two subsets, regulatory T cells, several subpopulations of dendritic cells and the two main populations of natural killer (NK) cells (CD56^{dim} and CD56^{bright}). We hypothesized that the greatest differences might be observed between the risk and the protective haplotypes because these varied most with respect to their putative effects on MS susceptibility. We found a significant, albeit small difference for CD56^{bright} NK cells (Figure 3). Risk haplotype carriers showed significantly lower IL-7RA surface expression compared with carriers of the protective haplotype (P -value = 0.014). Although there were consistent trends toward lower IL-7RA membrane expression of several immune cell populations of risk haplotype carriers, for example, in naive

CD4⁺ T cells and Tregs, these did not reach statistical significance (Supplementary Material, Supplementary Figure S2).

sIL-7RA inhibits STAT5 phosphorylation

Next, we questioned whether the sIL-7RA levels interfere with T-cell function by influencing the availability of cytokine for binding to the membrane-bound IL-7R, and hence IL-7R-mediated signaling. Previous studies have shown that sIL-7RA can bind IL-7.^{27,28} Although the affinity is lower^{29,30} than that of the membrane-bound form, the sIL-7RA (concentrations in risk haplotype carriers 50–185 ng ml⁻¹) may be capable of influencing the availability of IL-7 because of the fact that it is available in the serum at an up to 90 000-fold excess compared with the free cytokine IL-7 (levels 2–8 pg ml⁻¹). So far, most studies used the recombinant IL-7RA (R&D, Minneapolis, MN, USA, 306-IR-050) or sIL-7RA produced by WI26VA4 cells, which shed the IL-7RA upon IL-7 stimulation, to analyze the effect of sIL-7RA on IL-7 signaling. As the H6 isoform, which misses exon 6, is the most common sIL-7RA isoform in human plasma, we decided not to use the full-length (H20) or shed form of the IL-7RA, but express the soluble form lacking exon 6 and is generated in excess in risk allele carriers. We established an IL-7RA (isoform H6)-secreting HEK cell

Table 2. Haplotype analysis of IL7RA in 484 MS patients and 310 HDs from northern Germany using four adjacent SNPs that distinguish all common haplotypes

	Haplotype	Frequency	Frequency in individuals with MS	Frequency in controls	P-value
Haplotype 1 (risk Hap)	CCCT	0.34	0.35	0.34	0.78
Haplotype 2	TCTA	0.28	0.30	0.26	0.09
Haplotype 3 (protective Hap)	TTCA	0.25	0.24	0.27	0.20
Haplotype 4	TCCT	0.12	0.12	0.14	0.24

Abbreviations: HDs, healthy donors; IL7RA, interleukin-7 receptor alpha; MS, multiple sclerosis; SNPs, single-nucleotide polymorphisms.

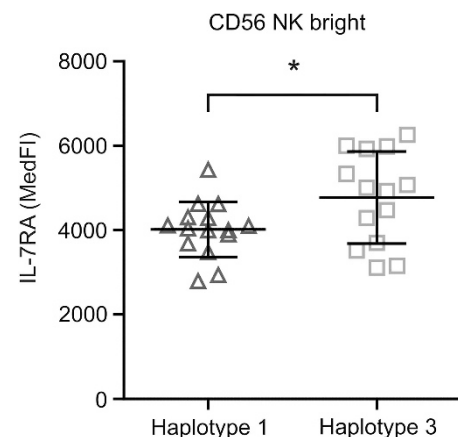


Figure 3. Haplotype differences in IL-7RA surface expression. PBMCs of MS patients homozygous for either haplotype 1 ($n = 15$) or haplotype 3 ($n = 14$) were specifically stained for CD56^{bright} cells and analyzed by flow cytometry. Results are presented as means \pm s.d.; * $P \leq 0.05$.

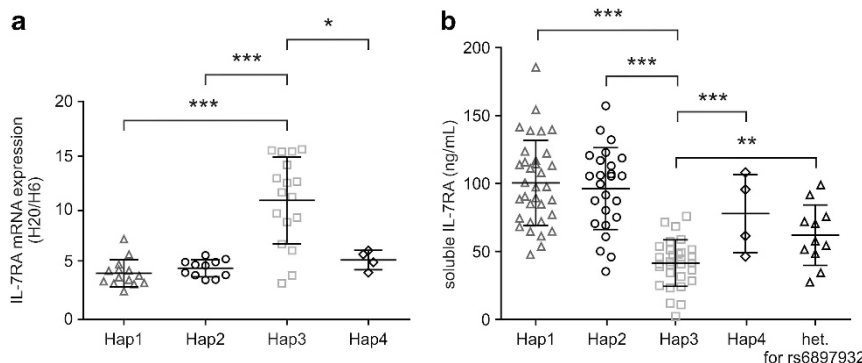


Figure 2. Haplotype-dependent differences in IL-7RA mRNA and protein expression. (a) IL-7RA mRNA expression from PBMCs of MS patients homozygous for haplotype 1–4 was determined by quantitative PCR and is shown as ratio of membrane bound to sIL-7RA mRNA isoform (sample size: Hap1 = 14, Hap2 = 11, Hap3 = 17, Hap4 = 4). (b) Differences in IL-7RA serum concentration were analyzed using sandwich enzyme-linked immunosorbent assay. The sIL-7RA serum levels were analyzed in samples from 32 haplotype 1, 25 haplotype 2, 30 haplotype 3, 4 haplotype 4 carrying homozygous and 11 heterozygous (for SNP rs6897932) MS patients. Results are presented as means \pm s.d.; * $P \leq 0.05$, ** $P \leq 0.005$ and *** $P \leq 0.001$.

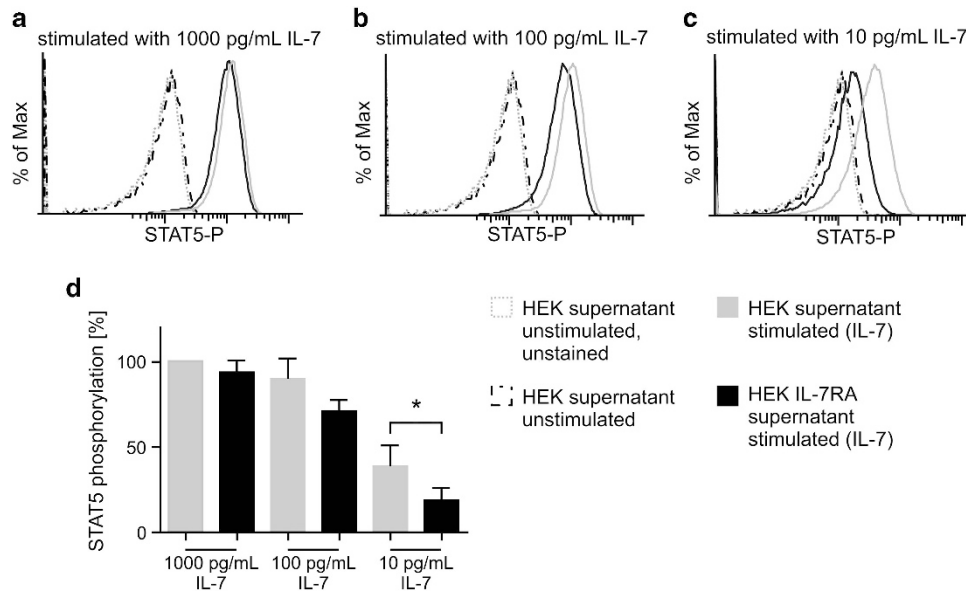


Figure 4. sIL-7RA is able to block IL-7-mediated signaling. IL-7-induced STAT5 phosphorylation in CD4⁺ T lymphocytes was measured by flow cytometry *in vitro*. (a–d) Histograms show the STAT5 phosphorylation in CD4⁺ T cells cultured in 10 times concentrated HEK cell supernatant, unstained unstimulated (dotted gray), unstimulated (dotted black), stimulated with 10–1000 pg ml^{−1} IL-7 (gray) and cultured in 10 times concentrated supernatant of a HEK cell line producing sIL-7RA stimulated with 10–1000 pg ml^{−1} IL-7 (black). Representative results from three independent experiments are shown as mean values ± s.d.; *P ≤ 0.05.

line. Using this sIL-7RA (H6), we analyzed the effect of sIL-7RA on IL-7R-mediated STAT5 phosphorylation in human CD4⁺ T lymphocytes using flow cytometry. We observed clear differences between CD4⁺ T lymphocytes stimulated in absence or presence of sIL-7RA with IL-7 (Figure 4a–d). The strongest effect was observed for CD4⁺ cells stimulated with 10 pg ml^{−1} IL-7 in presence of sIL-7RA (isoform H6), resulting in a 50% reduced STAT5 phosphorylation. According to our data, the strength of the observed effect correlates with the ratio between IL-7 and the sIL-7RA (Figure 4a–d). These data suggest a functional relevance of the increased sIL-7RA concentrations in carriers of the risk haplotype.

IL7RA haplotype-associated gene expression patterns

The above data demonstrate that carriers of haplotype 1, 2 and 4 have increased sIL-7RA serum levels because of differential splicing. Hence, it is remarkable that only haplotype 1 carriers show an increased risk for MS. We therefore wanted to address next, whether the *IL7RA* haplotypes differ in further aspects besides the sIL-7RA concentration. As CD4⁺ T cells are particularly critical for MS,¹ we performed global gene expression profiling of CD4⁺ T cells from MS patients that were homozygous for one of the three haplotypes (Hap1, Hap2 and Hap3). Because of a lack of patients carrying haplotype 4, which is also the rarest of all four haplotypes, we were not able to include this haplotype in the gene expression analysis. Purified CD4⁺ T cells were analyzed unstimulated and after challenge with IL-7 to specifically address the IL-7/IL-7R system. Concentrations of IL-7 and sampling times were chosen based on pilot experiments monitoring *BCL2* as major upregulated gene following IL-7R stimulation (not shown). The experimental design aimed at comparing gene expression profiles in carriers of the three haplotypes at basal level and more importantly also at examining haplotype-specific effects following IL-7R stimulation.

Resting condition. Expression levels in unstimulated cells showed a trend toward haplotype-specific differences when a fraction of genes selected solely by the criteria of showing minimal intra-group variance was inspected with multi-dimensional scaling (843

Table 3. Gene expression differences in CD4⁺ T cells of MS individuals homozygous for one of three haplotypes (Hap1, Hap2 and Hap3)

Ensembl ID	Symbol	P-value	LogFC Hap1 vs Hap3	LogFC Hap1 vs Hap2	LogFC Hap3 vs Hap2
ENSG00000202089	<i>U6 snRNA</i>	8.26E-04	−0.91	−2.38	−1.47
ENSG00000212527	<i>RN5563</i>	1.47E-04	0.99	−0.24	−1.23
ENSG00000200796	<i>U6 snRNA</i>	5.39E-04	−0.50	−1.04	−0.54
ENSG00000187172	<i>BAGE2</i>	1.43E-03	−0.64	0.34	0.97
ENSG00000206826	<i>U6 snRNA</i>	2.30E-06	−0.10	−0.94	−0.84
ENSG00000201813	<i>U6 snRNA</i>	9.35E-05	−0.17	−0.88	−0.71
ENSG00000120322	<i>PCDH8</i>	1.96E-04	−0.17	−0.87	−0.70
ENSG00000201088	<i>Y RNA</i>	8.18E-04	−0.22	−0.84	−0.63
ENSG00000125144	<i>MT1G</i>	7.88E-04	−0.09	0.70	0.79
ENSG00000201065	<i>U6 snRNA</i>	7.17E-04	−0.25	−0.79	−0.53
ENSG00000252574	<i>RNU5B-6P</i>	2.18E-05	−0.02	−0.70	−0.68

Abbreviations: MS, multiple sclerosis; snRNA, small nuclear RNA. Only genes whose expression levels vary maximally between haplotypes (top 0.1% divergent gene expression from F-test statistics, corresponding to P-value ≤ 1.8 E − 3) and show the largest distances (top 1% of effect values) are shown.

Ensembl gene IDs, Supplementary Material, Supplementary Table S1 and Supplementary Figure S3A). Although no gene reached statistical significance applying the widely used multiple test correction (Benjamini–Hochberg; two-group t-test as well as three-group F-tests; Table 3, Supplementary Material, Supplementary Table S1 and Supplementary Figure S3A), we noted that the gene list contains several spliceosomal RNAs and microRNAs. This may indicate that the different haplotypes are associated with varying capacity to react to cytokine stimulus at the transcriptional level especially for the extent to which splicing occurs, but also at the translational level for an immediate response to stimuli.

IL-7-stimulated condition. The treatment of purified CD4⁺ T cells with IL-7 induced a robust response with the majority of the most

Table 4. Genes induced by stimulating isolated CD4⁺ T cells with IL-7

Ensembl ID	Symbol	Adjusted P-value	logFC
ENSG00000114737	<i>CISH</i>	2.66E – 11	2.69
ENSG00000105810	<i>CDK6</i>	3.68E – 10	2.61
ENSG00000125384	<i>PTGER2</i>	3.36E – 09	2.55
ENSG00000137265	<i>IRF4</i>	3.02E – 10	2.48
ENSG00000171791	<i>BCL2</i>	8.51E – 12	2.40
ENSG00000079385	<i>CEACAM1</i>	2.45E – 09	2.33
ENSG00000067113	<i>PPAP2A</i>	1.25E – 09	1.56
ENSG00000157800	<i>SLC37A3</i>	3.36E – 09	1.23
ENSG00000074800	<i>ENO1</i>	3.03E – 09	1.16
ENSG00000171793	<i>CTPS</i>	3.36E – 09	1.13
ENSG00000123329	<i>ARHGAP9</i>	1.91E – 06	– 0.62
ENSG00000165071	<i>TMEM71</i>	4.86E – 06	– 0.74
ENSG00000137642	<i>SORL1</i>	2.66E – 06	– 0.83
ENSG00000167261	<i>DPEP2</i>	3.04E – 06	– 0.87
ENSG00000070731	<i>ST6GALNAC2</i>	7.64E – 06	– 0.87
ENSG00000150054	<i>MPP7</i>	3.06E – 06	– 0.90
ENSG00000106560	<i>GIMAP2</i>	7.11E – 06	– 0.91
ENSG00000162894	<i>FAIM3</i>	8.73E – 08	– 1.12
ENSG00000168685	<i>IL7R</i>	3.95E – 07	– 1.40
ENSG00000176928	<i>GCNT4</i>	1.42E – 06	– 1.47

Abbreviation: IL-7, interleukin-7. Top 1% of significantly regulated genes corresponding to adjusted P -value $\leq 1.6 \times 10^{-5}$ sorted by absolute log fold-change (10 entries each from strongly upregulated and downregulated genes, respectively). Expression differences were calculated by combining samples according to stimulus (either unstimulated or stimulated), irrespective of their haplotype assignment.

significantly responding genes being upregulated (203/225 genes, top 1% of significantly regulated genes corresponding to adjusted P -value $\leq 1.6 \times 10^{-5}$, total gene number at 0.05 level 3276). When combining individual samples by stimulus irrespective of haplotype assignment, particularly strong effects were seen for *BCL2*, *CISH*, *IRF4*, *CDK6*, *PPAP2A*, *CEACAM1*, *ENO1*, *PTGER2*, *SLC37A3*, *CTPS* (all upregulated) and *FAIM3*, *IL7R*, *GCNT4*, *ARHGAP9*, *SORL1*, *DPEP2*, *MPP7*, *TMEM71*, *GIMAP2*, *ST6GALNAC2* (all downregulated; Table 4, Supplementary Material, Supplementary Table S2 and Supplementary Figure S3B). For some of these genes, a direct link to the JAK/STAT pathway, which is employed as the IL-7/IL-7R signaling pathway, is already known or may be inferred from analysis of STAT5-occupied DNA sites (*CISH*, *BCL2*, *CDK6*, *CEACAM1*, *CTPS*, *GCNT4*, *PPAP2A*, *PTGER2*, *ST6GALNAC2*, *MPP7*).³¹

Analyzing individual responses considering the most stable signals for multi-dimensional scaling already showed grouping of individuals according to haplotypes (Figure 5a). It is noteworthy that this grouping was obtained from an exploratory data analysis with unsupervised clustering, that is, blind to haplotype assignment and cannot be accounted for by SNP rs6897932 alone. Further inspection made clear that a considerable number of genes that changed in expression after IL-7 stimulus did not show concordant changes or differed in effect size between haplotypes (Supplementary Material, Supplementary Table S3). Given the complex and overlapping participation of MS-associated genes in several signaling pathways, we applied an unsupervised clustering technique (non-negative matrix factorization, NMF) in order to extract haplotype-specific features from our high-dimensional gene expression data. NMF assigns weights to genes from a common input list according to their discriminatory power, which allows to distinguish a given group from the remaining ones and hereby creates group-specific profiles (metagenes).

We considered genes showing robust IL-7 responses (adjusted P -value ≤ 0.05) in combination with differences in response between haplotypes (F-test, P -value ≤ 0.05 , 209 genes, see Materials and methods, Supplementary Material, Supplementary Table S3) for NMF and observed a highly reproducible clustering of individuals as judged by the reported cophenetic coefficient

(peaking at factorization rank $k=3$) almost independent of the applied algorithm (Supplementary Material, Supplementary Figures S4A–D). The IL-7 response signals from a combination of top-ranked metagene members clearly show an overlapping pattern of differential reactivity. We note that haplotype 1 and 2, both comprising the rs6897932 risk allele, differ in their response to a considerable amount (Figure 5b, Supplementary Material, Supplementary Table S3).

In order to assess whether the top metagene-contributing genes are related to a specific signaling pathway and biologically plausible in the context of IL-7/IL-7R signaling, we queried the Reactome database. *IL7*, *IL7R*, *IL2RB*, *KRAS*, *IFNGR2* and *SUMO1* all participate in cytokine signaling in immune system networks (Reactome ID 75790, Figure 5c) and subnetworks. Combining this information with interaction, data from STRING (confidence score ≥ 0.7) identified even more genes from the input list to be associated with cytokine signaling. How and to which extent differential responses of those genes and potential additional partners might shape haplotype-associated responses is not clear, but it appears that despite the limited sample size the obtained interconnections indeed relate to a genome-defined response pattern to immunological stimuli. It is noteworthy that three cytokines and cytokine receptors, that is, IL-7, IL-7R and IL-2R that have been associated with MS risk appear as metagenes in this pathway (Figure 5c).

DISCUSSION

IL-7 is a critical factor for regulating T-lymphocyte development and homeostasis.³² The IL-7/IL-7R system has been described to have a role in MS as well as in other autoimmune diseases such as type 1 diabetes, rheumatoid arthritis and inflammatory bowel disease.^{24,33,34} There is compelling evidence from animal models of these diseases that blocking of the IL-7RA leads to an amelioration of the disease course by modulating inflammatory processes.^{22–24,33,34} For MS, we and others⁹ could demonstrate highly significant differences in the amount of sIL-7RA between the haplotypes. Carriers of the risk haplotype showed up to three times higher sIL-7RA serum concentrations compared with carriers of the protective haplotype. The observation that sIL-7RA is capable of reducing IL-7-mediated STAT5 phosphorylation in CD4⁺ T cells is in line with data of Crawley *et al.*,²⁸ who demonstrated that sIL-7RA reduces IL-7-mediated signaling in CD8⁺ T cells, although these authors did not use the sIL-7RA (H6), which is generated by differential splicing in MS risk haplotype carriers. Besides the JAK/STAT pathway, IL-7 also activates signaling pathways that influence cell metabolism as well as proliferative capacity.³⁵ Hence, the risk haplotype could result in reduced lymphocyte proliferation because of an inhibitory effect of the elevated sIL-7RA levels on IL-7-mediated signaling. An alternative interpretation is that the vast excess of sIL-7RA may serve as a sink or reservoir of IL-7, which could be released under conditions of, for example, lowered pH, which may occur in a local inflammatory environment. These scenarios are not mutually exclusive but may rather be relevant in different contexts such as homeostatic versus immune-activating conditions. Although our data do not exclude the latter hypothesis that increased sIL-7RA concentrations act immune-activating rather than compromising homeostatic turnover, the inhibition of STAT5 signaling and reduction of proliferation by sIL-7RA²¹ argue for the latter.

Autoimmunity involves breakdown of immune tolerance and among other factors an increased proliferation of autoreactive T lymphocytes. Thus, the increased sIL-7RA serum concentration and the reduced proliferation seem to contradict an increased risk of haplotype 1 carriers for MS. However, the immunomodulatory capacity of the excess of sIL-7RA might increase MS susceptibility in an indirect way. A recent study by Pellegrini *et al.*²⁵ demonstrated that IL-7 treatment reinvigorates the

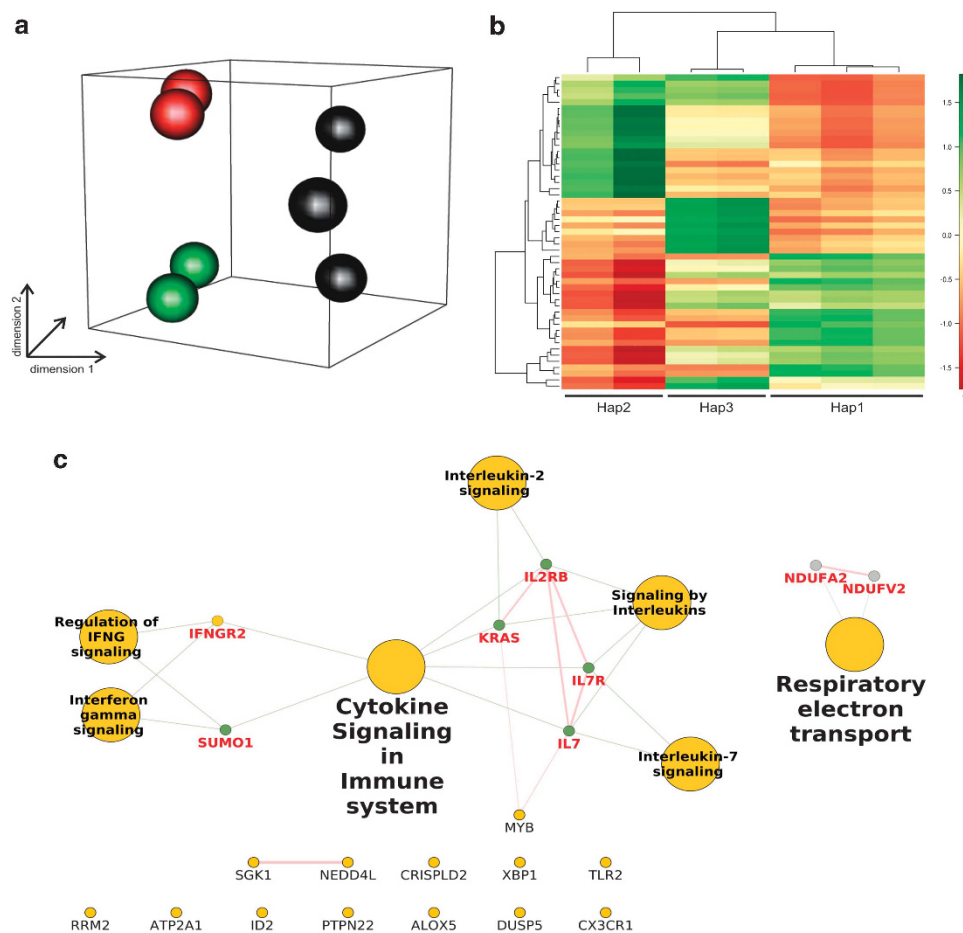


Figure 5. (a) Multi-dimensional scaling of individual response values (gene expression level after stimulation versus gene expression level in unstimulated cells). A subset (830 unique Ensembl gene IDs) corresponding to response values with low intra-group variability (lower third of median absolute difference per haplotype) was used. Color coding: haplotype 1, haplotype 2 and haplotype 3 are plotted as black, green and red spheres, respectively. (b) Heatmap of CD4⁺ T-cell responses to IL-7 stimulation for top-ranked metagenes. A subset of metagene-specific genes was selected from the top 10% contributing genes for each metagene and reconstructed values are shown (see Supplementary Material, Supplementary Table S3 for raw data). Columns represent individual samples and rows represent the selected genes. Ordering of both samples and genes was obtained from hierarchical clustering showing dendrograms on top and left side, respectively. Color coding uses row-wise scaling to emphasize between-haplotype differences. (c) Interaction network of top metagenes as obtained from Reactome and STRING. Genes based on Reactome association alone (red labels) and additional genes from input list found associated with STRING (yellow nodes). For clarity, only direct interactions between genes within the cytokine signaling in immune system network are shown.

immune response and improves protection against chronic viral infections. Furthermore, treatment with recombinant IL-7 promotes T-cell recovery after allogeneic stem cell transplantation³⁶ and also supports the elimination of John Cunningham (JC) polyoma virus in patients with progressive multifocal leukoencephalopathy, an opportunistic and often fatal infection of the brain in immunocompromised individuals (Sospedra *et al.*, unpublished). One possibility is that the lower bioavailability of IL-7 in carriers of the risk haplotype caused by sIL-7RA sequestration of IL-7 increases the susceptibility to viral infection. As viral infections are known to precede MS relapses and considering that Epstein–Barr virus is the most important environmental risk factor of MS,³⁷ the increased levels of sIL-7RA and lower IL-7 bioavailability may lead to an at least subtle compromise in controlling viral infections, which then indirectly might contribute to the onset of MS and/or occurrence of relapses/perpetuation once it has begun. This is supported by recent findings that the MS risk-conferring SNP rs6897932 is among three SNPs that confer poor outcome in human immunodeficiency virus infection.³⁸ Another line of supporting evidence is the reduced expression of IL-7RA on CD56^{bright} NK cells, and therefore possibly compromises in generating or maintaining

this subset of NK cells. CD56^{bright} NK cells are not only important for the control of latent/persistent viral infections³⁹ but also have immunoregulatory properties.⁴⁰ CD56^{bright} NK cells are reduced in numbers and compromised in function in MS patients,⁴¹ and treatment with the anti-CD25 monoclonal antibody daclizumab leads to significant expansion of CD56^{bright} NK cells, resulting in a decrease of inflammatory disease activity and of MS relapses.^{40,42}

When considering the influence of single quantitative trait loci, such as the MS risk-associated *IL7RA* SNP on a complex disease like MS, they appear to contribute only in a very minor way to MS risk, and their disease association could only be detected by comparing large numbers of patients and controls.⁵ One therefore expects that such quantitative trait loci act in concert with others and influence functional pathways that translate into increased pro-inflammatory cytokine secretion, T-cell activation and other immune mechanisms that functionally contribute to a disease like MS. As it is very difficult to dissect such interactions in genetically highly heterogeneous individuals, it is not surprising that with few exceptions⁴³ very little is known about the functional role of MS risk alleles. Also, one would not expect strong signals, for example, with respect to an important aspect of

immune function, in the context of common variants as this most likely would result in a readily observable phenotype. From a systems perspective, however, it is conceivable that even small differences in amount of sIL-7RA protein, bioavailability of IL-7 and/or transcriptional activity defined by *IL7RA* haplotypes shape the individual immune response and eventually sum up to discernible amounts. At the typical age of onset around 30 years, MS patients have undergone numerous immunological challenges. It is therefore highly interesting to note that Lundström *et al.*⁴⁴ found differences in IL-7 levels between rs6897932 genotypes in MS patients. Considering these aspects, our data on the complex pattern of differential response in CD4⁺ T cells in *IL7RA* haplotype homozygous individuals are not too unexpected and suggest that MS individuals differ in their response to immunological stimuli. The application of conservative statistical significance thresholds will in these instances tend to underestimate biologically relevant information.

As presented above, among the top induced changes in gene expression following stimulation of CD4⁺ cells with IL-7, there were no major haplotype-specific differences. In brief, the signal given by IL-7/IL-7R affects regulation of apoptosis, cell cycle progression and differentiation (upregulation of *CISH*, *BCL2*, *CDK6*, *CTPS*, *IRF4* and *ENO1*, and downregulation of *IL7R*, *FAIM3*, *GIMAP2* and *TMEM71*). In addition, substantial remodeling of cell–cell and cell–substrate interactions, adhesive properties and cellular skeleton is triggered (*CEACAM1*, *SORL1*, *MPP7*, *ARHGAP9*, *ST6GALNAC2*). Interestingly, genes involved in lipid uptake and metabolism also show considerable expression level changes (*PTGER2*, *DPEP2*). Of note, *PPAP2A* (phosphatidic acid phosphatase type 2A) exhibits high phosphatase activity toward FTY720 phosphate, the active form of the immunomodulating agent FTY720 (Fingolimod), which is an approved drug for the treatment of MS.⁴⁵ Among the many affected genes, the IL-7 response of a limited number of these differs between haplotypes and can be used to derive transcriptional signatures ('metagenes'), indicating variable processing of cytokine signals. Interestingly, genes in the first-line response to pathogens are top metagene-defining features. These include among others *IFNGR2*, the receptor for probably the most important pro-inflammatory cytokine in several autoimmune diseases including MS, *TLR2*, which has a fundamental role in pathogen recognition including also viruses like Epstein–Barr virus, *CX3CR1*, a chemokine acting as a coreceptor for human immunodeficiency virus-1 and *ALOX5*, a lipxygenase gene family member, for which overexpression in MS, both in relapse and remission, was reported recently. The transcription factor *XBP1*, which regulates major histocompatibility complex class II genes, acts as viral transactivator and also responds to endoplasmic reticulum stress, was placed among the top metagenes. The ability to respond to environmental stimuli is also defined by metabolic fitness and sensitivity to apoptosis of immune cells. Reflecting this, *NDUFA2* and *NDUFV2*, which participate in mitochondrial electron transport chain assembly, came up in our network analysis. Interestingly, it has been proposed that mitochondrial proteins, and especially haplotypic variants, may also influence progression of viral diseases like acquired immunodeficiency syndrome.⁴⁶ Of interest for a neurological disease like MS, compromise of mitochondrial function and metabolic compromise of neurons have been implicated in neurodegenerative processes.⁴⁷ Although speculative at this point, the expression of IL-7RA in the central nervous system^{48,49} may indicate that it exerts an influence not only in the immune system but also in the brain.

On the basis of our findings, we hypothesize that the *IL7RA* risk haplotype increases the susceptibility for viral infection, thereby enhancing the risk for MS. However, MS is a disease with a complex genetic trait in which the *IL7RA* is only one piece in the extending puzzle of MS genetics. It is most likely that the *IL7RA* acts in concert with other genes to trigger MS. Here we show that

by combining genetic information, specific cytokine stimulus, gene expression and network analysis, one can gain insight into the complex interactions of sets of haplotype-specific metagenes and involved signaling pathways, which together suggest an influence of *IL7RA* haplotypes not only on immune mechanisms but possibly also on metabolic function and an involvement in neurodegenerative aspects of MS. This approach is promising in addressing the functional implications of individual polymorphisms with small effects found in many genome-wide association studies and expression quantitative trait loci studies on autoimmune diseases.

MATERIALS AND METHODS

Subject and sample collection

Patient material was collected from 484 MS patients (29.8% men, 70.2% women, with a mean age of 45 years and a mean expanded disability status scale (EDSS) of 3.3) and 310 HDs (50% men, 50% women, with a mean age of 37 years) from the Hamburg cohort available at the University Medical Center Hamburg-Eppendorf. Informed consent was obtained from all MS patients and HDs. All patients had a clinically defined MS according to McDonald criteria.⁵⁰ Peripheral blood was collected in EDTA tubes (Sarstedt, Nümbrecht, Germany) and serum was collected in serum monovettes (Sarstedt).

Cell isolation

PBMCs were isolated using Ficoll (PAA, Pasching, Austria) gradient centrifugation, washed twice, resuspended in Roswell Park Memorial Institute 1640 supplemented with 10% dimethyl sulfoxide and 20% fetal calf serum and cryopreserved. CD4⁺ T cells were enriched from freshly isolated PBMCs by using the BD enrichment set DM (BD Biosciences, Franklin Lakes, NJ, USA) following manufacturer's protocol achieving a purity of at least 95%, verified by flow cytometry.

Genotyping

Genomic DNA was isolated from whole blood. Cells were lysed (1.6 M sucrose, 5% v/v Triton X-100, 25 mM MgCl₂ and 60 mM Tris-HCl, pH 7.5), centrifuged (1950 g), the pellet was resolved in H₂O supplemented with proteinase K and 20% SDS, incubated over night at 37 °C, centrifuged (3000 g) and precipitated with NaCl (6 M). After centrifugation (3000 g), the DNA was washed with 100 and 70% ethanol. Four SNPs in the *IL7RA* gene (rs987106, rs987107, rs1494555 and rs6897932) were analyzed using predesigned TaqMan probes (Applied Biosystems, Foster City, CA, USA). The reaction was performed in an Applied Biosystems 7900 Real-Time PCR system.

Association analysis

Genotype information was analyzed with Haploview 4.2 (ref. 51) and PLINK v1.07 (Shaun Purcell; <http://pngu.mgh.harvard.edu/~purcell/plink/>). None of the obtained genotype distributions did show significant deviation from the Hardy–Weinberg equilibrium.

Gene expression analysis of different IL-7RA isoforms

RNA was isolated from PBMC pellets using RNeasy kit (Qiagen, Hilden, Germany). cDNA synthesis was performed with Fermentas RevertAid H Minus first strand cDNA synthesis kit (Fermentas, St Leon-Rot, Germany). Three predesigned TaqMan (Applied Biosystems) probes (HS_00902334_m1, HS_00904815_m1 and HS_00902337_m1) were used to monitor membrane-bound (H20), the soluble (H6) IL-7RA isoform and the total amount of IL-7RA-encoding mRNA. The quantitative PCR reaction was performed in duplicates in an Applied Biosystems 7900 Real-Time PCR system.

Quantification of sIL-7RA

IL-7RA serum concentrations were determined using sandwich enzyme-linked immunosorbent assay with capture antibody (MAB306), detection antibody (BAF306), horseradish peroxidase-conjugated streptavidin (DY998) and substrate (DY997), all from R&D. Serum samples were diluted to 1/20 using phosphate-buffered saline supplemented with 10% fetal calf serum. Microtiter plates were analyzed using a Perkin-Elmer (Waltham, MA, USA) multilabel plate reader.

Cell surface expression of IL-7RA on immune cell populations

Quantification of IL-7RA surface expression was performed with frozen PBMCs using flow cytometry (LSRII, BD Biosciences). In all stainings duplicates were eliminated. CD4⁺ and CD8⁺ T-cells subtypes were identified by labeling the cells with antibodies specific for CD4 (Caltag Laboratories, Burlingame, CA, USA), CD8 (BioLegend, San Diego, CA, USA), CD27 (BD Biosciences), CD45RO (BioLegend) and CD127 (Beckman and Coulter, Indianapolis, IN, USA). IL-7RA expression on dendritic cells was analyzed by eliminating CD14⁺ (BD Biosciences) and CD19 (BD Biosciences)-positive cells and gating on CD1c⁺, CD141⁺ or CD303 (all from Miltenyi Biotec, Bergisch Gladbach, Germany)-positive cells. Natural killer (NK) cells were subdivided into CD56^{dim} and CD56^{bright} by using antibodies specific for CD3 (eBioscience, San Diego, CA, USA), CD16 (BioLegend), CD56 (eBioscience) and CD127 (BioLegend). Tregs were defined as CD3⁺, CD4⁺ and FoxP3⁺ and CD25⁺ high-expressing cells. The following antibodies were used: CD3 (BD Biosciences), CD4 and CD127 (BioLegend), CD25 (eBioscience) and intracellular FoxP3 and FoxP3 staining buffer set (Miltenyi Biotec). The stainings were performed according to manufacturers' protocols.

Cloning and stable expression of sIL-7RA

Human cDNA encoding the sIL-7RA (H6; AK301220) was amplified in two steps. In the first step, the regions encoded by exon 1–5 (forward primer: 5'-AATGACAATTCTAGGTACAACCTTTGGCATGGTTTTTC-3'; reverse primer: 5'-CGATAGGCTTAATCCTGAGCTATTATTG-3') and 7–8 (forward primer: 5'-CAATA ATAGCTCAGGATTAAGCCTATCG-3'; reverse primer: 5'-CTCACTTT TCTTGGT TTCTTACAAAGATGTTTC-3') were amplified and in a second step, the two constructs were combined and modified with *EcoRI*- and *XhoI*-binding sites (forward primer: 5'-GTAGAATTCGCCTCCATGACAATTCAGGT ACAACTTTGGCATGGTTTT-3', reverse primer: 5'-GACTCGAGCTCACTTTCT TGGTTTCTTAC-3'). The construct was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and transformed into *Escherichia coli* strain Top10F⁺.

Stable transfection of HEK293 cells was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. In short, HEK cells with a density of ~80% were transfected with 3 µg linearized DNA and grown under selection conditions in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum and 0.8 mg ml⁻¹ G418 (PAA). The efficiency of the transfected HEK cells to secrete IL-7RA was determined using enzyme-linked immunosorbent assay (see above).

STAT5 phosphorylation

The phosphorylation state of STAT5 was measured by flow cytometry. Freshly isolated CD4⁺ T cells were pre-incubated for 2 h at 37 °C in Roswell Park Memorial Institute 1640 Glutamax (PAA) supplemented with 10% fetal calf serum and then stimulated with the as well pre-incubated stimulus for 15 min. The following components were used for stimulation: IL-7 (Sigma-Aldrich, St Louis, MO, USA), concentrated HEK cell supernatant and concentrated supernatant of a HEK cell line producing sIL-7RA. Directly after stimulation, the cells were fixed and permeabilized using Cytofix Buffer and 1 × Perm Buffer III (both from BD Biosciences). Phospho-STAT5 (anti-STAT5-P (Y694), BD Biosciences) was measured by flow cytometry.

Transcriptome analysis

Transcriptomes from CD4⁺ T cells from patients with haplotype 1 (*n* = 3), haplotype 2 (*n* = 2) and haplotype 3 (*n* = 2) were recorded using Affymetrix human gene array chips (GeneChip Human Gene 1.0 ST Array, Affymetrix, Santa Clara, CA, USA) hybridized at Transkriptomanalyselabor, University of Göttingen, Germany (www.uni-bc.gwdg.de). In brief, RNA was isolated using the TRIzol Reagent (Invitrogen) and checked for quantity, purity and integrity using the Agilent 2100 bioanalyzer (Agilent Technologies) (Agilent Technologies, Santa Clara, CA, USA). Microarray hybridization and data extraction was done according to the manufacturer's specifications.

Raw microarray data were background corrected and normalized by applying robust multi-chip average and quantile normalization. Processed probe expression values were condensed into Affymetrix transcript clusters and filtered for genes containing Entrez gene identifier. Differential expression was calculated by fitting a linear model with moderated *t*-statistics to groups defined by haplotype, treatment and combinations of haplotype and treatment. All calculations were done in R/Bioconductor.⁵² For initial exploratory data analysis, expression levels of unstimulated cells were inspected for genes (only autosomal location) that showed small

median absolute deviation (lower third calculated per haplotype group) with a reasonable minimal expression (threshold set to 4.0). The resulting set corresponding to 843 Ensembl gene IDs was subjected to nonmetric multi-dimensional scaling (Sammon's nonlinear mapping) and plotted. Haplotype-specific effects were inspected in a similar way. For detailed analysis of haplotype-associated signatures, we used genes showing robust response to IL-7 treatment in at least one of the haplotype groups (Benjamini–Hochberg-adjusted *P*-value ≤ 0.05), which at the same time differed between haplotypes (F-test, *P*-value ≤ 0.05). Data with high intra-group variation taken as median absolute deviation ≥ 0.5 were removed. NMF on the resulting 207 differentially responding genes with autosomal location as input (completed with data for *IL7* and *IL7RA*) was used to extract subsets of haplotype-specific genes.⁵³ In NMF, the gene expression levels are treated as matrix *X* consisting of *N* genes in *M* samples that is factorized according to *X* = *W* × *H* into a metagene matrix *W* and a metagene expression profile (meta experiment) matrix *H*. Columns of *W* hold the contribution of genes to a metagene and columns of *H* describe the metagene composition corresponding to each sample. The optimal rank *k* (number of extracted basis components) was inferred from plotting the cophenetic correlation coefficient versus rank and was set to three. Initial tests showed that rank *k* = 3 gave optimal clustering and that nonsmooth NMF performed best (Supplementary Material, Supplementary Figure S4). The consensus matrix (average connectivity between metagenes and samples) obtained from using a NMF algorithm with random initialization after 1000 runs showed stable clustering of samples.⁵⁴ The interaction network of top metagenes was assembled in Cytoscape using Reactome and STRING databases. STRING-based associations of further genes (first neighbors only) were considered with combined score ≥ 0.7 (high confidence).

Data are available through Gene Expression Omnibus (GSE37584).

CONFLICT OF INTEREST

R. Martin is coinventor on a patent for the combined use of recombinant IL-7 and vaccination with JC polyoma virus capsid protein VP1 to treat progressive multifocal leukoencephalopathy. The remaining authors declare no conflict of interest.

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