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The IL-7Rα Pathway Is Quantitatively and Functionally Altered in CD8 T Cells in Multiple Sclerosis

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The IL-7Rα single nucleotide polymorphism rs6897932 is associated with an increased risk for multiple sclerosis (MS). IL-7Rα is a promising candidate to be involved in autoimmunity, because it regulates T cell homeostasis, proliferation, and antiapoptotic signaling. However, the exact underlying mechanisms in the pathogenesis of MS are poorly understood. We investigated whether CD4 and CD8 lymphocyte subsets differed in IL-7Rα expression and functionality in 78 MS patients compared with 59 healthy controls (HC). A significantly higher frequency of IL-7Rα+ CD8 effector memory (CD8EM) was found in MS. Moreover, IL-7Rα membrane expression was significantly increased in MS in naïve and memory CD8 (all p < 0.05) with a similar trend in CD8EM (p = 0.055). No correlation was found between the expression level or frequency of IL-7Rα+CD8+ and rs6897932 risk allele carriership. Upon IL-7 stimulation, MS patients had stronger STAT5 activation in CD8EM compared with HC. IL-7 stimulation had a differential effect on both mRNA and protein expression of granzyme A and granzyme B between MS and HC. Stainings of carriership. Upon IL-7 stimulation, MS patients had stronger STAT5 activation in CD8EM compared with HC. IL-7 stimulation in combination with the lower threshold for IL-7–induced cytotoxicity in MS may enhance the pathogenicity of these CD8 T cells. This is of special interest in light of the established demyelinating and cytotoxic actions of granzyme A. The Journal of Immunology, 2012, 188: 000–000.

Multiple sclerosis (MS) is a complex disease, presumed to be autoimmune mediated. The exact etiology is unknown, but a combination of genetic and environmental factors, including infections, is important in the development of the disease (1). Extensive research has focused on the role of CD4+ T cell subsets, notably Th1 and Th17 cells. However, recently increasing evidence for an important role of Ag-specific CD8+ cytotoxic T cells has emerged (2, 3).

For several decades, the only genetic association with an increased risk to develop MS was the class II HLA allele DRB1*15:01 (4). Recently, a protective effect of the HLA class I allele HLA A*02:01 has been shown and some additional HLA class I protective alleles have been suggested (5). Although many genetic studies have been performed, very few non-HLA genetic factors were found to be associated with MS. However, the International Multiple Sclerosis Genetics Consortium published the results of the first genome-wide association study in MS patients. The International Multiple Sclerosis Genetics Consortium validated the HLA class II association and in addition identified several single nucleotide polymorphisms (SNP) in genes associated with MS with modest odds ratios compared with the HLA class II association. Interestingly, most of the MS risk SNPs are involved in T cell homeostasis and differentiation (5, 6).

One of the most interesting risk SNP identified was rs6897932, located in IL-7Rα, also known as CD127 (5–8). CD127 is involved in homeostasis and longevity of T lymphocytes (9). Because autoreactive T cells are thought to play an important role in MS, a genetic variation within this important survival factor can be important for the disease (10). Moreover, early reports on the function of IL-7 implicated it to be involved in cytotoxicity (11), although the exact mechanisms (e.g., the specific cytotoxic pathway) have not been studied thoroughly.

IL-7Rα risk SNP rs6897932 is located in exon 6, encoding the transmembrane domain of the receptor. The SNP is presumed to cause a splice variant, which leads to increased levels of the soluble form of the receptor (8). Interestingly, this SNP is also significantly associated with type I diabetes mellitus (12) and rheumatoid arthritis (13), other autoimmune-mediated diseases where autoreactive lymphocytes play an important role. Additionally, these diseases have a considerable overlap in genetic associations with MS (14). Currently, no studies are available about putative cellular differences in the IL-7Rα axis and downstream signaling aberrations in MS.

The present study focused on the role of IL-7Rα expression on lymphocytes of MS patients. We show that the frequency of IL-7Rα+ CD8 effector memory (CD8EM) T cells is significantly higher in MS patients. Moreover, IL-7 stimulation resulted in an enhanced phosphorylation of STAT5 in CD8EM T cells, which in MS patients leads to dysregulated changes in the expression of the
cytotoxic molecules granzymes A and B, which have been implicated for their demyelinating effects. Our results cannot be explained by the genetic association solely and shed new light on the importance of the IL-7Rα pathway in MS patients.

Materials and Methods

Patients and controls

Fifty-one consecutive relapsing-remitting MS patients, diagnosed according to the McDonald criteria (15), and seven patients with clinically isolated syndrome, aged between 18 and 75 y and seen on regular basis in the Multiple Sclerosis Center ErasMS in Rotterdam, were included in the study. Exclusion criteria were an exacerbation and/or the use of methylprednisolone 3 mo prior to sampling. In the first phase, the use of immunomodulating therapy was an additional exclusion criterion. In the second phase of this study, 15 patients treated with IFN-β1a and one with glatiramer acetate were included. In phase three, also 10 primary progressive and 10 secondary progressive patients were included. Healthy controls (HC), aged 18–75 y old, were relatives of the MS patients who accompanied a patient to the outpatient clinic in our hospital. Exclusion criteria were 1) diagnosis of MS or 2) prior symptoms suggestive of CNS demyelination, such as optic neuritis and myelitis, and 3) use of any immunomodulating therapy for an autoimmune disease. This study was approved by the Medical Ethical Committee of the Erasmus Medical Center, and written informed consent was obtained from all patients and controls.

Flow cytometry for IL-7Rα expression on lymphocyte subsets

PBMCs were collected in CPT tubes and processed according to the manufacturer’s protocol (BD Biosciences). After collection, the PBMCs were cryopreserved and stored in liquid nitrogen. For flow cytometric analysis 1 × 10^6 lymphocytes were stained with CD3 FITC, CD4 PE-Cy7, CD8 allophycocyanin-Cy7, CD27 allophycocyanin, CD45RA PE-Cy5.5 (eBioscience), and CD127 eFluor 450 (eBioscience) for 20 min. In the validation experiments for Western blotting and quantitative PCR, cells was determined with titration experiments (see Supplemental Fig. 1). In further reactions were performed in duplicate.

Cell stimulation experiments

For flow cytometry assay 1 × 10^6 PBMCs and for Western blot 2 × 10^5 PBMCs were cultured in RPMI 1640 serum-free medium with or without 5 ng/ml IL-7 (PeproTech) or 50 U/ml IFN-γ (Boehringer Ingelheim) at 37°C with 5% CO2 for 15 min. For quantitative PCR, 1 × 10^6 T cells or PBMCs per well were cultured in RPMI 1640 serum-free medium with or without 5 ng/ml IL-7 (PeproTech) or 50 U/ml IFN-γ (Boehringer Ingelheim) at 37°C with 5% CO2 for 2 h and thereafter coincubated with IL-7 or IFN-γ.

Phosphorylated STAT1 and phosphorylated STAT5 expression by lymphocyte subsets upon IL-7 stimulation

Stimulated and unstimulated cells were fixed with Cytofix at 37°C for 10 min, permeabilized with Perm III buffer for 30 min on ice, and then stained with CD3 PerCP, CD4 Amcyan, CD8 FITC, CD27 allophycocyanin, CD45RA PE-Cy7, phosphorylated STAT (pSTAT)1 Pacific Blue, and pSTAT5 PE for 30 min at 4°C. Because IL-7 stimulation leads to phosphorylation of STAT1 and not STAT5, pSTAT1 was used as a negative control for IL-7 stimulation. Moreover, because IFN-γ stimulation leads to phosphorylation of STAT1, but not STAT5, IFN-γ was used as negative control for pSTAT5 to ascertain the specificity of the effects. The differences in percentage and mean fluorescence intensity (MFI) of pSTAT1 and pSTAT5 cells between the unstimulated and the 15 min stimulation condition were calculated and compared between MS patients and controls.

Intracellular expression of granzymes A and B upon IL-7 stimulation

Baseline expression and expression of granzymes A and B upon 22 h IL-7 stimulation was assessed. PBMCs were extracellularly stained for CD3 V500, CD8 PE-Cy7, CD27 allopocycocyanin-H7, CD45RA PerCP-Cy5.5 (eBioscience), and CD127 eFluor 450 (eBioscience) for 30 min at 4°C. Next, the cells were fixed and permeabilized with a FOXP3 staining buffer set (eBioscience) and intracellularly stained for granzyme A PE (R&D Systems) and granzyme B Alexa Fluor 647 (R&D Systems) for 1 h at 4°C. Unstained cells and isotype controls were used as negative controls.

All Abs and buffers for flow cytometric analyses were obtained from BD Biosciences unless stated otherwise. Flow cytometry was performed on an LSR II flow cytometer (BD Biosciences). Data analysis was performed with FACSDiva software version 6.1.2 (BD Biosciences).

Confirmation of pSTAT1 and pSTAT5 expression with Western blot

For analysis of STAT1 and STAT5 protein expression and phosphorylation of STAT1 and STAT5, PBMCs were lysed in 50 μl radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid) supplemented with phosphatase and protease inhibitors (Roche). Insoluble material was removed by centrifugation at 10,000 × g for 10 min at 4°C, and the soluble fraction was assayed for total protein. Briefly, proteins were separated on a 10% SDS polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Millipore). The membranes were incubated with Abs specific for STAT1, pSTAT1 (pY701), STAT5, and pSTAT5 (pY694) (BD Biosciences). Protein loading was analyzed by reprobing blots with an Ab against β-actin (Abcam). Detection of immunoreactive bands was performed using Western Lightning Plus-ECL substrate (PerkinElmer). Quantification was performed using ImageJ software version 1.44p (http://rsb.info.nih.gov/ij/).

IL-7 proliferation assay

PBMCs (1 × 10^5) were cultured in RPMI 1640 medium with 10% FCS and 10% penicillin/streptomycin at 37°C with 5% CO2 for 72 h in three conditions: 1) unstimulated, 2) with 5 ng/ml IL-7, and 3) with 10 ng/ml phytohemagglutinin (Sigma-Aldrich) as positive control. After 1 d, proliferation was determined by incorporation of 0.5 μCi/well of [3H]thymidine (GE Healthcare) for 18 h. All culture conditions were performed in triplicate.

Gene expression

Total RNA was extracted from PBMCs or sorted T cells using the GenElute Mammalian Total RNA kit (Sigma-Aldrich). RNA samples were treated with DNase I (Invitrogen) to remove any contaminating DNA. Using 1 μg of the total RNA as template, cDNA was prepared using SuperScript II (Invitrogen). Target gene mRNA expression was determined by real-time quantitative reverse transcription PCR using TaqMan technology on a 7000HT PCR machine (Applied Biosystems) with a final step of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Expression levels were calculated with the obtained Ct values compared with standard curves of the gene of interest. Target gene expression levels were corrected for 18S RNA levels. Sequences and probes were selected using the universal probe library Assay Design Center from Roche. Sequences of the PCR primers are shown in Supplemental Table I, and fluorogenic probes were obtained from Roche. All quantitative PCR reactions were performed in duplicate.

MACS

To assure that the results obtained from the quantitative PCR were T cell specific, T cells were negatively isolated with MACS beads (Miltenyi Biotec) according to the manufacturer’s protocol. Purity of the cells was routinely >95% as assessed by flow cytometry with staining for CD3 FITC, CD4 PE-Cy7, CD8 allopocycocyanin-Cy7, CD14 allopocycocyanin, CD19 PerCP-Cy5.5, and CD56 PE on positive and negative sorted fractions.

Genotyping

rs6897932 genotyping was performed as previously described with either a Sequenom platform (16) or TaqMan assay (17). Both MS patients (p = 0.98) and HC (p = 0.89) were in Hardy–Weinberg equilibrium.

Collection of autopsy tissue and immunohistochemistry

Human autopsy brain tissues from five MS patients and five nondemented controls (NDC) were obtained from The Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam. All material was collected from donors from whom a written informed consent for brain autopsy and the use of the material and clinical information for research purposes had been obtained by The Netherlands Brain Bank. The immunohistochemical methods used in this study were described in detail before (18). In brief, 6-μm frozen sections of human brain white matter were thaw-mounted on gelatin-coated slides and kept overnight at room temperature (RT) in humidified atmosphere. After air-drying for 1 h, slides were fixed at room temperature in fresh acetic containing 0.02%
(v/v) H2O2 for blocking endogenous activity. After washing in PBS, slides were incubated with optimally diluted primary Abs overnight at 4°C in humidified atmosphere. Incubations with HRP-labeled rabbit anti-mouse Ig (Dako) or in case of a three-step staining with secondary biotin-labeled rabbit anti-mouse Ig (Dako) and tertiary HRP-labeled avidin/biotin complex (Dako) were performed for 1 h at RT. HRP activity was revealed by incubation with 3-amin-9-ethylcarbazole (Sigma-Aldrich) for 10 min at RT, leading to a bright red precipitate. Nuclei were counterstained by hematoxylin (Merck). Slides were embedded with glycerol gelatin. Reactive human tonsil sections were included in each staining procedure as positive control tissue. Incubation with irrelevant isotype control Abs and omission of primary Ab were used as negative control stainings.

For detection of IL-7, tyramide signal amplification with HRP-streptavidin and biotin-XX tyramide (Molecular Probes) was used according to the manufacturer’s instructions.

The Abs used in this study were directed against IL-7 (BioSource International), IL-7Rα (R&D Systems), CD4, CD8, CD68, and HLA-avidin/biotin complex Ag (all from Dako). Isotype controls used were mouse IgG1 and mouse IgG2a (both from R&D Systems).

Double staining of IL-7Rα with CD8 or CD68 was performed as described before (19). For double staining of IL-7Rα with CD8, IL-7Rα was first stained. To detect infiltrating monocytes, macrophages, and microglia cells, acid phosphatase, a lysosomal enzyme, is a useful marker. Activated cells can be detected with an Ab against HLA-ABC (Sigma-Aldrich) and Fast Blue BB base (Sigma-Aldrich) at 37°C for 30 min, resulting in a blue precipitate.

For double staining of IL-7Rα and CD68, IL-7Rα was detected in blue and therefore AP-labeled goat anti-mouse-IgG (Dako) and AP-labeled rabbit anti-goat IgG (SouthernBiotech) were used. After blocking with 10% normal mouse serum for 15 min, CD68 Ab was then incubated for 1 h at RT, followed by incubation with alkaline phosphatase (AP)-labeled goat anti-mouse IgG1 at RT for 1 h. AP activity was revealed with naphthol-AS-MX phosphate (Sigma-Aldrich) and Fast Blue BB base (Sigma-Aldrich) at 37°C for 30 min, resulting in a blue precipitate.

As observed by others, both CD4+ and CD8+ memory T cell subsets were increased in frequency (p = 0.01 and p = 0.02, respectively) in MS patients compared with HC (data not shown).

**IL-7 stimulation leads to a higher increase of pSTAT5 in CD8 T cells of MS patients**

Next, we questioned whether the significantly increased CD127 expression would lead to an increased phosphorylation of STAT5, the downstream target of CD127. At baseline, no differences in unphosphorylated STAT5 or pSTAT5 were observed between age-matched MS and HC (Fig. 2A). After stimulation of PBMCs from MS patients and HC with 5 ng/ml IL-7 for 15 min, the increase of pSTAT5 compared with the unstimulated cells was measured using seven-color flow cytometry with the illustrated gating strategy (Fig. 1A, 2B). In MS patients, a significantly higher percentage of pSTAT5+ CD4+ memory T cells compared with those of HC was found upon IL-7 stimulation. Furthermore, a higher increase in pSTAT5 in total CD8+ T cells (p = 0.05) and CD8EM T cells of MS patients was observed (p = 0.02). A similar trend was found for CD8 memory T cells, although this did not reach statistical significance (Fig. 2C). These findings were confirmed by Western blotting, with one representative blot being shown as well as quantification for 12 HC and 12 MS patients (Fig. 2D). As expected, IFN-γ stimulation, which phosphorylates STAT1 and not STAT5, did not increase pSTAT5 levels. Additionally, no upregulation of pSTAT1 was observed upon IL-7 stimulation (data not shown). Interestingly, in MS patients a significant correlation was found between the MFI of CD127 and the increased MFI of pSTAT5 upon IL-7 stimulation. In contrast, no correlation between these two parameters was found in HC (Fig. 2E).

**No differences in IL-7–induced proliferation**

In view of the role of IL-7 in homeostasis, proliferation, and antiapoptotic signaling in T cells and its increased receptor expression on most CD8 T cell subsets in MS patients, we next questioned whether this increased expression influenced the proliferative response in MS patients. No significant difference in IL-7–induced proliferation in PBMCs was found between 25 MS patients and 24 HC (Supplemental Fig. 2).

**Differential upregulation of cytotoxicity-related genes upon IL-7 stimulation in MS patients**

One of the earliest reports on the function of IL-7 implicated it as an important factor for cytotoxicity, although the exact mechanism of action was not investigated. Because most differences in the IL-
7Rα pathway in MS patients were observed in CD8+ T cells, we investigated the effect of IL-7 stimulation in PBMCs on genes involved in cytotoxicity. LAMP1 (CD107a), a marker for degranulation, and FASL (CD178) were not affected by IL-7 stimulation, whereas perforin 1 was comparably upregulated in MS and HC. Granzyme B was also upregulated in both MS and HC upon IL-7 stimulation. Interestingly, granzyme A was only upregulated in MS patients and not in HC. Finally, we tested TNF-α and observed a significant increase in TNF-α mRNA in HC and the same trend in MS, although not significant (Fig. 3A).

The observed mRNA upregulation of the cytotoxic molecules was modest. To further validate and more specifically study cytotoxic gene expression and regulation, we used a secondary cohort of patients and controls. In this validation study, we used a Jak inhibitor to block the downstream effects of IL-7 on STAT5 phosphorylation. All changes in gene expression were validated in this independent group of patients and controls. Moreover, the combination of the Jak inhibitor with IL-7 stimulation resulted in a partial downregulation of genes induced by IL-7 stimulation (Fig. 3B). As expected, the Jak inhibitor also led to a partial inhibition of phosphorylation of STAT5 (Fig. 3C). To further ascertain that IL-7 induces the expression of granzymes A and B, we stimulated PBMCs with IL-7 for 22 h and determined protein expression of granzymes A and B and compared this with baseline expression in functional CD8 T cell subsets coexpressing IL-7Rα. For granzyme A in MS, a 4- to 5-fold increase in protein expression was found in naive CD8 T cells, whereas in CD8EMRA T cells a slight increase was observed. A significant, although very slight decrease in granzyme A expression in MS patients was observed in CD8EM T cells (Fig. 3D), which might be a result of activation and subsequent degranulation, a phenomenon that has previously been shown for granzyme B (23). In HC, only in naive and CD8EMRA cells, a significant increase in granzyme A was observed. Granzyme B increased in MS patients in all subsets analyzed, being most pronounced in the naive CD8 T cells. In HC, the increase in granzyme B was restricted to the naive and memory CD8 subsets (Fig. 3E).

Next, we reasoned that lymphocyte trafficking, including CD8+ EM T cells, into the CNS (24) is mediated by very late Ag 4 (VLA-4). This trafficking is a major process in MS (25). A recent study showed that IL-4–mediated STAT6 phosphorylation inhibited VLA-4 expression (26). Because STAT6 is negatively dysregulated in CD8 T cells in MS.

Table I. Clinical characteristics of patients included in IL-7Rα expression and function study in PBMC

<table>
<thead>
<tr>
<th></th>
<th>HC (n = 59)</th>
<th>CIS (n = 7)</th>
<th>RRMS (n = 51)</th>
<th>SPMS (n = 10)</th>
<th>PPMS (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at onset (SD)</td>
<td>NA</td>
<td>36 (12)</td>
<td>31 (11)</td>
<td>29 (8)</td>
<td>41 (9)</td>
</tr>
<tr>
<td>Age at sampling (SD)</td>
<td>39 (13)</td>
<td>39 (11)</td>
<td>41 (10)</td>
<td>51 (9)</td>
<td>53 (11)</td>
</tr>
<tr>
<td>Female, %</td>
<td>64</td>
<td>100</td>
<td>78</td>
<td>90</td>
<td>70</td>
</tr>
<tr>
<td>Disease duration, y</td>
<td>NA</td>
<td>NA</td>
<td>8.8 (5.1)</td>
<td>21.7 (10)</td>
<td>12.4 (8.1)</td>
</tr>
<tr>
<td>Presenting symptoms</td>
<td>NA</td>
<td>NA</td>
<td>17.1 (5.1)</td>
<td>21.7 (10)</td>
<td>12.4 (8.1)</td>
</tr>
<tr>
<td>Optic nerve</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Brainstem/cerebellum</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Multifocal</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

None of the samples of the CIS patients was taken during the acute phase of the CIS attack. Four of the seven CIS patients eventually developed relapsing-remitting MS; the remaining CIS patients all fulfilled the criteria for dissemination in space, but not dissemination in time. The remaining CIS patients were followed for at least 24 mo (range, 24-59 mo). For relapsing-remitting MS, 15 patients were treated with immunomodulating therapy (14 with IFN-β1a, 1 with glatiramer acetate). For secondary progressive MS, one patient was treated with IFN-β1a.

NA. Not applicable; PPMS, primary progressive MS; RRMS, relapsing-remitting MS; SPMS, secondary progressive MS.
regulated by STAT5 via suppressor of cytokine signaling 1, and we observed a significant increased pSTAT5 upon IL-7 stimulation, we hypothesized that IL-7 might induce VLA-4 expression. Indeed, upon 24 h IL-7 stimulation, both MS patients and HC upregulated expression of VLA-4 gene (CD49D subunit) as shown in Fig. 3B.

To demonstrate that the observed effects were T cell specific, we sorted CD3+ T cells, which were subsequently stimulated according to the same protocol. A similar upregulation of genes involved in cytotoxicity was observed (data not shown). Because IL-7 can induce the production of IFN-γ, we finally investigated whether the observed effects could be IFN-γ mediated instead of IL-7. PBMCs and sorted CD3+ T cells were stimulated with IFN-γ, which did not lead to changes in the expression of cytotoxic genes (data not shown), confirming that the effects were indeed IL-7 mediated.

No association between IL-7Ra risk SNP rs6897932 and increased expression of IL-7Ra

MS patients have a higher frequency of the risk allele of the SNP rs6897932. In this study we observed both a higher percentage of CD127+ CD8EM T cells and CD8EM T cells from MS patients and HC. *p < 0.05, **p < 0.01, ***p < 0.001.
and homozygous non-risk carriership (HC, \( n = 5 \); MS, \( n = 5 \)) for both the frequency of CD127\(^+\) CD8\(^{EM} \) (Fig. 4A) and expression of CD127\(^+\) on CD8\(^{+} \) T cell subsets (Fig. 4B). CD127 expression on the remaining CD8\(^{+} \) and CD4\(^{+} \) subsets stratified to rs6897932 can be found in Supplemental Fig. 3. Pooled analysis of both MS patients and HC showed no significant differences between the different genotypes, indicating that a power problem was highly unlikely to have occurred (data not shown).

**CD8\(^{+}\)IL-7R\(_{a}\)\(^{+}\) T cells are mainly localized in preactive MS lesions**

Using immunohistochemistry, we addressed the question of whether CD8\(^{+}\) T cells coexpressing IL-7R\(_{a}\) are present in preactive and active lesions in the brain of MS patients (for clinical details of the MS patients and NDC, see Table II). Interestingly, only in preactive lesions in the perivascular space could CD8\(^{+}\)IL-7R\(_{a}\)\(^{+}\) T cells be detected. In active lesions, only CD8\(^{+}\) T cells lacking IL-7R\(_{a}\) were found in white matter tissue of five MS patients. Expression of IL-7 could only be detected in preactive MS lesions and not in active demyelinating lesions. As expected, no CD8\(^{+}\) T cells or IL-7 expression could be detected in normal appearing white matter. Interestingly, also a number of microglia cells (based on morphology and CD68 expression; data not shown) coexpressed IL-7R\(_{a}\) and these microglia cells were observed in both preactive and active lesions. HLA class I expression was predominantly found in preactive lesions with the highest

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**FIGURE 3.** Differential changes in IL-7–induced gene expression in MS patients and HC. Gene expression is shown of an activation marker and molecules involved in cytotoxicity upon 24 h 5 ng/ml IL-7 stimulation in PBMCs. A. Fold changes of molecules involved in the three major cytotoxic pathways. The number of MS patients ranged from 8 to 14 and of HC from 7 to 14, dependent on the gene studied. Note that comparisons in A and B are within the groups compared with baseline. B. Genes with a modest significant increase upon IL-7 stimulation were tested in an independent group of 8–10 patients and 12–15 HC for upregulation upon IL-7 stimulation. Additionally, changes in VLA-4 expression upon IL-7 stimulation were tested. Moreover, to show that the effect was IL-7 pSTAT5-dependent, a JAK inhibitor (25 µM AG-490 with preincubation of 2 h before IL-7 was added) was used to inhibit the pSTAT5 signal. C. Representative Western blot for (phosphorylated) STAT5 upon IL-7 stimulation with simultaneous addition of a JAK inhibitor. Quantification was performed with densitometry. IFN-\( \gamma \) served as a negative control for the upregulation of pSTAT5. D and E. Protein expression of granzyme A and granzyme B, respectively, was assessed before and after IL-7 stimulation by intracellular FACS in 10 HC and 10 MS patients and compared with a Wilcoxon matched-pairs signed-rank test. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).
intensity compared with active lesions. In active demyelinating lesions and normal appearing white matter, only the blood vessel endothelium and some microglia cells expressed HLA class I (Fig. 5). In brain materials from five NDC, no CD8 T cells were found. IL-7 was detected only in one NDC brain sample. As expected, in NDC, HLA class I could only be detected on endothelial cells of blood vessels (data not shown).

Discussion

Classically, MS has been presumed to be a CD4-mediated disease, although the role of the different CD4 effector T cells (e.g., Th1 and Th17 cells) is currently heavily debated (27). In contrast, the number of articles about the role of CD8+ T cells in MS is relatively limited, although most studies report that CD8+ T cells outnumber CD4+ T cells in MS lesions (28). Of note is the

Table II. Clinical characteristics of patients included in postmortem IL-7/IL-7Rα expression in white matter study

<table>
<thead>
<tr>
<th>Age at Death, y</th>
<th>Gender</th>
<th>Age at Onset</th>
<th>Presenting Symptom</th>
<th>MS Disease Form</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDC1 68</td>
<td>Female</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Metastasized mamma carcinoma</td>
</tr>
<tr>
<td>NDC2 76</td>
<td>Female</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Ischemic cerebrovascular accident</td>
</tr>
<tr>
<td>NDC3 78</td>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td>Pulmonary emphysema and cardiac insufficiency</td>
</tr>
<tr>
<td>NDC4 91</td>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>NDC5 73</td>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td>Colon carcinoma with liver metastases, without brain metastases</td>
</tr>
<tr>
<td>NDC6 73</td>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td>Lung emboli</td>
</tr>
<tr>
<td>MS1 40</td>
<td>Female</td>
<td>26</td>
<td>Optic neuritis</td>
<td>SP</td>
<td>Dehydration</td>
</tr>
<tr>
<td>MS2 59</td>
<td>Female</td>
<td>38</td>
<td>Optic neuritis</td>
<td>SP</td>
<td>Respiratory insufficiency</td>
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<tr>
<td>MS3 71</td>
<td>Female</td>
<td>43</td>
<td>Optic neuritis</td>
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<td>Pneumonia</td>
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<tr>
<td>MS4 46</td>
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<td>23</td>
<td>Optic neuritis</td>
<td>SP</td>
<td>Carcinoma of bladder and intestine without brain metastases</td>
</tr>
<tr>
<td>MS5 72</td>
<td>Male</td>
<td>50</td>
<td>Optic neuritis</td>
<td>SP</td>
<td>Carcinoma of bladder and intestine without brain metastases</td>
</tr>
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NA, not applicable; SP, secondary progressive.
FIGURE 5. CD8 T cells in preactive white matter lesions express IL-7Rα. Double staining to detect CD8 T cells coexpressing IL-7Rα and single staining for IL-7 and HLA class I expression were performed in preactive and active demyelinating lesions and in normal-appearing white matter. Figures show representative stainings from five patients. In columns, the different lesion types are shown. Original magnification ×400. Stains: upper row, blue CD8, red IL-Rα; middle row, red IL-7; lower row, red HLA-I. Nuclei were counterstained with hematoxylin.

presence of clonally expanded CD8 T cells in postmortem tissue of active MS lesions (29) and the demonstration of oligoclonally expanded CD8 T cells in the CSF of MS patients (30).

The homeostasis of CD8 T cells is maintained by signals from IL-7Rα, among others (10). Moreover, a functional role of IL-7Rα in MS pathogenesis is implied by the fact that a genetic variant enhances the risk for MS (5–8) and this makes it an interesting candidate for further study.

Therefore, we addressed the question of whether IL-7Rα expression and functioning in both CD4+ and CD8+ T cell subsets is altered in MS patients. We demonstrate that the frequency of CD127+CD8EM and the expression levels of CD127 on most CD8+ subsets are increased in MS patients. Upon IL-7 stimulation, a differential effect on the expression of granzymes A and B in MS patients was found and these effects may further enhance the cytotoxicity of these CD8EM T cells, which are candidates to function as Ag-specific CD8+ T cells, because they have an EM phenotype (31).

Recently, there is accumulating attention for a regulatory subset of CD8 T cells that coexpress CD25 and FOXP3. The question occurs whether differences observed in this study are caused by differences in frequency of this specific subset. Note that no numerical differences in CD8 regulatory T cells between MS patients and HC have been described (32), nor have functional defects in this subset been shown (33). Although we previously found a significant increased frequency of CD8 regulatory T cells coexpressing IL-7Rα in MS patients (34), this increase was only ~5% of the total increase in IL-7Rα expression on CD8 T cells observed in this study. Therefore, an increase of IL-7Rα+CD8 regulatory T cells cannot explain the abundance in IL-7Rα expression on CD8EM T cells observed in this study.

In the periphery, IL-7 is expressed on stromal cells in the bone marrow and in lymphoid tissues (35). It is thought that autoreactive T cells in MS patients are activated in secondary lymphoid organs and then migrate across the blood–brain barrier into the brain (36). This potential transmigration is underlined by our observation that IL-7 induces VLA-4, an important marker for T cell activation and transmigration into the brain by binding to VCAM-1 on activated brain endothelial cells and epithelial cells of the choroid plexus (37). Natalizumab (Tysabri), a blocking monoclonal blocking Ab against VLA-4 is an approved treatment for MS (38). Moreover, CD8EM T cells have the capacity to migrate to nonlymphoid tissues (39). In the case of MS, they potentially migrate into the CNS, where they re-encounter IL-7 (Fig. 5), which is produced by reactive astrocytes in MS lesions (40). This intrathecal production IL-7 might further activate and increase the cytotoxic potential of these T cells by the upregulation of genes involved in cytotoxicity, as we have shown in this study in vitro (Fig. 3A, 3B). Moreover, it has been shown that both granzymes A and B are upregulated during a relapse compared with patients in remission and controls (41), further emphasizing the importance of these molecules in the MS disease course.

Granzyme A is of particular interest, because in contrast to granzyme B, it is capable of causing direct myelin damage via degradation of myelin basic protein (42). Moreover, oligodendrocytes are sensitive to granzyme/perforin-mediated killing, whereas other neural cells are less sensitive to granzyme-mediated effects (43). Our finding that granzyme A is not upregulated in HC is in accordance with a previous report (44). It is particularly interesting that this pathway is dysregulated in MS patients.

Importantly, the observed differences cannot be attributed to the genetic association in IL-7Rα. No correlation with the rs6897932 SNP and expression of IL-7Rα was detected. Other factors must contribute to the increased expression of IL-7Rα and dysregulation of this pathway in MS. It is conceivable that rare genetic polymorphisms with high odds ratios that are currently not detected in genome-wide association studies (45) contribute to this phenomenon, but also environmental factors can be important. One interesting environmental factor might be EBV, because infectious mononucleosis is a well-known risk factor for MS (46). An EBV infection alters the expression of IL-7Rα and IL-15R, an IL-7Rα family member. During acute infection, almost all CD8+ T cells lose their IL-7Rα expression, which quickly recovers after infection (47). Moreover, Ag-specific CD8EM T cells can persist in inflamed tissue (48) for a long time after clearance of viral Ags. The observed pattern of expression in the different types of lesions of MS patients suggests that in the preactive lesions all requirements are locally met to initiate a strong antiviral immune response with cytotoxic potential. In contrast, fully activated CD8+ T cells present in active demyelinating lesions do not require IL-7 and therefore lack IL-7Rα expression. Alternatively, it cannot be excluded that the CD8+ T cells expressing IL-7Rα have bound IL-7, prohibiting detection.

In our study, we initially included only untreated relapsing-remitting MS patients. The main advantage of this approach is
that the results are not be influenced by the use of immune-modulating therapy. Conversely, this approach might have introduced a potential bias, because untreated patients are generally more mildly affected and differences between relapsing-remitting and primary progressive patients cannot be assessed. We are confident that the severity bias has been eliminated by the inclusion of 15 treated patients in the second phase of this study, confirming the initial findings. Moreover, the inclusion of both primary and secondary progressive MS patients has likely prevented the second disadvantage, especially because no differences between subgroups were detected.

The IL-7Rα/IL-7 pathway is also implicated in other autoimmune diseases. For example, IL-7Rα expression by lymphoid cells in the joint in rheumatoid arthritis patients is increased compared with osteoarthritis patients (49). Treatment response to methotrexate in rheumatoid arthritis patients correlated with decreased IL-7 levels (50). In a mouse model for rheumatoid arthritis, anti–IL-7 treatment completely inhibited autoimmune arthritis (51). Moreover, IL-7 expression is increased in the inflamed salivary glands in primary Sjögren’s syndrome compared with non-Sjögren’s sicca syndrome patients (52).

In conclusion, MS is associated with increased expression of IL-7Rα on most CD8+ T cell subsets and with higher frequencies of CD8EM cells coexpressing IL-7Rα. Functional experiments showed a lower threshold for IL-7–induced CD8+ cytotoxicity in MS patients. We also confirmed earlier reports that IL-7 is produced in the MS lesions of MS patients, strongly suggesting that IL-7 and its receptor are a functionally relevant couple at the site of the pathology. Differences between patients and controls could not be explained by the rs6897932 MS risk SNP. A recent study showed that IL-7Rα knockout mice are almost completely protected against experimental autoimmune encephalomyelitis (53), the experimental model of MS. Taken together, the IL-7R/IL-7Rα pathway appears to be an interesting target for therapeutic interventions in MS.

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The authors have no financial conflicts of interest.

References


