doi:10.1093/brain/awt108 Brain 2013: 136; 1783–1798 | 1783

BRAIN
A JOURNAL OF NEUROLOGY

HLA-DR15-derived self-peptides are involved in increased autologous T cell proliferation in multiple sclerosis

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The HLA-DR15 haplotype confers the largest part of the genetic risk to develop multiple sclerosis, a prototypic CD4+ T cell-mediated autoimmune disease. The mechanisms how certain HLA-class II molecules functionally contribute to autoimmune diseases are still poorly understood, but probably involve shaping an autoimmune-prone T cell repertoire during central tolerance in the thymus and subsequently maintaining or even expanding it in the peripheral immune system. Self-peptides that are presented by disease-associated HLA-class II molecules most likely play important roles during both processes. Here, we examined the functional involvement of the HLA-DR15 haplotype in autologous proliferation in multiple sclerosis and the contribution of HLA-DR15 haplotype-derived self-peptides in an *in vitro* system. We observe increased autologous T cell proliferation in patients with multiple sclerosis in relation to the multiple sclerosis risk-associated HLA-DR15 haplotype. Assuming that the spectrum of self-peptides that is presented by the two HLA-DR15 allelic products is important for sustaining autologous proliferation we performed peptide elution and identification experiments from the multiple sclerosis-associated DR15 molecules and a systematic analysis of a DR15 haplotype-derived self-peptide library. We identify HLA-derived self-peptides as potential mediators of altered autologous proliferation. Our data provide novel insights about perturbed T cell repertoire dynamics and the functional involvement of the major genetic risk factor, the HLA-DR15 haplotype, in multiple sclerosis.

Keywords: HLA-DR15; autologous T cell proliferation; multiple sclerosis; self-peptides; homeostatic proliferation

Abbreviations: HLA = human leukocyte antigen; IL = interleukin; MHC = major histocompatibility complex; PBMCs = peripheral blood mononuclear cells

Received November 28, 2012. Revised January 30, 2013. Accepted March 13, 2013

Introduction

Multiple sclerosis is considered a CD4+ T cell-mediated autoimmune disorder of the CNS characterized by inflammation, demyelination, glial scarring and axonal/neuronal loss (Sospedra and Martin, 2005). Regarding disease aetiology, genome-wide association studies have identified a large number of genetic loci, which, together with multiple environmental factors, confer multiple sclerosis risk (Sawcer *et al.*, 2011). The association between human leucocyte antigen (HLA)-DR15 (formerly called DR2) and multiple sclerosis was first noticed in 1973 (Jersild *et al.*, 1973), and since then has been among the most reproduced findings in major histocompatibility complex (MHC) genetics (Oksenberg *et al.*, 2008). Furthermore, HLA-DR15 also influences clinical aspects like disease onset and phenotype (Hensiek *et al.*, 2002). However, the mechanisms of how the presence of a specific HLA-DR type, other multiple sclerosis risk alleles and environmental factors translate to disease are largely unknown. Data from animal models and immunological studies in patients with multiple sclerosis point at a role for autoreactive CD4+ T cells in multiple sclerosis (Sospedra and Martin, 2005). The most important, albeit indirect finding, supporting this notion is the fact that ~10–60% of the genetic risk stems from the HLA-DR15 haplotype consisting of two DR alleles, DRB1*15:01 (the heterodimer of DRA1*01:01/DRB1*15:01, also referred to as DR2b) and DRB5*01:01 (the heterodimer of DRA1*01:01/DRB5*01:01, also referred to as DR2a) (Oksenberg *et al.*, 2008). Interestingly, the influence of the environmental risk factors that have been identified for multiple sclerosis thus far, i.e. infection with Epstein-Barr virus, low vitamin D levels, and smoking, is enhanced in DR15+ patients with multiple sclerosis (Kakalacheva *et al.*, 2011).

HLA-class II molecules are expressed by antigen-presenting cells. They present peptides derived from processing of self or exogenous proteins, and the complexes of self HLA-class II and antigenic peptides serve as recognition structures for CD4+ T cells. In the thymus HLA-class II molecules are involved in shaping the CD4+ T cell receptor repertoire through central tolerance mechanisms of positive and negative selection of T cells (Ashton-Rickardt *et al.*, 1994). Once in the peripheral immune system the T cell repertoire is maintained by homeostatic proliferation, and interactions between CD4+ T cells and their T cell receptors with HLA-class II/self-peptide complexes as well as the cytokines interleukin 7 (IL-7) and IL-15 are the most important stimuli during this process (Sprent and Surh, 2011). Despite the well-known role of HLA-class II in T cell receptor repertoire generation and maintenance, our understanding about the functional contribution of HLA-class II molecules to human autoimmune diseases in general and also to multiple sclerosis is, however, still limited.

Regarding possible mechanisms several hypotheses have been developed including preferential presentation of self-peptides with similarities to foreign antigens but also tissue-specific autoantigens (Wucherpfennig and Strominger, 1995), less complete thymic deletion of autoreactive T cells (Klein *et al.*, 2000), and differential HLA-class II expression in certain immune cells and the brain (Prat *et al.*, 2005). Another interesting possibility, which has not been examined thus far in multiple sclerosis, is that distinct structural features of the HLA-DR15 haplotype, i.e. of HLA-DR molecules themselves or the complexes between HLA molecule and self-peptide, operate through homeostatic proliferation and/or autologous expansion to shape an autoimmunity-prone T cell repertoire (Haegert, 2011). Homeostatic T cell proliferation depends on interactions between T cell receptor and self-peptides presented by HLA molecules and the cytokines IL-7 and IL-15 (Sprent and Surh, 2011). Interestingly, genes coding for components of these and related molecules, i.e. DR15, IL7RA, IL7 and IL2RA, are associated with multiple sclerosis risk (Sawcer *et al.*, 2011).

Evidence for alterations in CD4+ T cell selection and/or peripheral T cell repertoire maintenance in multiple sclerosis are: myelin-reactive T cells with a proinflammatory phenotype, lack of CD28 expression, and higher antigen avidity are increased in patients with multiple sclerosis (Martin *et al.*, 1990; Ota *et al.*, 1990; Pette *et al.*, 1990; Markovic-Plese *et al.*, 2001; Bielekova *et al.*, 2004). Further, reduced thymic output of CD4+ T cells has been observed in multiple sclerosis (Hug *et al.*, 2003; Duszczyszyn *et al.*, 2006; Haegert *et al.*, 2011) as well as reduced numbers and function of CD4+ T regulatory cells (Viglietta *et al.*, 2004). Studies of the T cell repertoire by complementary region determining region 3 spectratyping indicate reduced diversity in patients with multiple sclerosis compared with age-matched controls (Laplaud *et al.*, 2004). These observations indicate alterations of the peripheral proliferation and survival/expansion of CD4+ T cells, which might affect antigen avidity and frequency of autoreactive T cells. Hence, alterations in T cell homeostasis could contribute to T cell repertoire perturbations in multiple sclerosis.

Given the prominent role of HLA-DR15 for multiple sclerosis risk, we wanted to investigate its functional involvement in autologous T cell expansion in multiple sclerosis. To this end, we devised an *in vitro* method to assess autologous proliferation. Different from the previously well examined autologous mixed lymphocyte reaction of human peripheral blood mononuclear cells (PBMCs), which is reduced in multiple sclerosis and other autoimmune diseases (Hafler *et al.*, 1985), we did not expose autologous responder (T lymphocytes) cells to autologous, gamma-irradiated non-T cells as stimulators, but rather seeded unmanipulated PBMCs in a serum-free media. Hereby we addressed first, if autologous T cell proliferation is altered in patients with multiple sclerosis in general and if there is a relation to the DR15 haplotype. Next, we dissected the relative role of self-peptides bound to DR2a and DR2b by two approaches: (i) elution and identification of self-peptides bound to DR2a or DR2b; and (ii) based on the fact that HLA-derived-peptides themselves are frequently presented by HLA-class II alleles, we used overlapping peptides spanning the sequences of the two DR- and the DQ molecules of the DR15 haplotype, i.e. the non-polymorphic DRα chain DRA1*01:01, the two DRβ chains DRB1*15:01 and DRB5*01:01, and the tightly linked DQw6 molecule consisting of DQA1*01:02 and DQB1*06:02, as antigens. Our data demonstrate that autologous proliferation is increased in patients with multiple sclerosis, which is related to the presence and dose of HLA-DR15 expression, that this phenomenon is observed with both DR alleles expressed in the DR15 haplotype and that HLA-class II-derived self-peptides are likely involved as antigens.

Materials and methods

Patients and healthy donors

Patients with multiple sclerosis had clinically definite multiple sclerosis by clinical and/or McDonald criteria. We examined 69 untreated patients with multiple sclerosis, eight patients with clinically isolated syndrome, a pre-stage of relapsing-remitting multiple sclerosis that often evolves into multiple sclerosis, based on MRI- and clinical findings; 37 patients with relapsing-remitting multiple sclerosis (eight of these in exacerbation), eight patients with secondary-progressive multiple sclerosis , i.e. the multiple sclerosis form that evolves from relapsing-remitting multiple sclerosis; and finally eight patients with primary-progressive multiple sclerosis. Signed informed consent was obtained from all patients and donors under a protocol that was approved by the Ethics Committee of the Hamburg Board of Physicians (No. 2758). Samples were typed for HLA class II (DRB1*, DRB3*, DRB4*, DRB5*, DQA1* and DQB1*) at the Department of Transfusion Medicine, UKE Hamburg by a reverse sequence-specific oligos in-house test. In some cases the results were verified by a commercial reverse sequence-specific oligos test (Dynal RELI-SSO, Invitrogen) or by Atria SBT (Sequence Based Typing, Abbott). HLA-DR15+ indicates the haplotype HLA-DRB1*15:01, HLA-DRB5*01:01, HLA-DQA1*01:02 and HLA-DQB1*06:02. *X indicates another HLA class II haplotype, and *protective indicates HLA-DRB1*14 or HLA-DRB*11 haplotypes (Ramagopalan *et al.*, 2007). PBMCs were freshly isolated from EDTA-containing blood tubes or from buffy coats by Ficoll® density gradient centrifugation (PAA). All isolated PBMCs were cryopreserved in media containing 10% dimethyl sulfoxide (DMSO; AppliChem) and stored at −180°C.

Proliferative assays

After thawing 2×10^5 PBMCs/well (average of 22 wells per donor) were cultured with serum-free AIM-V medium (GIBCO, Invitrogen), containing 2 mM L-glutamine, 50 μg/ml streptomycin sulphate, 10 μg/ml gentamicin sulphate and human albumin, in 96-well U-bottom microtitre plates (Greiner Bio-One) at 37°C, 5% CO_2. Depending on the experiment, wells were pulsed at Day 0, 2 and 6 cells for 15 h with 1 μCi of methyl-^{3}H-thymidine (Hartmann Analytic) and incorporation was measured by β-scintillation counting (Wallac 1450, Perkin Elmer) (Supplementary Fig. 1A). Phytohaemagglutinin-L (1 μg/ml, Sigma) was used as positive control.

HLA-DR15 haplotype-derived self-peptides were either tested in pools or individually at a 2 μM concentration (10 μM total pool concentration) in 7-day proliferation assays. Stimulatory indices were calculated as follows: stimulatory indices = mean counts per minute (peptide) / mean counts per minute (unstimulated). Statistical analysis was performed using a student's *t*-test (two-tailed, unpaired) using Prism 5.0 software (GraphPad). Analysis of the mean standard deviations has been verified with tests for the homogeneity of variance (Fligner-Killeen, Bartlett, Levene), which have been performed using R (v 2.15.1) with libraries stats and car.

Human leucocyte antigen restriction

CD4+ T cells were negatively enriched from thawed PBMCs of HLA-DR15+ healthy donors using the IMag system (BD Biosciences). 25.000 CD4+ T cells (purity > 95%) were co-cultured for 3 days with 6.5×10^4 irradiated (200 Gy) bare lymphocyte syndrome cells in AIM-V serum-free medium. Restriction was tested with bare lymphocyte syndrome cells transfected with a single HLA-class II molecule, DRB1*15:01 (DR2b: DRB1*15:01, DRA*01:01), DRB5*01:01 (DR2a: DRB5*01:01, DRA*01:01), DQw6 (DQA1*01:02, DQB1*06:02). Bare lymphocyte syndrome cells were kindly provided by G. Nepom and W. Kwok (University of Washington, Seattle, USA). Proliferation was assessed as described above.

Blocking of autologous proliferation

Autologous T cell proliferation was blocked by incubating PBMCs with anti-HLA-class I (HLA-A, -B, -C; w6/32) and anti-HLA-DR (L243) antibodies at the depicted concentrations and compared with a purified mouse IgG2a$_\kappa$ low endotoxin, azide-free isotype control (BioLegend, MOPC-173) in a 3- and 7-day proliferation assay.

Flow cytometry analysis

Samples were thawed, Fc-blocked with mouse IgG and directly stained for surface expression using following antibodies: anti-CD3 (PE-Cy7, UCHT1, eBioscience), anti-CD4 (APC, RPA-T4, eBioscience), anti-CD8 (PacificBlue, DK25, Dako), anti-CD14 (PacificBlue, MφP9, BD Pharmingen), anti-CD19 (PacificBlue, HIB19, BD Pharmingen), anti-CD303 (APC, AC144, Miltenyi Biotech), anti-panHLA-class II (DR, DQ, DP) (FITC, Tü39, BD Pharmingen). Analyses were performed on a LSRII (BD Biosciences) flow cytometer using FACS Diva 5.0 software.

Elution of human leucocyte-presented peptides

HLA-bound peptides were obtained by immunoprecipitation of HLA molecules from the bare lymphocyte syndrome cells expressing only DR2a or DR2b according to standard protocols (Falk *et al.*, 1991) using the DRB5*01:01-specific antibody (clone: H0596) for DR2a and the DRB1*15:01-specific antibody (clone: H0427A) for the DR2b cells. Both were provided by Jar-How Lee (One Lambda Incorporation). This was followed by a second HLA-precipitation by the pan-HLA-DR specific antibody L243 for each cell lysate.

About 3 ml cell pellet per cell line were gently shaken in 3 ml PBS containing 1.2% (w/v) CHAPS in the presence of protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche Applied Science) for 1 h followed by sonication of the lysate on ice with subsequent shaking for 1 h. The lysate was centrifuged and the supernatant was passed through a 0.20 μm sterile filter. Subsequently, HLA molecules and bound peptides were isolated with the solid-phase bound monoclonal antibody by immunoaffinity chromatography (overnight at 4°C). HLA ligands were eluted with 0.2% trifluoroacetic acid, ultrafiltrated through a 10-kDa cut-off Amicon ultracentrifuge filter and lyophilized.

Liquid chromatography-mass spectrometry

Lyophilized samples were resuspended in 30 μl of solvent A [0.1 (v/v) formic acid in 2% (v/v) acetonitrile] and loaded onto a C_{18} precolumn (Dionex) for concentration and desalting with a flow rate of 20 μl/min. The precolumn was placed in line for separation by a 250 × 0.075 mm fused silica microcapillary column packed with C_{18} reversed-phase material (Acclaim PepMap 100, Dionex). A binary gradient of 0 to 55% solvent B was performed within 120 min, applying a flow rate of 300 nl/min.

The eluted peptides were analysed by electrospray ionization (ESI) mass spectrometry on a linear trap quadrupole (LTQ) Orbitrap XL

mass spectrometer (Thermo Fisher Scientific) coupled to nanoLC-2D (Eksigent) nano-HPLC system through a nanospray-ESI source. A gold-coated fused-silica glass capillary Picotip™ Emitter (SilicaTip™, FS360-20-10 Coating P200P, New Objective) was used for the introduction into the nanospray-ESI source. The heated capillary temperature and spray voltage were held at 200°C and 1.9 kV, respectively, creating an electrospray between the Picotip and the transfer capillary resulting in a sample flow rate of 300 nl/min. Parent ions were measured in the Fourier transform Orbitrap analyser. Fragment ions were generated by collision-induced dissociation with normalized collision energy of 35 V and recorded in the LTQ. The activation time for collision-induced dissociation was 30 ms. Mass spectrometry-survey scans were acquired at a resolution of 60 000 at 400 m/z. The 'lock mass' option (Olsen *et al.*, 2005) was enabled for the Fourier transform-mass spectrometry scans using the 445.120025 m/z ion for real time internal calibrations. The five most abundant parent ions from the Fourier transform survey scan were selected for fragmentation and excluded from further sequencing for 90 s. The mass spectrometer was operated in positive ion mode and parent ions with an unknown or higher charge state than three were excluded from tandem mass spectrometry.

Mass spectrometrical data analysis

Tandem mass spectrometry data were analysed with the Thermo Proteome Discoverer 1.1 (Thermo Fisher Scientific) software employing Mascot v2.2 (Matrix Science) as a search engine for peptide identification using a target-decoy database search in the Swiss-Prot protein sequence database (*Homo sapiens*, release, 2010_10). For database searches a mass tolerance of 5 ppm were used for Fourier transform-mass spectrometry scans whereas 0.5 Da were accepted for LTQ-MS/MS scans. The false discovery rate was set to $\leqslant 1\%$. Phosphorylation of serine (S), threonine (T) and tyrosine (Y) as well as oxidation of methionine to methionine sulphoxide (m) were enabled as dynamic modifications. The software was allowed to group spectra if the retention time difference did not exceed 24 s.

Peptide library generation and *in silico* peptide binding prediction

One hundred and twenty-two overlapping (five amino acid overlap) 15-mer and a few 16-mer peptides from DRB1*15:01, DRB5*01:01, DRA*01:01, DQA1*01:02, DQB1*06:02 (http://hla.alleles.org) were synthesized and provided by peptides & elephants GmbH. DRβ-chain-derived peptides with identical sequences were only synthesized once. Lyophilized peptides were resuspended in 100% dimethyl sulphoxide and stored at −80°C.

In silico peptide binding predictions were performed by the consensus approach provided by the Immune Epitope Database (www.iedb.org, Wang *et al.*, 2008). Gene Ontology annotation analysis of biological pathways was performed with the DAVID functional annotation analysis webpage (http://david.abcc.ncifcrf.gov/).

Results

Autologous proliferation is altered in patients with multiple sclerosis

We first characterized and compared in a standard thymidine incorporation assay the proliferation of PBMCs from 69 untreated patients with multiple sclerosis and 36 healthy donors (for demographics see Supplementary Table 1) over 7 days in serum-free media. Below, we will refer to this condition in the absence of a stimulus as autologous proliferation. Because there is no background control and a stimulation index cannot be calculated, we expressed the strength of proliferation by the mean counts per minute of all wells (Fig. 1A) and the variation or heterogeneity by the mean, amongst multiple donors within each group, e.g. multiple sclerosis or controls, of the standard deviation of all wells within each donor (Fig. 1B; schematic depiction in Fig. 1C). PBMCs from patients with multiple sclerosis incorporated significantly more thymidine (mean counts per minute ± SEM, 5337 ± 548) than healthy donors (3606 ± 503, $P = 0.04$), whereas no differences in strength of proliferation were observed after phytohaemagglutinin stimulation (Fig. 1A). PBMCs from patients with multiple sclerosis not only incorporated more thymidine, but also the variation between wells was significantly higher or heterogeneous (SD ± SEM, 2099 ± 217) than in healthy donors (1422 ± 180, $P = 0.04$) (Fig. 1B), and again no differences in heterogeneity were observed after phytohaemagglutinin stimulation (data not shown). Some wells from patients with multiple sclerosis proliferated as vigorously as usually observed upon stimulation with nominal antigen (Fig. 1C), suggesting that some T cells from patients with multiple sclerosis are activated in the absence of a stimulus. We then compared patients with different forms of multiple sclerosis, i.e. clinically isolated syndrome, a pre-stage of multiple sclerosis, relapsing-remitting multiple sclerosis, relapsing-remitting multiple sclerosis in remission or in exacerbation (relapsing-remitting multiple sclerosis-relapse), secondary-progressive multiple sclerosis and primary-progressive multiple sclerosis. The differences of the strength of proliferation were similar in the subgroups, whereas the heterogeneity measure was higher for patients with clinically isolated syndrome and patients with relapsing-remitting multiple sclerosis in relapse (Supplementary Fig. 1B). In order to exclude that a leuco- or lymphopenic condition of the donors/patients with increased autologous proliferation elicited homeostatic proliferation, i.e. growth of T lymphocytes to fill an empty or partially emptied niche, we performed differential blood counts on all donors. Only two healthy donors showed lymphocyte counts slightly below normal values, and there was no correlation between peripheral blood white cell or lymphocyte counts and the degree of autologous proliferation (data not shown).

Increased autologous proliferation is related to the multiple sclerosis-associated DR15 haplotype

We next addressed the involvement of the HLA-DR15 haplotype in the above findings. As shown in Fig. 2A, the strength of autologous proliferation (mean counts per minute) was higher in DR15 + individuals than in DR15 − when comparing all studied individuals and also in patients with multiple sclerosis versus healthy donors, although differences were only significant for DR15 − healthy donors versus DR15 + patients with multiple sclerosis ($P = 0.045$). The differences were more pronounced for

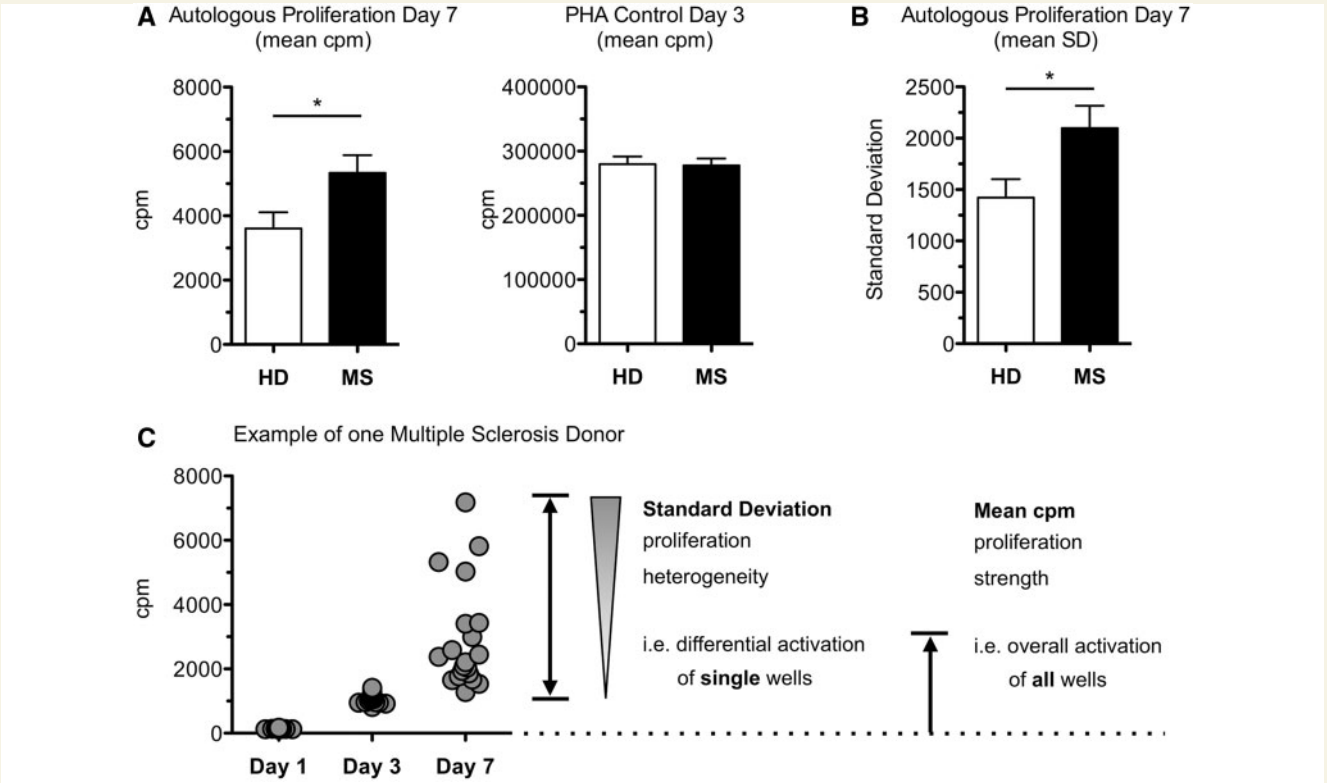

Figure 1 Comparison of autologous proliferation of peripheral mononuclear cells. Significant differences in proliferation strength (**A**) [mean counts per minute (cpm) ± SEM] and heterogeneity (**B**) (SD ± SEM) are observed between multiple sclerosis (MS) and healthy donors (HD) at Day 7 (*$P < 0.05$). (**A**) Right bar graph depicting the phytohaemagglutinin (PHA) positive control. (**C**) Example of one multiple sclerosis donor showing all unstimulated wells over a 7-day proliferation assay with adjacent scheme explaining measures of proliferation strength and heterogeneity.

the proliferation heterogeneity (mean SD) of all studied DR15+ versus DR15− individuals ($P = 0.004$) and for both DR15− versus DR15+ healthy donors ($P = 0.047$) and DR15− versus DR15+ patients with multiple sclerosis ($P = 0.036$) indicating that autologous proliferation strength and heterogeneity increase with disease and HLA-DR15 status, which is particularly clear from the 'single-well' view of PBMCs (Fig. 2B). Here, each column represents a single donor with all seeded wells. Each well is represented by a white dot. Donors were sorted by the well with the highest counts per minute and according to disease and HLA status. Supplementary Table 1 summarizes the results of Fig. 2A and B.

Characterization of autologous proliferating cultures

In order to characterize which cell population in the peripheral blood mononuclear cells is responsible for autologous proliferation we determined the percentage of CD4+, CD8+ and memory CD4+ T cells before and after 7 days of culture. As expected autologous proliferation was primarily mediated by CD4+ T cells, since their percentages were high at the time of starting the assay and increased further until Day 7 (Fig. 3A), whereas the initially low numbers of CD8+ T cells dropped (Fig. 3A) indicating that under uninhibited culture conditions CD8+ T cells did not proliferate to a significant extent. Further, we did not observe major changes regarding the naïve/memory CD4+ T cell population composition (Fig. 3A). However, the slight but consistent decrease of CD4+CD45RO+ T cells indicates that autologous proliferation is mediated to a larger extent by naïve cells, i.e. CD4+CD45RO−, then by memory T cells.

Next we wanted to address the potential contribution of the level of baseline HLA-DR expression or its upregulation during the culture period. As shown in Fig. 3A, DR expression on B cells increased significantly, supporting a putative role of HLA-DR in the above phenomena. We then asked whether a higher baseline HLA-DR/DR15 expression on antigen presenting cells is a consequence of for example a proinflammatory environment in multiple sclerosis and then causes increased homeostatic proliferation. Towards this aim we examined the HLA-class II expression (DR, DP and DQ) on plasmacytoid dendritic cells, monocytes and B cells in healthy donors and patients with multiple sclerosis homozygote or heterozygote for DR15 as indicated by Cella *et al.* (1997). HLA-class II expression did not differ significantly between donors with the exception of lower levels of HLA-DR expression on monocytes of patients with multiple sclerosis (Fig. 3B). Also, there was no DR-related gene dosage effect in DR15 homozygous versus heterozygous patients with multiple sclerosis or healthy donors. Finally, blocking antibodies against HLA-class II alone

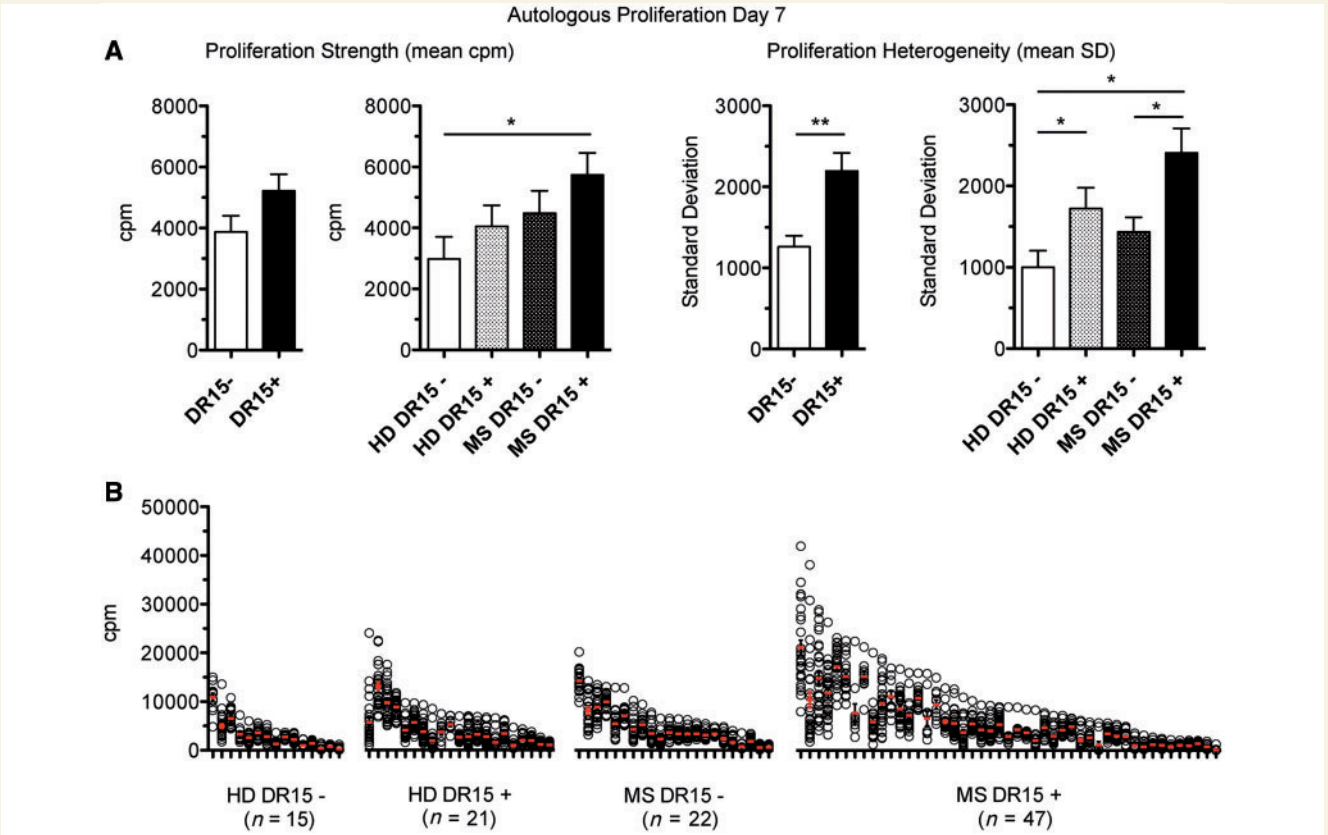

Figure 2 Influence of HLA-DR15 haplotype on autologous proliferation. (**A**) Proliferation strength is shown for multiple sclerosis (MS) and healthy donors (HD) together (*left* graph) or subdivided into HLA-DR15 haplotype-positive and -negative patients and healthy donors (*right* graph) (mean counts per minute ± SEM). (**B**) Proliferation heterogeneity is shown for multiple sclerosis and healthy donors together (*left* graph) or subdivided into HLA-DR15 haplotype-positive and -negative patients and healthy donors (standard deviation ± SEM). (**B**) Each column represents one donor with dots displaying every single unstimulated well (thymidine incorporation at Day 7; $*P < 0.05$, average wells per donor = 22).

(Fig. 3C) and against both HLA-class I and class II (Fig. 3C) reduced the autologous proliferation in a dose-dependent manner at Days 3 and 7 (Fig. 3C). Together these data indicate that interactions between CD4+ T cells and their T cell receptor with self HLA-class II molecules are involved in autologous proliferation, that B cells may play a role in this phenomenon *in vitro* and that different levels of HLA-class II expression at baseline are unlikely to be involved.

Elution of HLA-DR-derived self-peptides from the DR alleles expressed in the multiple sclerosis-associated DR15 haplotype

The above observations point at an involvement of the two DR15 alleles, DRB1*15:01 (DR2b) or DRB5*01:01 (DR2a) and the self-peptides they display in increased autologous T cell proliferation in DR15+ patients with multiple sclerosis. Multiple factors could contribute to this observation including a genetically determined lower activation threshold of T cells in multiple sclerosis and a T cell receptor repertoire that is shaped in DR15+ individuals by central tolerance mechanisms, which has higher affinity for DR15/self-peptide complexes. With respect to the latter, structural features of the DR molecules, i.e. their contact surfaces with the T cell receptor of CD4+ T cells, or the spectrum of bound self-peptides or both could play a role. To address the latter aspect we next examined the nature of self-peptides and their potential role in increased autologous proliferation by characterizing self-peptides that are bound to DR2a and DR2b by peptide elution and sequencing by mass spectrometry (schematically shown in Fig. 4A). In a previous study, we employed Epstein-Barr virus-transformed B cell lines that co-expressed all alleles of the DR15 haplotype using a monoclonal antibody against monomorphic determinants of DR alleles (Vogt *et al.*, 1994). To focus on DR2a and DR2b directly, we here used bare lymphocyte syndrome patient-derived B cell lines that had been transfected with either DR2a or DR2b and did not express any other HLA-class II molecule. Furthermore, for precipitation of DR2a and DR2b molecules we used allele-specific monoclonal antibodies against DR2a or DR2b, respectively. Owing to the lower precipitation efficiency of the latter IgM antibodies, a second sequential immunoprecipitation was performed with the L243 antibody. Since bare lymphocyte syndrome transfectants only express one class II allele, the

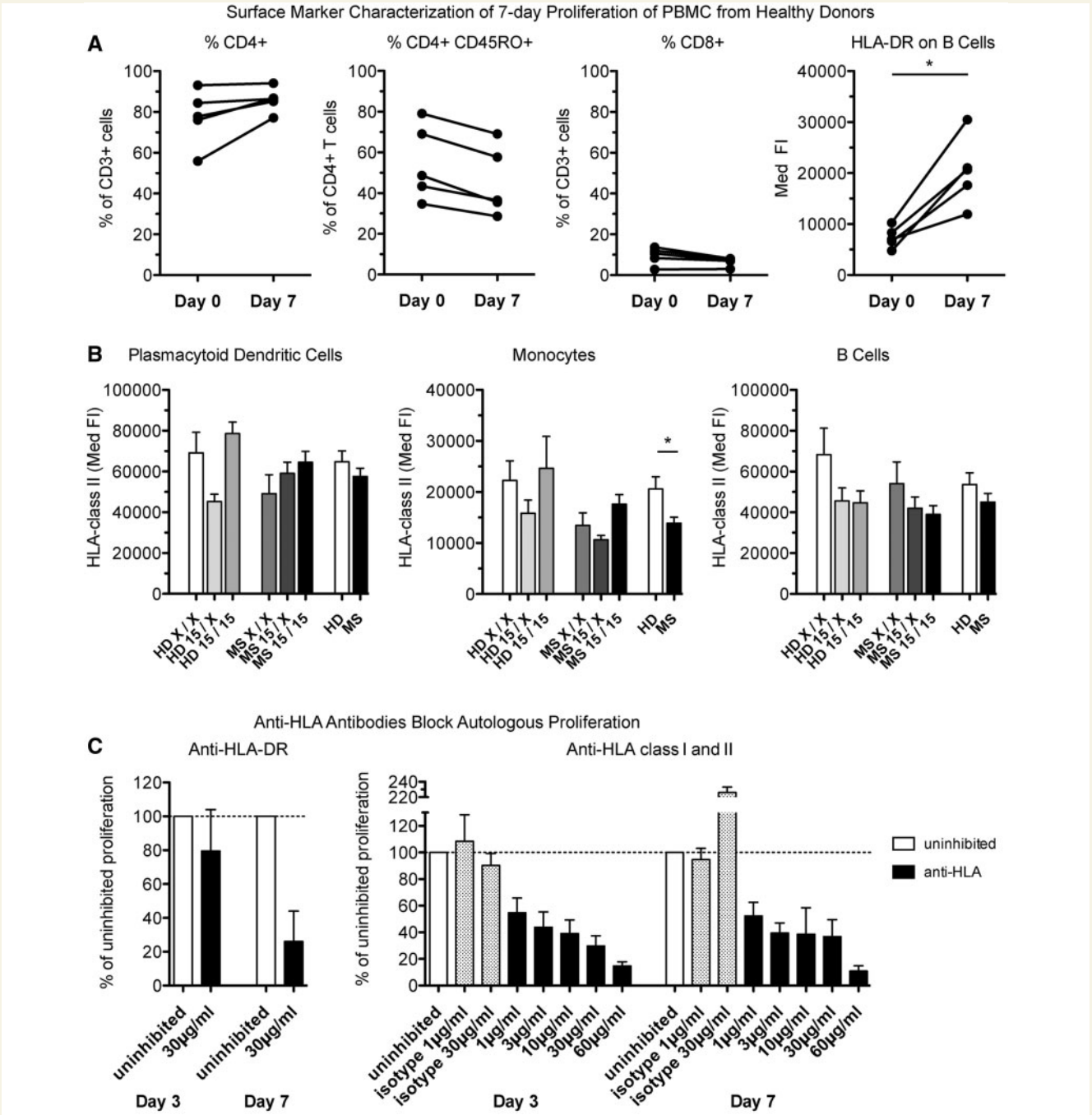

Figure 3 T cell subpopulations and HLA-DR expression. (**A**) FACS analysis of CD4 +, CD4 + CD45RO + and CD8 + surface expression on CD3 + T cells as well as the HLA-DR expression changes on B cells during 7 days of unstimulated PBMCs proliferation of five healthy donors (HD). (**B**) HLA-class II expression (median fluorescent intensity; Med FI) on different antigen presenting cells of HLA-DR15-positive and -negative patients with multiple sclerosis (MS) and healthy donors: CD303 + plasmacytoid dendritic cells, CD14 + monocytes and CD19 + B cells. (**C**) Blocking of autologous proliferation using anti-HLA class I- and -DR-specific antibodies. Inhibition of proliferation was measured on Days 3 and 7 and is shown as per cent of uninhibited proliferation.

peptide elution presumably underestimates the relative presence of HLA-class II-derived peptides by limiting the number of available class II molecules. We eluted and identified a total of 154 peptides from DR2a and DR2b molecules (Fig. 4A and Supplementary Table 2), and 7.14% (without considering multiple protein hits) of these peptides were derived from DRα (DRA1*01:01) or the β chains of DR2b (DRB1*15:01) or DR2a (DRB5*01:01). Hence, HLA-class II molecule-derived self-peptides form a substantial fraction of the spectrum of self-peptides that are bound to the multiple sclerosis-associated DR15 alleles. Biological pathway gene ontology annotation of the eluted peptides demonstrated that many of them are assigned to the antigen presentation pathway (Supplementary

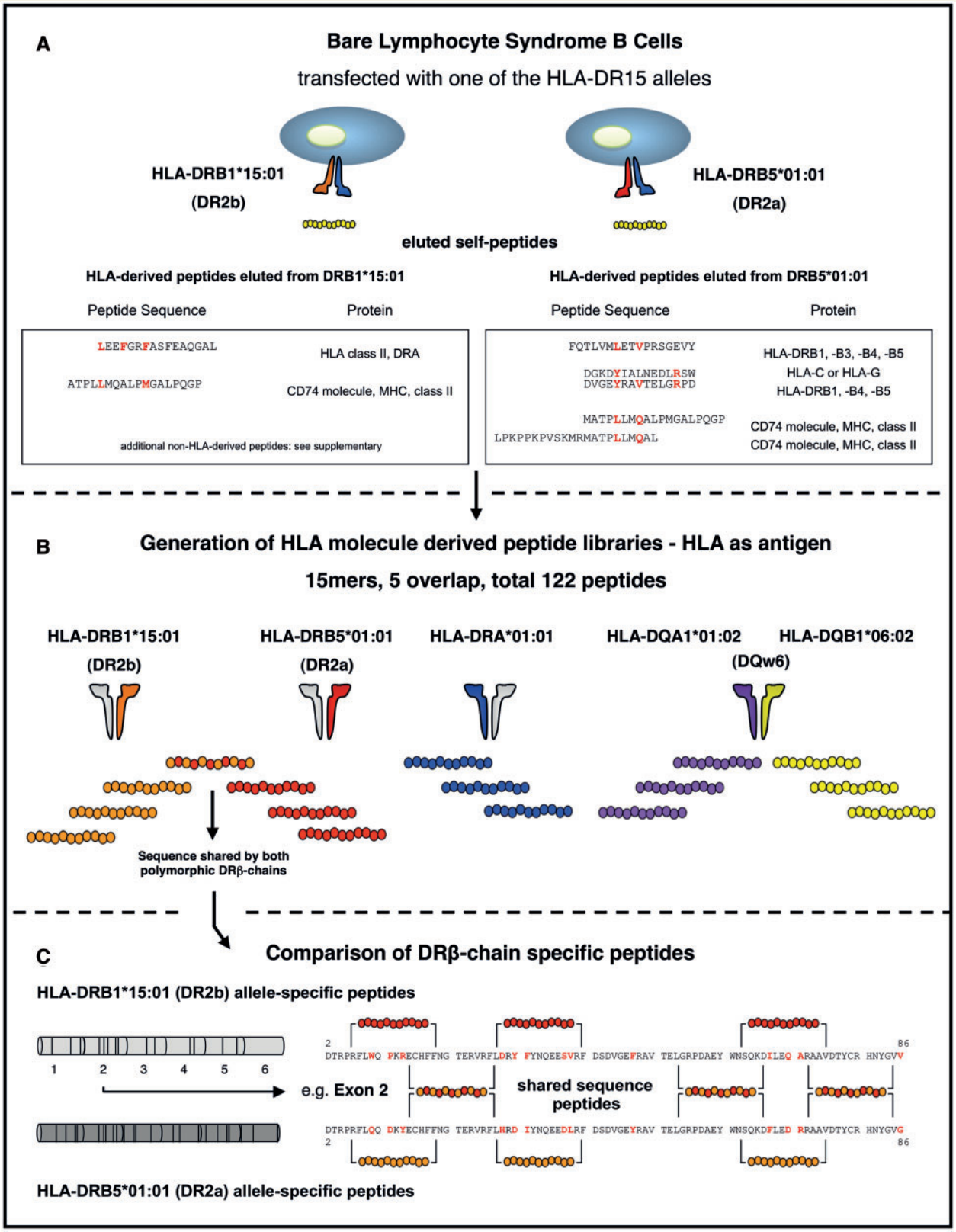

Figure 4 HLA-DR2a and –DR2b-specific peptide elution and characterization, and HLA-DR15 haplotype-derived self-peptide library. (**A**) Schematic drawing of peptide elution from bare lymphocyte syndrome cells expressing only DRB1*15:01 (DR2b) or DRB5*01:01 (DR2a), respectively. (**B**) Pictogram illustrating the composition of the HLA-DR15 haplotype-derived library of self-peptides (15-mers). (**C**) Exon 2 sequences of DRB1*15:01 and DRB5*01:01. Origin of allele-specific DRβ chain-derived peptides and peptides with shared sequences are depicted with single- and red and orange colours.

Table 3). Herewith we confirm and broaden our earlier results that HLA-class II-derived self-peptides make up a considerable portion of the peptide spectrum presented by DR2a and DR2b (Vogt *et al.*, 1994).

Reactivity to HLA-DR15 haplotype-derived self-peptides is related to expression of the haplotype itself

The above data indicate that HLA-derived self-peptides probably participate in autologous proliferation and also in T cell activation/stimulation beyond autologous turnover. To address this point we generated a HLA-DR15 haplotype-derived self-peptide library consisting of 15-mer peptides with five amino acid overlaps spanning the whole HLA-DR15 haplotype protein sequence (Fig. 4B). The resulting 122 peptides were derived from five proteins, i.e. the polymorphic chains HLA-DRB1*15:01 (DR2b), HLA-DRB5*01:01 (DR2a), the conserved HLA-DRA*01:01, and the HLA-DQw6 molecule consisting of HLA-DQA1*01:02 and HLA-DQB1*06:02 (Fig. 4B and Supplementary Table 4). HLA-DRβ chains share most of their sequences and are only polymorphic in certain areas, and therefore peptides derived from shared sequences were only synthesized once (Fig. 4B and C).

Since the relative genetic risk conferred by the multiple sclerosis-associated HLA-DR15 haplotype correlates not only with DR15 carrier status, but also with its dose, i.e. homo- versus heterozygosity (Barcellos *et al.*, 2003; Cournu-Rebeix *et al.*, 2008) we used the entire HLA-derived peptide library, i.e. 24 pools of five peptides each, to stimulate PBMCs from patients with multiple sclerosis/healthy donors (20 donors each) that were stratified according to DR15 homo- and heterozygosity (Fig. 5A). Since large genetic studies had indicated that there are also HLA-DR alleles that are 'protective' for multiple sclerosis, e.g. the HLA-DR14 haplotype (Ramagopalan *et al.*, 2007), we included a HLA-DR15/'protective' cohort (HLA-DRB1*14, HLA-DRB1*11), which should have the lowest risk for multiple sclerosis development regarding HLA background. We did not find these 'protective' DR alleles among our patients with multiple sclerosis, and therefore only tested healthy donors. Figure 5A depicts the per cent of wells with an stimulatory indices > 2.0 of all peptide pools tested for the different donors. Although the differences did not reach statistical significance (Fig. 5A), there is a clear trend that the reactivity increases from protective DR allele carriers to DR15 heterozygous to homozygous DR15 carriers. Owing to the higher background/autologous proliferation in patients with multiple sclerosis (see above), which affects the calculation of the stimulatory index, effects are more pronounced in healthy donors. In addition, we tried to capture not only proliferating cells according to standard criteria, i.e. surpassing background proliferation by at least a factor of two (stimulatory indices > 2), but also those showing a 'low-grade' proliferation (defined as stimulatory indices > 1.4). Since we added here nominal peptide in distinction to the above unstimulated condition, which we referred to as autologous proliferation, we will from now on refer to the peptide-induced 'low-grade' proliferation as 'tonic' stimulation. As shown in Fig. 5A, the tonic stimulation also increases in relation to DR15 haplotype and homozygosity.

Potential role of HLA-DR15 haplotype-derived self-peptides in autologous T cell proliferation

Next, we assessed the stimulatory or inhibitory potential of the HLA-derived self-peptides (Fig. 5B). Selected individual HLA-DR15-derived self-peptides were tested after analysing the proliferation of 35 donors (20 patients with multiple sclerosis, 15 healthy donors) to the 24 pools of self-peptides (Supplementary Fig. 2). Additionally, because they are most likely to be presented *in vivo*, we also tested two peptides that had been eluted. Peptide 8 (SDVGEFRAVTELGRP) and its DR2a homologue have been eluted twice, once by Vogt *et al*. (1994) and again from DR2a in the present study. Peptide 54 (EEFGRFASFEAQGAL) was eluted from HLA-DR2b. Like peptides 14, 15, 20 and 45, both elicited strong T cell proliferation (Fig. 5B). These experiments demonstrate that distinct DR15 haplotype-derived self-peptides are stimulatory to bulk PBMCs, whereas other peptides seem to inhibit activation or proliferation (stimulatory indices > 2.0). As described in Fig. 5A, we used the thresholds of stimulatory indices > 2.0 and > 1.4, respectively, to discriminate between activation and tonic proliferation. When looking at one specific peptide as an example, peptide 45, 12.5% of all wells proliferated with stimulatory indices > 2.0 (Fig. 5C) and 66% with stimulatory indices > 1.4 (not shown) indicating that a large number of wells responds with a tonic proliferation pattern, and a fraction of cells is fully activated. Interestingly, peptide 45 showed a marked T cell response despite originating from the inhibitory pool number 9 supporting the consideration that inhibitory and stimulatory peptides within each pool interact, consequently explaining the low overall proliferative response to the peptides pools (Supplementary Fig. 2).

We further asked if the stimulatory HLA-DR15-derived self-peptides could, if pooled together, evoke a stable T cell proliferation and if so, which concentrations are sufficient to induce proliferation. For this purpose different doses between 0.1 and 20 μM of a pool of five strongly stimulatory peptides were used. Peptide 54 had been eluted, peptides 8 and 20 were analogues of eluted peptides, and peptides 14 and 45 demonstrated the strongest tonic proliferation stimulus in the above proliferative assays with individual peptides. As shown in Fig. 5C, the pool of stimulatory peptides led to full stimulation and also tonic proliferation over a wide range of concentrations with a peak at 1.0 μM (Fig. 5C).

Next, we used the above five stimulatory peptides and examined the HLA-class II restriction using purified CD4+ T cells and bare lymphocyte syndrome transfectants expressing either DR2b, DR2a or DQw6. Most of the peptides elicited their stimulatory effect when presented by HLA-DR2a (Fig. 5D). Interestingly, when they were added to antigen presenting cells expressing HLA-DQw6, most peptides showed inhibitory rather than stimulatory effects.

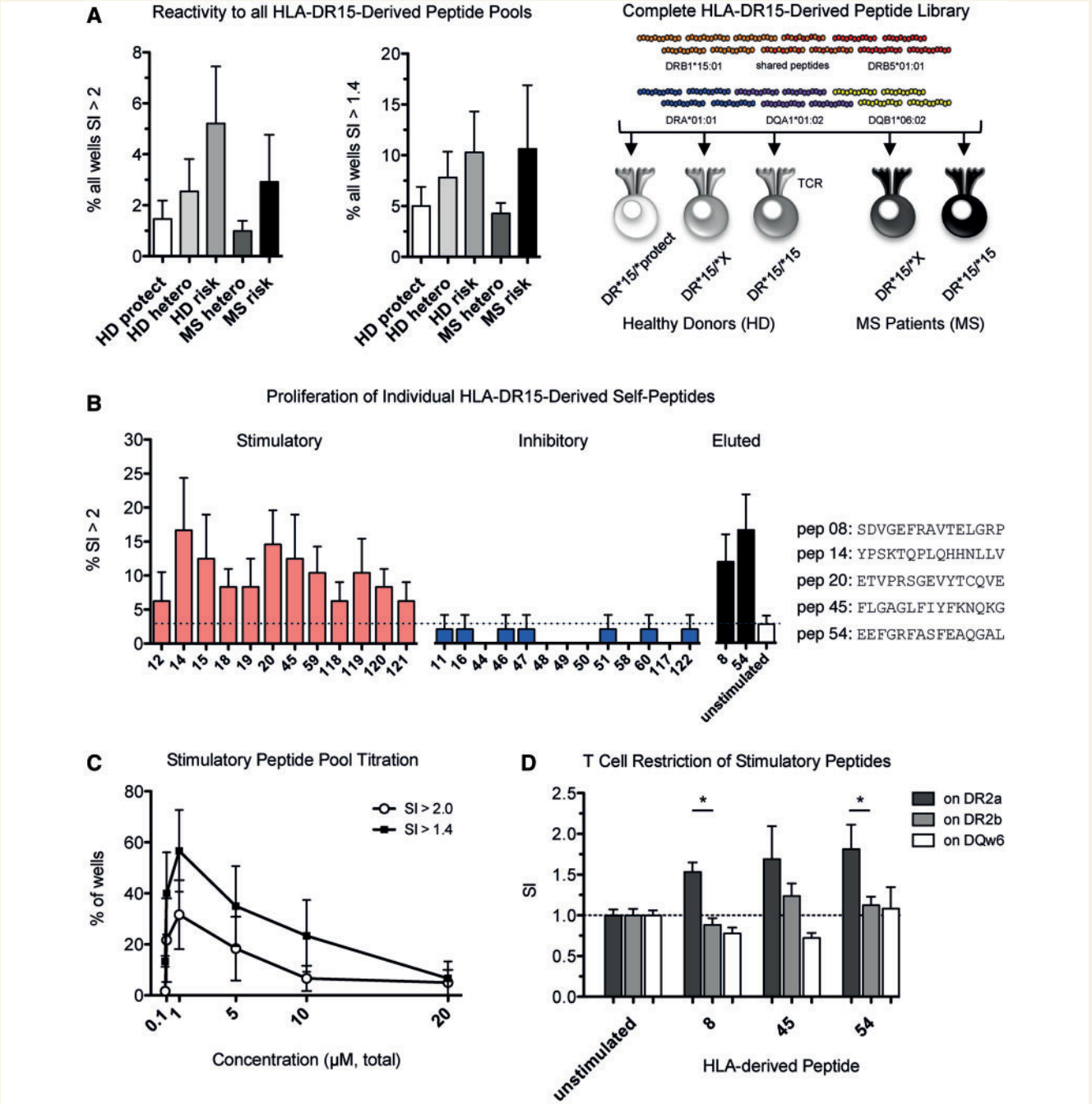

Figure 5 Proliferation of peripheral blood mononuclear cells upon stimulation with HLA-DR15 haplotype-derived self-peptides. (**A**) Per cent positive wells tested against 24 pools of HLA-DR15-derived self-peptides. Patients with multiple sclerosis (MS) (MS risk $n = 10$, MS hetero $n = 10$) and healthy donors (HD) (HD risk $n = 7$, HD hetero $n = 8$, HD protective $n = 5$) were stratified according to HLA-DR15 status as depicted by the schematic on the right. Risk indicates homozygosity for HLA-DR*15, hetero indicates HLA-DR*15/*X, and donors with protective alleles carry HLA-DR*15/*14 or -*11 haplotypes. Per cent positive wells with stimulatory indices > 2.0 (*left* graph) and stimulatory indices > 1.4 (*right* graph). (**B**) Proliferation of PBMCs to individual HLA-DR15-derived self-peptides (% of wells > stimulatory indices 2.0, $n = 6$) grouped by stimulatory, inhibitory and eluted peptides. (**C**) The most stimulatory peptides from **B** (peptide 8, 14, 20, 45, 54) were pooled and tested in a dose titration experiment. Per cent wells with stimulatory indices > 2 and stimulatory indices > 1.4 are shown ($n = 6$). (**D**) Proliferation of isolated bulk CD4+ T cells in response to five HLA-derived peptides presented by antigen presenting cells (bare lymphocyte syndrome transfectants) expressing only DR2a, DR2b, or DQw6 respectively [mean stimulatory index (SI) with SEM using five donors, six wells each].

Characterization of polymorphic HLA-DRβ-derived peptides

Finally, we compared corresponding peptides derived from the polymorphic regions of the two DRβ chains (Fig. 6; see also Fig. 4C). We found significant differences in 5 of 10 pairs (Fig. 6). In four of the five pairs DR2b-derived peptides (Fig. 6, light grey) were more stimulatory, whereas we found the reverse, i.e. that the DR2a-derived peptide was more stimulatory, only for peptide 44 versus peptide 22. Peptide 4 (DTRPRFLWQPKRECH, DR2b-derived) gave the strongest proliferative response of all polymorphic peptides. Tables 1 and 2 summarize all allele-specific DRβ chain-derived peptides, which we compared in Figure 6. In order to understand better, if these peptides bind to either of the two DR15 molecules, DR2a and DR2b, or to DQw6, we performed well-established *in silico* peptide binding predictions. The predicted binding affinities (% consensus rank; low values indicate better binding; highest affinity highlighted in green) are summarized in Table 1, and we further mention if the respective peptide was eluted from DR15 molecules in this or our prior study (Vogt *et al.*, 1994). Table 2 additionally analyzes peptides that have been eluted or shown to be stimulatory in our experiments. Both tables depict the peptide origin and the probability of being presented by an HLA-class II molecule of the DR15 haplotype.

Discussion

Despite substantial progress in immunology we still know remarkably little about how certain HLA-class II alleles contribute to autoimmune diseases (Gronski *et al.*, 2004; McFarland and Martin, 2007). HLA-DR15 as major risk factor for multiple sclerosis is one of the most striking examples for this notion. The mechanisms as to how HLA-DR15 shapes and maintains an autoreactive T cell repertoire that leads to CNS autoimmunity are still elusive despite longstanding efforts by a number of groups including our own, which focused primarily on studying the antigen specificity, function and HLA restriction of autoreactive T cell clones. In the present study we explored a hypothesis that had been discussed earlier in the context of autoimmune liver disease (Burroughs *et al.*, 1992) and oligoarticular juvenile idiopathic arthritis (Massa *et al.*, 2002) but not for multiple sclerosis so far. These studies postulate that self-peptides derived from and presented by abnormally expressed HLA-class II molecules potentially mimic microbial/viral peptides and may serve as target for the autoimmune T cell responses. Based on the consideration that interactions between HLA-class II molecules and the T cell receptors of CD4+ T cells are, besides IL-7, the most important factor to keep peripheral T cells alive, we examined the functional involvement of the HLA-DR15 haplotype in shaping and/or maintaining the peripheral T cell repertoire in multiple sclerosis through autologous proliferation. It has to be clarified, however, that we cannot assess by our simplified *in vitro* system the complex *in vivo* interactions that lead to T cell repertoire maintenance. Different from the steady-state *in vivo* homeostatic proliferation, i.e. in the absence of lymphopenia, which involves HLA/MHC/self-peptide complexes and/or IL-7 depending on the cell type (Baccala and Theofilopoulos, 2005; Cox *et al.*, 2005), our assay system most likely depicts a combination of autologous activation and subsequent proliferation in the absence of any stimulus. As stated above, this setting is different from the well-examined autologous mixed lymphocyte reaction, where antigens that are generated by apoptosis of the

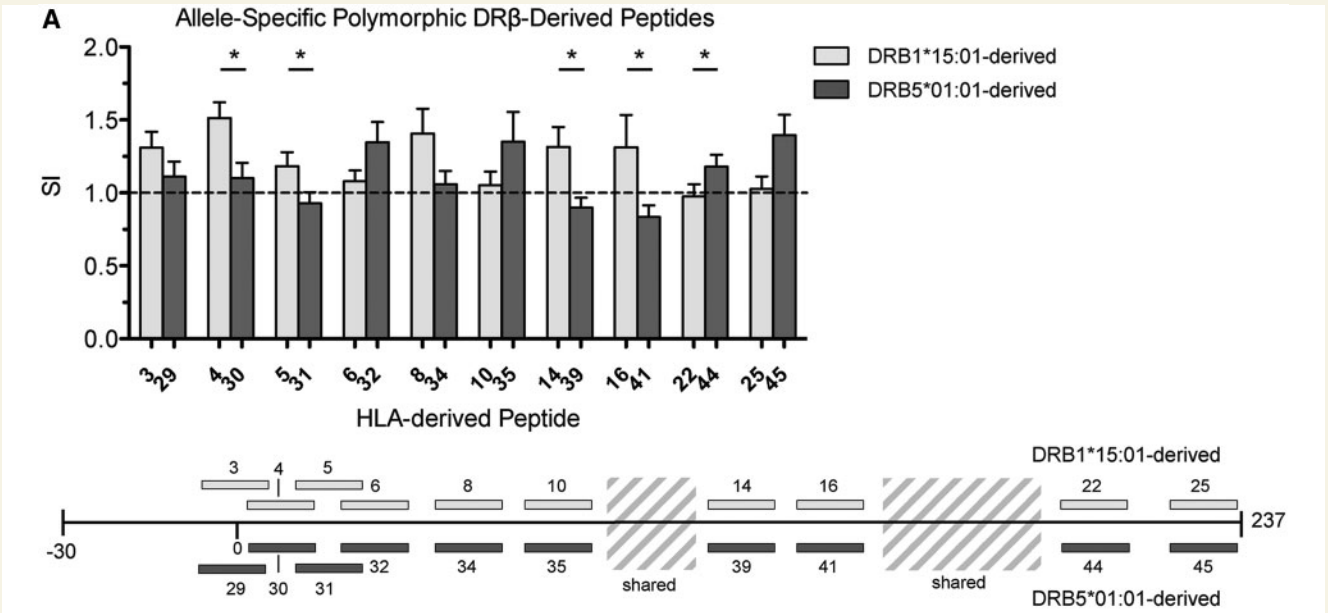

Figure 6 Reactivity to HLA-DRβ chain-derived, allele-specific self-peptides. Comparison of 20 peptides derived from the polymorphic HLA-DRβ chains of DRB1*15:01 and DRB5*01:01 (five donors, eight wells each; *P-value < 0.05). Scheme depicting origin of allele-specific peptides and shared peptide regions of the DRβ-derived sequence.

Table 1 Comparison of the allele-specific DRβ chain-derived peptides

HLA library peptide	Origin of peptide	Position in sequence	Sequence	to DR2b	to DR2a	to DQw6	Remarks
				binding affinity of peptides % consensus rank			
3	DRB1*15:01 (DR2b)	(−)9 to 6	VLSSP LALSG DTRPR	51,16	20,79	24,93	
29	DRB5*01:01 (DR2a)		VLSSP LALAG DTRPR	54,26	21,62	11,64	
4*	DRB1*15:01 (DR2b)	2 to 16	DTRPR FLWQP KRECH	44,19	8,23	85,72	Polymorphic region incl. mutation described by Zipp *et al*. (2000)
30*	DRB5*01:01 (DR2a)		DTRPR FLQQD KYECH	65,06	19,57	85,72	
5*	DRB1*15:01 (DR2b)	12 to 26	KRECH FFNGT ERVRF	29,81	5,79	84,80	
31*	DRB5*01:01 (DR2a)		KYECH FFNGT ERVRF	30,83	5,79	86,38	
6	DRB1*15:01 (DR2b)	22 to 26	ERVRF LDRYF YNQEE	2,01	14,87	94,16	
32	DRB5*01:01 (DR2a)		ERVRF LHRDI YNQEE	26,40	27,88	80,89	
8	DRB1*15:01 (DR2b)	42 to 56	SDVGE FRAVT ELGRP	43,62	0,38	37,84	Eluted by Vogt *et al*. (1994)
34	DRB5*01:01 (DR2a)		SDVGE YRAVT ELGRP	46,97	0,49	39,70	Eluted from DR2a
10	DRB1*15:01 (DR2b)	62 to 76	NSQKD ILEQA RAAVD	22,57	27,55	21,18	
35	DRB5*01:01 (DR2a)		NSQKD FLEDR RAAVD	41,36	31,98	81,95	
14*	DRB1*15:01 (DR2b)	102 to 116	YPSKT QPLQH HNLLV	36,20	46,73	78,73	
39*	DRB5*01:01 (DR2a)		YPART QTLQH HNLLV	34,29	35	56,98	
16*	DRB1*15:01 (DR2b)	112 to 126	FYPGS IEVRW FLNGQ	49,58	39,37	49,00	
41*	DRB5*01:01 (DR2a)		FYPGS IEVRW FRNSQ	51,21	29,98	55,42	
22*	DRB1*15:01 (DR2b)	182 to 196	SPLTV EWRAR SESAQ	51,07	25,72	69,38	
44*	DRB5*01:01 (DR2a)		SPLTV EWRAQ SESAQ	62,42	23,55	62,53	
25	DRB1*15:01 (DR2b)	212 to 226	FLGAG LFIYF RNQKG	0,51	1,69	41,68	
45	DRB5*01:01 (DR2a)		FLGAG LFIYF KNQKG	0,89	1,69	49,87	

Polymorphic amino acids are marked in red, the highest predicted binding affinity (consensus rank < 20%) to one of the alleles with green shading, and the peptide giving stronger responses in proliferation testing is indicated by grey shading.

Table 2 Summary of four peptides that had been eluted from either DR2a or DR2b

HLA library peptide	Origin of peptide	Position in sequence	Sequence	to DR2b	to DR2a	to DQw6	Remarks
				binding affinity of peptides % consensus rank			
19	shared DRβ	152 to 166	DWTFQ TLVML ETVPR	12,25	1,43	8,32	Eluted from DR2a
20	shared DRβ	162 to 176	ETVPR SGEVY TCQVE	81,63	64,18	62,29	Euted from DR2a
54	DRA*01:01	56 to 70	EEFGR FASFE AQGAL	2,64	5,42	15,95	Eluted by Vogt *et al*. (1994)., eluted from DR2b
104	DQB1*06:02	39 to 53	RFDSD VGVYR AVTPQ	28,86	20,38	32,67	Eluted by Vogt *et al*. (1994), analogue eluted from DR2a

irradiated stimulator cells are recognized by autologous T cells (Amel Kashipaz *et al.*, 2002), which probably also explains the divergent results between autologous mixed lymphocyte reaction in multiple sclerosis, i.e. decreased proliferation (Hafler *et al.*, 1985), and our observation of increased autologous proliferation.

With advanced age thymic output decreases, and hence compensatory peripheral homeostatic proliferation becomes more important for maintaining a functional T cell repertoire (Naylor *et al.*, 2005). Previous studies have described earlier thymic involution as well as reduced generation of CD4+ recent thymic emigrants in multiple sclerosis (Duszczyszyn *et al.*, 2010). Here, we show elevated autologous T cell proliferation in patients with multiple sclerosis compared with healthy donors in our experimental *in vitro* system. The autologous proliferation is more heterogeneous in that a substantial fraction of cells shows signs of full activation in patients with multiple sclerosis, and it is related to the multiple sclerosis-associated HLA-DR15 haplotype. Based on the importance of HLA-class II molecules, presented self-peptides and the T cell receptor avidity (Sprent and Surh, 2011) for this process, we assume that the HLA-DR15 haplotype is involved in altered homeostatic T cell proliferation in multiple sclerosis. Because the patients with multiple sclerosis of this study were not lymphopenic, i.e. did not have a relative depletion of the lymphocyte niche, it appears that lymphocytes are not numerically reduced, but rather that their repertoire is more narrow with respect to different specifities, but enriched for T cells with higher affinity for self-peptide. Such a repertoire is then expected to be activated easier in an autologous setting, which is simulated by our *in vitro* system.

Although immune mechanisms exist that sustain a T cell repertoire of great diversity (Min *et al.*, 2004) previous studies have reported that the peripheral maintenance of naïve T cells is not random (Rudd *et al.*, 2011). Under homeostatic conditions T cell receptor affinity/avidity appears to regulate T cell survival (Kieper *et al.*, 2004). In line with these concepts, we have previously shown that the presentation of self-peptides by mature dendritic cells can lead to a state of preactivation of T cells (Kondo *et al.*, 2001). Together, these studies emphasize that homeostatic/autologous proliferation supports mechanisms of selection and preferential activation of certain parts of the T cell repertoire. Therefore, the heterogeneous autologous proliferation with some cells showing a tonic response and others signs of full activation could indicate a process of selection through predominant proliferation of certain sets of T cells expressing T cell receptors with higher avidities for self-MHC/self-peptide complexes.

During T cell activation the T cell receptor contacts both antigenic peptide and HLA backbone. The two alpha-helices of the HLA backbone represent over 60% of the T cell receptor:MHC/peptide interaction surface (Sundberg *et al.*, 2007). When dissecting the trimolecular complex (T cell receptor:MHC/peptide), the following considerations are important. First, the higher the contribution of the HLA backbone to overall avidity the higher the number of peptides that can be recognized by the T cell receptor. Second, because T cell activation (or prevention of death) is related to T cell receptor affinity, selection is a consequence of homeostatic T cell proliferation. Third, the composition of self-peptides that are presented by a given HLA molecule is constrained by structural aspects of its binding pockets. Fourth, the presenting HLA molecule itself, which passes through the main processing compartments, is an important source of peptides that are displayed in its own binding pockets (Dengjel *et al.*, 2005). Therefore, HLA haplotypes present a haplotype-specific diverse set of self-peptides, which probably participates in shaping the peripheral T cell repertoire. As our *in vitro* studies show that the heterogeneity of proliferation was enhanced by the HLA-DR15 haplotype, we hypothesize that either the structural configuration of the HLA backbone, the self-peptides presented by the HLA-DR15 haplotype, or a combination of both are at least in part responsible for the observed proliferation patterns.

Most of the time surface HLA-class II molecules are loaded with self-peptides (Schild *et al.*, 1990). To analyse the potential role of self-peptides derived from the multiple sclerosis-associated HLA-DR haplotype and thus address some of the above possibilities, we eluted peptides presented by the HLA-DR15 haplotype. Consistent with previous work including ours (Vogt *et al.*, 1994; Dengjel *et al.*, 2005) we identified self-peptides from various sources and mostly from processing- and antigen presentation-related molecules. Among the latter a substantial fraction was derived from molecules of the HLA-DR15 haplotype itself. Our data indicate that HLA-derived self-peptides play an important role in the observed HLA haplotype-specific differences in autologous proliferation and that they can promote T cell proliferation. By using systematically generated sets of peptides spanning all DR- and DQ α- and β chains, we demonstrate that relatively high frequencies of peripheral T cells recognize certain HLA-derived self-peptides. Although T cell proliferation is often tonic, full activation is not a rare event even without any antigenic stimulus in PBMCs from patients with multiple sclerosis. These data indicate that HLA-DR15-derived self-peptides are able to provide a low-level stimulus or fully activate parts of the peripheral T cell repertoire. Furthermore, the observed HLA-derived peptide reactivity correlated with the genetic risk conferred by the HLA-DR haplotype. In individuals, who are haploidentical with respect to HLA-class II type, the combined effects of stimulatory and inhibitory self-peptides derived from multiple sclerosis risk-conferring and/or risk-lowering HLA alleles probably participates in setting the activation thresholds of T cells.

Positively selecting self-peptides in the context of CD4+ T cells have recently been described in murine models (Lo *et al.*, 2009), and the most important conclusions from these studies were the high specificity in the recognition of positively selecting ligands and that the same self-peptides can support peripheral homeostatic proliferation or serve as coagonists in the recognition of nominal ligand (Krogsgaard *et al.*, 2007; Wucherpfennig and Gagnon, 2009). Because the number of self-peptides that are involved in thymic selection processes is considered to be small, and many of these are presented both on thymic- and peripheral antigen presenting cells, they are probably relevant in both contexts, i.e. during central selection and peripheral homeostasis. Although thymic T cell repertoire selection appears to depend primarily on T cell receptor:MHC/peptide affinity, the peripheral maintenance and selection is determined by T cell receptor cross-reactivity (Hao *et al.*, 2006). Regarding the latter, the T cell receptor cannot distinguish between self- and non-self

peptides (Ashton-Rickardt *et al.*, 1994; Krogsgaard *et al.*, 2007), and therefore self-peptides presumably are a major factor in shaping and maintaining the composition of the peripheral T cell repertoire. Since HLA molecules bind different sets of peptides based on the structural requirements of their binding pockets (Falk *et al.*, 1994), different self-peptide repertoires will be presented according to haplotype. These factors together with the structural characteristics of the exposed surface of HLA molecules most likely lead to HLA haplotype-specific shaping of the T cell receptor repertoire.

Besides these structure-related possibilities our data point at yet another one, which involves peptides derived from the non-polymorphic or polymorphic stretches of HLA molecules as antigens themselves. Interestingly, an earlier study on the role of polymorphic regions of different DRB1* alleles including DRB1*15:01 observed significant multiple sclerosis-association of the amino acids proline at position 11, arginine at position 13, and alanine at position 71 of HLA-DRB1*15:01 (Zipp *et al.*, 2000). This study did not examine the DRB5* allele nor the abovementioned structural contributions or role of a polymorphic DR-derived peptide as antigen. In our present study, HLA-derived peptide number 4 contains both proline at position 11 and arginine at position 13, and therefore this peptide could contribute to increased autologous proliferation by shaping the binding pocket of DR2b, or conversely it could be an antigen itself. Peptide 4 was the most stimulatory of the polymorphic areas (see Fig. 6B), whereas the homologous peptide 30 derived from the same area of DRB5*01:01 was not stimulatory.

If one attempts to link these findings to multiple sclerosis, it is currently believed that autoreactive T cell responses in multiple sclerosis are directed against myelin proteins including myelin basic protein (MBP) (Sospedra and Martin, 2005). Early studies by others and us have found MBP-specific T cells in patients with multiple sclerosis, but also in healthy controls (Martin *et al.*, 1990; Ota *et al.*, 1990; Pette *et al.*, 1990). Despite higher precursor frequencies in patients with multiple sclerosis in some studies this observation at first seemed to argue against a role of these cells in multiple sclerosis, but later studies not only described higher frequencies, but also other potentially relevant characteristics such as higher functional T cell receptor avidity against several myelin peptides derived from MBP, myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP) (Bielekova *et al.*, 2004), populations that are independent of costimulation (Markovic-Plese *et al.*, 2001), signs of activation (Bielekova *et al.*, 1999) and enrichment in the naïve CD4+ T cell compartment (Muraro *et al.*, 2000). An interesting question is if the increased autologous turnover that we describe here, also leads to conversion to memory phenotype in autoreactive T cells, which would explain the increased numbers of MBP-specific memory cells in patients with multiple sclerosis (Burns *et al.*, 1999). In line with this speculation, investigations of the CD8+ T cell compartment show that T cell numbers and diversity decrease with age, surviving T cells undergo faster rates of homeostatic proliferation, are selected for high T cell receptor avidities, and preferably acquire 'memory-like' phenotype (Rudd *et al.*, 2011). The relatively early thymic involution that was described in multiple sclerosis (Duszczyszyn *et al.*, 2010) could lead to an increased demand for compensatory peripheral homeostasis in the CD4+ compartment as observed in the autologous proliferation setting. A combination of the presented HLA haplotype-specific self-peptides and structural features of the HLA-DR15 haplotype may lead to alterations in the overall strength and quality of proliferation with the consequence of selecting an 'autoimmune-prone' T cell repertoire.

Another important aspect is the question if only DRB1*15:01 or also DRB5*01:01 determines multiple sclerosis risk. A number of studies indicate the former, i.e. that risk is only conferred by DRB1*15:01 (Caillier *et al.*, 2008). We favour that both alleles contribute jointly to multiple sclerosis risk based on the similarities of the peptide binding motifs (Vogt *et al.*, 1994; Wucherpfennig *et al.*, 1994), which lead to presentation of similar peptides including the immunodominant $MBP_{83–99}$ peptide, which can be presented equally well by both alleles (Martin *et al.*, 1991; Valli *et al.*, 1993; Wucherpfennig *et al.*, 1994). Also, humanized transgenic mice expressing a multiple sclerosis patient-derived $MBP_{83–99}$-specific T cell receptor and DR2a (DRB5*01:01) develop spontaneous experimental autoimmune encephalomyelitis (Quandt *et al.*, 2012) as do transgenic mice expressing a $MBP_{84–102}$-specific T cell receptor and DR2b (DRB1*15:01) (Madsen *et al.*, 1999). Furthermore, myelin-specific- and virus-specific T cell clones, which recognize the same and different peptides in the context of both DR2a and DR2b, have been described (Lang *et al.*, 2002; Sospedra *et al.*, 2005), and that their cross-restriction leads to higher antigen avidity (Sospedra *et al.*, 2006; Yousef *et al.*, 2012). Here, we observe that the majority of HLA-derived polymorphic peptides that stimulate T cell proliferation are derived from DR2b (e.g. peptides 4 and 8), however, when examining their peptide binding to the two DR molecules, both show stronger affinity, are restricted by and have been eluted from DR2a (Fig. 6). We observed the reverse with peptide 45. Finally, we found non-polymorphic peptides, i.e. peptide 54 derived from DRA1*, which is predicted to bind better to DR2b and which we had also previously eluted from DR2b (Fig. 6C), although the bulk CD4+ T cells recognize the peptide when presented by DR2a (Fig. 6A). These data do not answer definitively the above question, which of the two alleles is more relevant for shaping an autoimmune-prone T cell repertoire in DR15+ patients with multiple sclerosis, but they support the notion that both are involved.

Taken together, our observations are in line with previous reports describing early thymic involution and repertoire narrowing in patients with multiple sclerosis. The subsequent demand for an increased peripheral maintenance and the accompanying effects on T cell receptor repertoire selection are reflected not only in the enhanced-, but also in a more heterogeneous proliferation pattern. Further, we demonstrate that the HLA-DR15 haplotype-derived self-peptides and/or structural features of the two HLA-DR molecules of the DR15 haplotype are likely involved in increased autologous proliferation. It remains to be shown if T cells that are expanded by autologous proliferation have the propensity to cross-react with myelin- or CNS autoantigens as indicated by (Cai and Hafler, 2007), can be activated by peptides from foreign agents and if the autologous proliferation leads to preferential differentiation into proinflammatory T cells. There are probably

additional factors that contribute to the proliferation patterns including cytokine receptors, cytokines, costimulatory molecules and their ligands, CD4-HLA-class II co-receptor interactions, and integrin/adhesion molecules, which may all be involved in regulating the threshold for T cell activation, expansion or functional differentiation. Interestingly, single nucleotide polymorphisms of a number of these (IL7RA, IL2RA, IL7, CD58, CBLB, CD40, IL12B, IL22RA2, and others) have been found to be involved in multiple sclerosis risk (Sawcer *et al.*, 2011). Based on our observations and the genetic data, we propose the following model. The two DR alleles in the DR15 haplotype and as yet unknown self-peptides select by central tolerance mechanisms a T cell receptor repertoire that has the inherent propensity to recognize CNS autoantigens and lead to a CNS-specific autoimmune T cell response. Positively selected T cells are maintained and/or expanded by peripheral homeostatic proliferation, which again involves the two DR15 alleles and according to our data among the spectrum of self-peptides also those derived from the DR15 haplotype themselves. Both a genetically predetermined lower activation threshold and certain environmental triggers then finally lead to full activation and expansion of CNS-autoreactive T cells, their proinflammatory differentiation and eventually the development of multiple sclerosis.

Acknowledgements

We thank Marianne Mohme for continuous encouragement, Vera Rosbroj for excellent technical support, Stefan Gold, PhD, for helpful discussions, Ivan Jelcic, PhD, for carefully reading the manuscript, the Gemeinnützige Hertie-Stiftung for supporting the inims.

Funding

The Clinical Research Priority Program MS ($CRPP^{MS}$), University Hospital- and University Zurich, supports M. Okoniewski at the Department of Neuroimmunology and MS Research, University of Zurich.

Supplementary material

Supplementary material is available from *Brain* online.

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