Identification of CD4 T-Cell Epitopes in Soluble Liver Antigen/Liver Pancreas Autoantigen in Autoimmune Hepatitis

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See Al-Chalabi T et al on page 1389 in CGH.

Background & Aims: Autoimmune hepatitis (AIH) is a chronic inflammatory liver disease associated with autoantibodies and liver-infiltrating lymphocytes. Although autoantibodies are tested routinely to diagnose and classify AIH, liver-infiltrating lymphocytes are regarded as the primary factor for disease pathogenesis. The purpose of this study was to identify and characterize autoantigenic peptides within human AIH-specific soluble liver antigen/liver pancreas antigen (SLA/LP) that are targeted by CD4+ T cells and restricted by the disease susceptibility gene HLA-DRB1*0301.

Methods: HLA-DRB1*0301 transgenic mice were immunized with SLA/LP. Antibody and T-cell responses were analyzed with SLA/LP-overlapping peptides in enzyme immunoassay, proliferation, and enzyme-linked immunospot (ELISpot) assays. Minimal optimal T-cell epitopes were identified, characterized with cloned T-cell hybridomas, and confirmed in tetramer and ELISpot assays with AIH patients’ peripheral blood mononuclear cells.

Results: All mice developed SLA/LP-specific IgG1/IgG2a antibodies against the same SLA/LP peptides as human beings. T cells targeted several peptides within SLA/LP, 2 of which were DR3-restricted and one overlapped the sequence recognized by human autoantibodies. Minimal optimal epitopes were mapped, DRB1*0301/epitope-tetramers were generated, and the frequency and function of HLA-DRB1*0301-restricted autoantigen-specific T cells in AIH patients were analyzed with tetramer and interferon-γ ELISpot assays.

Conclusions: This study identified T-cell epitopes within SLA/LP, restricted by the disease susceptibility gene DRB1*0301 and in close proximity to the human autoantibody epitope. These results and the generated reagents now provide the opportunity to directly monitor autoreactive T cells in AIH patients in clinical studies.

Autoimmune hepatitis (AIH) is a chronic inflammatory liver disease that has a prevalence of approximately 1 in 10,000 in the United States and Europe and accounts for 2%–6% of all liver transplantations.1 AIH is diagnosed by increased alanine aminotransferase levels, an intrahepatic lymphocytic mononuclear cell infiltrate, hypergammaglobulinemia, and autoantibodies, and the absence of liver disease of viral, toxic, or metabolic etiology.2,3

Autoantibodies target nuclear antigens (antinuclear antibodies), smooth muscle antigens, liver-kidney microsomes, and/or soluble liver antigen/liver pancreas antigen (SLA/LP). Autoantibodies to SLA/LP are detectable in about 20% of AIH cases,4,5 and are unique in that they are the only autoantibodies specific for AIH.4 The target of SLA/LP autoantibodies has been cloned6 and named SEPEC5 by the Nomenclature Commission of the Human Genome Organization. However, even though autoantibodies are well accepted as serologic markers for diagnosis and classification of AIH, it is still unclear whether they play any role in disease pathogenesis.7

The degree of the intrahepatic lymphocytic infiltrate, the histologic hallmark of AIH, correlates well with disease progression and is therefore thought to contribute directly to disease pathogenesis. The clonal restriction of the intrahepatic T-cell population and the observation that HLA-DBR1*0301 and HLA-DBR1*0401 predispense to AIH and influence disease severity,8,9 have led to the hypothesis that intrahepatic CD4+ T cells recognize self-antigens in the context of HLA-DBR1*0301 and HLA-DBR1*0401.2 HLA-DRB1*0301, the principal AIH susceptibility allele among white Europeans and Americans,8,9 and HLA-DRB1*0401 may even present the same autoantigen to CD4+ T cells because both alleles share an

Abbreviations used in this paper: aa, amino acid; AIH, autoimmune hepatitis; ELISpot, enzyme-linked immunospot; EMA, ethidium monooxide; IL, interleukin; PBMC, peripheral blood mononuclear cell; SLA/LP, soluble liver antigen/liver pancreas antigen.

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amino acid motif in their antigen binding groove (shared binding hypothesis).\textsuperscript{10}

Despite much effort, it still remains unknown which autoantigens HLA-DRB1*0301 and HLA-DRB1*0401-restricted CD4\textsuperscript{+} T cells recognize in AIH. This question has been declared a high priority investigational challenge,\textsuperscript{11} but has proven difficult to answer for the following reasons. As in other autoimmune diseases,\textsuperscript{12} the frequency of autoreactive CD4\textsuperscript{+} T cells in the peripheral blood is too low to screen a large number of candidate peptides for T-cell recognition. Autoreactive CD4\textsuperscript{+} T cells are presumably more frequent at the site of inflammation, but only a small piece of tissue and thereby only a small number of lymphocytes can be obtained by liver biopsy. It therefore remains necessary to expand and/or clone liver-biopsy–derived T cells in vitro before they can be studied in functional assays.\textsuperscript{13} This approach renders an ex vivo assessment of the number and function of autoantigen-specific T cells impossible. Furthermore, assays that solely rely on the function of autoantigen-specific T cells are often compromised because of immunosuppressive therapy of AIH.

We therefore immunized humanized mice, which are transgenic for HLA-DRB1*0301 and do not express murine major histocompatibility complex class II,\textsuperscript{14} with the recently cloned SLA/LP autoantigen\textsuperscript{6} and established T-cell hybridomas to identify the optimal DRB1*0301-restricted sequences (epitopes) within SLA/LP (Figure 1). These epitopes then were used in enzyme-linked immuno-spot (ELISpot) assays and in flow cytometry assays with custom-made fluorescently labeled tetrameric DRB1*0301 complexes to monitor AIH-specific T-cell responses in the blood of patients with AIH. The identification of these T-cell epitopes also may be a step towards the identification of those immunogenic self- and/or cross-reactive foreign antigens that set off the initial autoimmune reaction.

### Materials and Methods

**SLA/LP Protein, Peptides, Tetramer, and Antibodies**

Recombinant human SLA/LP (AAD33963) was synthesized and purified as previously described.\textsuperscript{6} Twenty-mer peptides overlapping by 12 amino acids (aa) and spanning aa 1–422 of human SLA/LP (Mimotopes, Clayton, Australia), shorter SLA/LP peptides, and the DRB1*0301-restricted hepatitis C virus (HCV) peptide...
HCV-261-269 (VLVNLPSVA)\textsuperscript{15} (Facility for Biotechnology Resources, CBER, FDA, Bethesda, MD) were resuspended at 20 mg/mL in dimethyl sulfoxide and diluted with phosphate-buffered saline solution (PBS) to 2 mg/mL. Tetramers DRB1*0301-SLA/LP\textsubscript{84-198} and DRB1*0301-SLA/LP\textsubscript{373-386} were custom-made by Beckman Coulter (Fullerton, CA).

Anti-mouse CD4-phycoerythrin (PE) or -fluorescein isothiocyanate (APC)-Cy7 (clone 129.19), CD3e-allophycocyanin (APC)-Cy7 (clone 145-2C11), CD19-PE-Cy5 or -PerCP (clone HIB 19), as well as purified antibodies to HLA-DR, -DP, -DQ (clone 3G8), HLA-DR-fluorescein isothiocyanate (clone RPA T-8), CD14-PerCP (clone MUR38), and CD16-PE-Cy5 (clone 70-1, from BD Pharmingen) were purchased from BD (San Diego, CA). Anti-human CD14-PE-Cy5 or -peridinin chlorophyll protein (PerCP) (clone SK3), CD25-APC-Cy7 (clone MA 251), CD8-PE-Cy5 or -fluorescein isothiocyanate (clone P9), CD16-PE-Cy5 (clone H9021), and CD19-PE (clone 1D3), CD11a/CD11c (clone H57-597; eBiosciences, San Diego, CA) was added per well. Cells were harvested with a Packard Filtermate 96-well-harvester (Perkin Elmer, Wellesley, MA) and incorporated radioactivity was measured with a Packard Topcount (Perkin Elmer).

**Analysis of the Humoral Immune Response**

SLA/LP-specific antibodies were detected with the QUANTA Lite SLA/LP enzyme immunoassay (EIA) kit (Inova Diagnostics, San Diego, CA) according to the manufacturer’s instructions except for the use of a horseradish-peroxidase–labeled polyclonal goat anti-mouse Ig (BD Pharmingen, dilution 1:2000) for detection. Isotyping was performed with a mouse immunoglobulin screening/isotyping kit (Zymed Laboratories, San Francisco, CA). Fine mapping of the antibody specificity was performed by EIA with 1/100 dilutions of sera using microtiter plates (Falcon) coated with SLA/LP-overlapping peptides (10 μg/mL).

**Mouse Immunization**

C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) and HLA-DRB1*0301-transgenic mice that do not express endogenous class II at detectable levels (DR3.AB0)\textsuperscript{14} were maintained in a clean conventional facility. Experiments were approved by the Animal Care and Use Committee of the National Institute of Diabetes and Digestive and Kidney Diseases.

Eight- to 12-week-old mice were immunized at the base of the tail and both hind foot pads with the following: (1) 40 μg of human recombinant SLA/LP (11 mice) (2) 50 μg of peptides SLA/LP\textsubscript{84-100} SLA/LP\textsubscript{89-108}, and SLA/LP\textsubscript{97-116} (5 mice), (3) 50 μg of peptides SLA/LP\textsubscript{177-196} and SLA/LP\textsubscript{185-204} (5 mice), or (4) 50 μg of peptides SLA/LP\textsubscript{34-41} and SLA/LP\textsubscript{68-48} (5 mice), in 100–200 μL PBS emulsified with an equal amount of AS01B adjuvant (GlaxoSmithKline, Rixensart, Belgium).\textsuperscript{16} Biotin-conjugated anti-mouse IL-2 antibody (2 μg/mL, clone JES6-1A12; BD Pharmingen). Biotin-conjugated anti-mouse IL-2 antibody (2 μg/mL, clone JES6-SH4; BD Pharmingen) and 3-amino-9-ethylcarbazole substrate solution (Sigma–Aldrich) were used for detection. Spots were counted with a KS ELISpot Reader (Carl Zeiss Inc, Thornwood, NY).

**Tetramer analysis.** CD4\textsuperscript{+} T cells were negatively isolated from spleen and lymph node cells with magnetic beads and magnetic cell sorting columns (Miltenyi Biotec, Auburn, CA), incubated for 10 minutes with anti-CD16/anti-CD32 as Fc-block (clone 2.4G2; BD Pharmingen), washed, and stained with 2 μg/mL anti-mouse interleukin-2 (IL-2) (clone JES6-1A12; BD Pharmingen). Biotin-conjugated anti-mouse IL-2 antibody (2 μg/mL, clone JES6-SH4; BD Pharmingen) and 3-amino-9-ethylcarbazole substrate solution (Sigma–Aldrich) were used for detection. Spots were counted with a KS ELISpot Reader (Carl Zeiss Inc, Thornwood, NY).
**Generation of T-cell hybridomas.** Sixty million spleen cells of SLA/LP-immunized mice were stimulated with 5 μg/mL SLA/LP or 10 μg/mL of the immunization peptides in 5 mL culture medium. Four days later, cells were fused to BWZ.36 (kindly provided by Nilabh Shastri, University of California, Berkeley, CA), a TCRαβ- cell line transfected with the β-galactosidase (LacZ) reporter gene under the control of the NFAT enhancer element of the IL-2 gene. SLA/LP-specific hybridomas were subcloned at 0.3 cells/well.

**Hybridoma activation and LacZ assays.** Duplicate or triplicate cultures of 0.2–1 × 10⁶ T-cell hybridomas were stimulated overnight with 2 × 10⁵ irradiated (3000 rad) spleen cells from HLA-DRB1*0301 transgenic mice with or without SLA/LP peptides at the indicated concentrations and with or without 2.5 or 10 μg anti-I-A/anti-I-E (clone 2G9l BD Pharmingen), anti-HLA-DR, anti-HLA-DP, anti-HLA-DQ (TU39; BD Pharmingen), or isotype control antibodies. TCR activation resulted in activation of the NFAT enhancer element of the IL-2 gene and transcription of the LacZ reporter gene. To measure LacZ activity, cultures were washed extensively and lysed with 100 μL buffer (1 mmol/L MgCl₂, 0.125% NP-40 [Calbiochem, La Jolla, CA] and 0.15 mmol/L chlorophenol red β-galactoside [Roche] in PBS). After 8 hours at 37°C, the reaction was stopped with 100 μL buffer (300 mmol/L glycine and 15 mmol/L Na₂–ethylenediaminetetraacetic acid in water, pH 12). Absorption was read at 575 nm with a 96-well Benchmark Microplate reader (Bio–Rad, Hercules, CA) with 620 nm as the reference wavelength.

**Detection of SLA/LP-Specific CD4⁺ T Cells in Human Beings**

Peripheral blood mononuclear cells (PBMCs) were isolated from 9 patients with anti-SLA/LP AIH and, as negative controls, from an HLA-DRB1*0301 patient with anti-SLA/LP AIH and 2 HLA-DRB1*0301, anti-SLA/LP patients with a past history of hepatitis B (anti-hepatitis B surface antigen positive, anti–hepatitis B e antigen positive, hepatitis B surface antigen negative, hepatitis B e antigen negative). Patients were followed up either at Johannes Gutenberg Universität (Mainz, Germany), Universitätsklinikum Hamburg Eppendorf (Hamburg, Germany), Medizinische Universitätsklinik (Freiburg, Germany), or the Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (Bethesda, MD), and provided informed consent under protocols approved by the Ethics Committee and Institutional Review Board of the respective institutions.

Thawed PBMCs were stained with 2.5 μg/mL EMA under a fluorescent light source for 10 minutes, washed in PBS, and stained with 10 μL of either the DRB1*0301-SLA/LP373-386 tetramer or the DRB1*0301-SLA/LP184-196 tetramer (both coupled to PE) in complete RPMI medium for 30 minutes at 37°C. After washing in PBS, cells were stained with antibodies against CD3, CD8, CD14, and CD19 and CD4 antibodies for 20 minutes at 4°C, washed, and subsequently treated with anti-PE magnetic beads (Miltenyi Biotec) for 15 minutes at 4°C. Ten per-

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**Figure 2.** Humoral immune response of SLA/LP-immunized HLA-DRB1*0301-transgenic mice. (A and B) HLA-DRB1*0301 transgenic mice (n = 15) were immunized with adjuvanted SLA/LP on days 0 and 21. (A) SLA/LP-specific antibody responses were quantified at the indicated time points and (B) studied for isotype distribution on day 42. The dashed horizontal line indicates the cut-off level of positivity. (C) Fine mapping of the antibody response of immunized HLA-DRB1*0301 transgenic (filled bars) and C57BL/6 mice (open bars). Mean and SD of the results of 3 mice are shown in B and C. A P value of less than .05 (Student t test) was considered significant.
cent of the cells were analyzed directly by flow cytometry (pre-enrichment sample), the remaining 90% were applied to MS columns (Miltenyi Biotec) to enrich PE-conjugated tetramer+ cells (postenrichment sample). After exclusion of doublets by flow cytometry, forward and side-scatter gating of lymphocytes and exclusion of EMA/CD8/CD14/CD19+ events, the CD3+ T-cell population was analyzed for expression of CD4 and for tetramer binding.

For proliferation assays, \(10^7\) PBMCs were stained with 1 \(\mu\)mol/L carboxyfluorescein diacetate succinimidylester (Molecular Probes, Eugene, OR) in 2 mL PBS for 5 minutes at room temperature. After addition of 5 mL fetal calf serum and 3 washes in PBS, cells were stimulated with 10 \(\mu\)g/mL DRB1*0301-restricted SLA/LP373–386 peptide or the DRB1*0301-restricted HCV261–269 control peptide,15 respectively. Forty U/mL of recombinant IL-2 was added on days 4 and 7. On day 10, cells were stained with EMA, the respective DRB1*0301/epitope-tetramer and antibodies against cell surface proteins, and analyzed by flow cytometry. After EMA/CD8/CD14/CD16/CD19 exclusion, CD3+CD4+ lymphocytes were analyzed for tetramer binding and carboxyfluorescein diacetate succinimidylester content.

For interferon-\(\gamma\) ELISpot assays, CD4+ T cells were isolated with the Miltenyi Multisort Kit (Miltenyi Biotec), released from the beads, stained with the DRB1*0301-SLA/LP373–386 tetramer or the DRB1*0301-SLA/LP184–196 tetramer as described earlier, and sorted manually with anti-PE beads (Miltenyi Biotec) following the manufacturer’s protocol. Part of the tetramer-enriched cell population was used directly in an ELISpot, the other part was expanded with 40 ng/mL anti-CD3 under a twice-weekly addition of 200 U/mL IL-2, 10 ng/mL IL-7, and 100 ng/mL IL-15 before ELISpot analysis. Interferon-\(\gamma\) ELISpot assays were performed as described19 using 60,000–300,000 tetramer-enriched cells and 200,000 irradiated (3000 rad) autologous tetramer-negative PBMCs as feeder cells in the presence or absence of 10 \(\mu\)g/mL peptide.

Results

Humoral Immune Response of HLA-DRB1*0301 Transgenic Mice to Human Recombinant SLA/LP

Sixteen DRB1*0301-transgenic mice were immunized subcutaneously with human recombinant SLA/LP and AS01B adjuvant.16 As shown in Figure 2A, SLA/LP-
specific antibodies were detectable after a single immunization and increased further after a booster immunization. Isotyping revealed a large IgG1, IgG2b, and IgG3 fraction (Figure 2B) consistent with a strong Th2 CD4+ T-cell response in the context of this adjuvant.16 Because the humoral immune response of patients with AIH is targeted against the carboxyterminus of SLA/LP,4 antibodies against this region were mapped with overlapping 20mer peptides. As shown in Figure 2C, the 20mer peptide spanning aa 403–422 elicited the most vigorous response in HLA-DRB1*0301-transgenic mice. This peptide was recognized significantly less vigorously by nontransgenic C57BL/6 mice, suggesting a role of HLA-DR-restricted CD4+ T-cell help in the generation of these autoantibodies.

Cellular Immune Response to SLA/LP in HLA-DRB1*0301 Transgenic Mice

Spleen-derived T cells of SLA/LP-immunized HLA-DRB1*0301-transgenic mice proliferated (Figure 3A) and produced IL-2 (Figure 3B) in a dose-dependent manner in response to SLA/LP. When T-cell responses of SLA/LP-immunized mice were assessed with a panel of overlapping 20mer peptides, positions 1–28, 49–76, 81–116, 137–156, 177–220, 233–284, 289–316, 329–356, 361–404, and 400–422 were identified as immunogenic regions within the SLA/LP protein (Figure 3C).

To fine-map the immunodominant regions, T cells of SLA/LP-immunized HLA-DRB1*0301-transgenic mice were fused with the lymphoma cell line BWZ.36. The generated CD4+ T-cell hybridomas recognized peptides spanning SLA/LP positions 1–20, 257–276, and 369–388, respectively, indicating that these sequences were immunodominant (Figure 2C). To increase the efficiency of T-cell hybridoma generation against sequences that were recognized less vigorously, additional groups of 5 HLA-DRB1*0301-transgenic mice subsequently were immunized with peptide mix SLA/LP81–100/SLA/LP89–108/SLA/LP97–116, peptide mix SLA/LP177–196/SLA/LP185–204, and peptide mix SLA/LP403–416/SLA/LP408–418, respectively. This strategy generated CD4+ T-cell hybridomas against SLA/LP177–196/SLA/LP185–204. No T-cell hybridomas were generated for SLA/LP81–116 and SLA/LP400–422, indicating that CD4 T cells with these specificities were at low frequency or did not respond well to in vitro stimulation.

Fine Specificity of the SLA/LP-Specific T Cells

Stimulation of T-cell hybridoma with their cognate peptide activated the β-galactosidase reporter gene and resulted in a color reaction. The immunogenic 20mer peptide SLA/LP369–388 was of specific interest (Figure 4A, top bar) because it overlapped with the SLA/LP sequence that is recognized by antibodies of patients with AIH.4 T-cell recognition increased further when 3 amino acids were removed from the aminoterminus and 2 or 3 amino acids were removed from the carboxyterminus (Figure 4A). T-cell recognition was abrogated, however, on further stepwise truncation of the peptide and peptide SLA/LP373–386 NRLDRCLVKVR was identified as the optimal epitope (Figure 4A, underlined sequence) and con-
firmed in dose-titration experiments (Supplementary Figure 1A; see Supplementary material online at www.gastrojournal.org). To analyze major histocompatibility complex restriction, the same T-cell hybridoma then was stimulated with the optimal peptide and DRB1*0301+ spleen cells in the context of I-A/I-E antibody, HLA-DR antibody, or an isotype control. Recognition of peptide SLA/LP184–197 was inhibited by HLA-DR antibodies, but not by I-A/I-E antibodies and isotype controls (Figure 4B), indicating that it was restricted by HLA-DR.

A second DRB1*0301-restricted optimal epitope was mapped in a similar way within the sequence of SLA/LP184–204 (Figure 4C). Stepwise amino- and carboxyterminal truncation identified peptide SLA/LP186–197 LIQQGARVGRID as the optimal epitope (Figure 4A, Supplementary Figure 1B; see Supplementary material online at www.gastrojournal.org) because deletion of either its aminoterminal lysine or its carboxyterminal aspartic acid reduced recognition. Antibody-blocking experiments revealed that this epitope also was restricted by HLA-DR (Figure 4D).

Next, we mapped the fine specificity of SLA/LP257–276–specific hybridomas (Supplementary Figure 2A, top bar; see Supplementary material online at www.gastrojournal.org). Analysis of the 2 shorter overlapping peptides SLA/LP257–268 and SLA/LP262–276 clearly showed that immunogenicity resided in the carboxyterminal sequence (Supplementary Figure 2A, third bar). Removal of an arginine and lysine from the aminoterminus and a lysine from the carboxyterminal did not significantly reduce activation of the T-cell hybridomas. Recognition was abolished by further removal of glutamic acid from the aminoterminus, revealing SLA/LP264–275 EMFSYSNQIKK as the optimal epitope (Supplementary Figure 2A and B; see Supplementary material online at www.gastrojournal.org). In contrast to the 2 epitopes identified in Figure 4, this epitope was not restricted by HLA-DR because anti–I-A/anti–I-E rather than anti–HLA-DR antibodies blocked its recognition. Although DR3.AB0 transgenic mice do not express endogenous class II at a detectable level, they do express the murine Eb gene.14 It is therefore possible that low-level expression of a DRα1/EB fusion molecule was responsible for the presentation of this peptide.

Finally, using the same technique, the immunogenic epitope within SLA/LP1–20 was identified as SLA/LP7–18 CFWRRIFHGIGR (not shown). The sequence of this epitope is not present in the major SLA/LP splice variant (NM_153825), but only in the less-expressed splice variant derived from a Jurkat-cell complementary DNA library (NM_016955).6 Because expression of the minor variant in liver is uncertain and the relevance of its recognition by liver-derived T cells thus is questionable, this epitope was not evaluated further.

In summary, the use of a humanized, HLA-DRB1*0301-transgenic mouse model resulted in the identification of 2 HLA-DR–restricted SLA/LP epitopes that are recognized by CD4+ T cells and one of these epitopes (Figure 4A and B) overlapped the sequence of the major B-cell autoantigen that is recognized by patients with anti-SLA/LP+ AIH.

Figure 5. Verification of the DRB1*0301-SLA/LP373–386 tetramer and DRB1*0301-SLA/LP184–198 tetramer. (A) DRB1*0301-SLA/LP373–386 tetramer and (B) DRB1*0301-SLA/LP184–198 tetramer. CD4+ T cells were detectable in spleens of SLA/LP-immunized, but not SLA/LP-nonimmunized, DRB1*0301-transgenic mice, CD62L and CD11a expression were compared on tetramer+ and tetramer− CD4+ T cells. Numbers indicate the percentage of positive cells.
Generation of DRB1*0301-SLA/LP373–386 and DRB1*0301-SLA/LP184–198 Tetramers to Detect SLA/LP-Specific CD4+ T Cells

Next, we investigated whether the identified HLA-DR–restricted epitopes could be used to detect SLA/LP-specific CD4+ T cells with fluorescently labeled tetrameric DRB1*0301 complexes. DRB1*0301-tetramers were synthesized for epitope SLA/LP373–386 and attempted for epitope SLA/LP184–198. Because the latter tetramer did not fold with the minimal epitope

Figure 6. Detection of tetramer+ SLA/LP-specific CD4+ T cells in human beings. (A) Gating strategy. After exclusion of doublets (left graph), gates were set on lymphocytes based on forward and side scatter (second graph from left), and on CD3+ cells after exclusion of CD8+ T cells, CD16+ monocytes, and CD19+ B cells (second gate from right). Staining of (B) CD4+ tetramer+ T cells ex vivo and (C) after enrichment with anti-PE–coupled magnetic beads. PBMCs were carboxyfluorescein diacetate succinimidylester–labeled and stimulated with either the (D) SLA/LP373–383 peptide or an (E) DRB1*0301-restricted HCV peptide. Proliferation of DRB1*0301-SLA/LP373–386+ and DRB1*0301-SLA/LP184–198+ cells were compared. Numbers indicate the percentage of events in the respective gate or quadrant.
SLA/LP<sub>186-197</sub>, we chose a longer precursor peptide, SLA/LP<sub>184-198</sub>, instead.

Both the DRB1*0301-SLA/LP<sub>373-386</sub>-tetramer and the DRB1*0301-SLA/LP<sub>184-198</sub>-tetramer were first titrated and validated with CD4<sup>+</sup> T cells of immunized HLA-DRB1*0301-transgenic mice. A large DRB1*0301-SLA/LP<sub>373-386</sub>-tetramer CD4<sup>+</sup> T-cell population was identified in the spleens of 2 representative DRB1*0301-transgenic, immunized mice, but not in a DRB1*0301-transgenic, nonimmunized control mouse (Figure 5A). A CD4<sup>+</sup> T-cell population of similar size was detected with the second DRB1*0301-SLA/LP<sub>184-198</sub>-tetramer (Figure 5B). Both DRB1*0301-tetramer<sup>+</sup> CD4<sup>+</sup> T-cell populations expressed CD11a and, when compared with DRB1*0301/epitope-tetramer<sup>+</sup> CD4<sup>+</sup> T cells, had down-regulated CD62L expression, indicating loss of their lymph node homing potential as expected for antigen-induced effector T cells. Serum alanine aminotransferase levels reflected homing potential as expected for antigen-induced effector T cells. Serum alanine aminotransferase levels reflected and binding of the respective tetramer (Figure 6A). Both the DRB1*0301-SLA/LP<sub>373-386</sub>-tetramer and the DRB1*0301-tetramer<sup>+</sup> CD4<sup>+</sup> T cells had down-regulated CD62L expression, indicating loss of their lymph node homing potential as expected for antigen-induced effector T cells. Serum alanine aminotransferase levels remained within the upper limit of normal and no significant lymphocyte infiltrate was detected in the liver (not shown).

**Detection of SLA/LP-Specific CD4<sup>+</sup> T Cells in the Blood of AIH Patients**

As a proof of principle, we also studied PBMCs from patients with anti-SLA/LP<sup>+</sup> AIH using the DRB1*0301-SLA/LP<sub>373-386</sub> and DRB1*0301-SLA/LP<sub>184-198</sub>-tetramers. As specificity controls, PBMCs from an HLA-DRB1*0301-patient with anti-SLA/LP<sup>+</sup> AIH and 2 HLA-DRB1*0301-anti-SLA/LP<sup>+</sup> patients with a past history of hepatitis B were studied in parallel. After gating on single cells and lymphocytes, EMA<sup>+</sup>/CD8<sup>+</sup>/CD14<sup>+</sup>/CD19<sup>+</sup> cells were excluded and the CD3<sup>+</sup> cells were analyzed for CD4 expression and binding of the respective tetramer (Figure 6A and B). Magnetic bead enrichment concentrated tetramer<sup>+</sup> cells by a factor of 23-1042 (Table 1). Overall, tetramer<sup>+</sup> CD4<sup>+</sup> T cells were detected in PBMCs of 7 of 9 AIH patients after enrichment with anti-PE-labeled magnetic beads as compared with 0 of 3 (0%) control patients ($P = 0.045$, Fisher exact test, Table 1). When carboxyfluorescein diacetate succinimidylester–labeled PBMCs were stimulated with the cognate SLA/LP<sub>373-386</sub> peptide more tetramer<sup>+</sup> cells than tetramer<sup>+</sup> cells proliferated (47.0% vs 6.2%). In contrast, only 3.7% tetramer<sup>+</sup> cells proliferated on stimulation with the unrelated, DRB1*0301-restricted HCV peptide HCV<sub>261-269</sub> (Figure 6E).<sup>15</sup>

Finally, the newly identified CD4<sup>+</sup>T-cell epitopes also were tested in interferon-γ ELISpot assays. As shown in Figure 7, tetramer-enriched CD4<sup>+</sup> T cells of HLA-DRB1*0301-positive AIH patients recognized both epitopes and produced interferon-γ, but tetramer-enriched CD4<sup>+</sup> T cells of HLA-DRB1*0301-negative AIH patients or DRB1*0301-positive controls without AIH did not. These cells were tested ex vivo and after several weeks of antigen-nonspecific in vitro expansion with 40 ng/mL anti-CD3, 200 U/mL IL-2, 10 ng/mL IL-7, and 100 ng/mL IL-15. The rapid, antigen-nonspecific expansion protocol was chosen over a peptide-specific expansion protocol because of the very low number (<10<sup>5</sup>) of tetramer-positive cells with which the expansion was started. ELISpot assays with the nonspecifically expanded T-cell lines (Figure 7B, filled symbols) confirmed the ex vivo ELISpot results (Figure 7B, open symbols).

**Discussion**

We here report that SLA/LP is not only targeted by humoral but also by cellular immune responses and

### Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Sex</th>
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<th>AST level (U/L)</th>
<th>IgG level</th>
<th>Treatment</th>
<th>Ex vivo</th>
<th>Enriched&lt;sup&gt;a&lt;/sup&gt; Factor&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ex vivo</th>
<th>Enriched&lt;sup&gt;a&lt;/sup&gt; Factor&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Patients with anti-SLA&lt;sup&gt;+&lt;/sup&gt;AIH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>23</td>
<td>anti-SLA/LP&lt;sup&gt;+&lt;/sup&gt; anti-LKM1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>F</td>
<td>18</td>
<td>16</td>
<td>11.5</td>
<td>Tacrolimus, mycophenolate</td>
<td>0.01%</td>
<td>0.40%</td>
<td>51</td>
<td>0.00%</td>
</tr>
<tr>
<td>2</td>
<td>56</td>
<td>anti-SLA/LP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>F</td>
<td>23</td>
<td>19</td>
<td>17.9</td>
<td>Predisilone</td>
<td>0.00%</td>
<td>0.12%</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>3</td>
<td>77</td>
<td>anti-SLA/LP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>F</td>
<td>72</td>
<td>32</td>
<td>20.3</td>
<td>Predisilone</td>
<td>0.00%</td>
<td>0.05%</td>
<td>53</td>
<td>0.01%</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>anti-SLA/LP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>F</td>
<td>65</td>
<td>41</td>
<td>11.7</td>
<td>Predisilone</td>
<td>0.01%</td>
<td>1.32%</td>
<td>152</td>
<td>0.01%</td>
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<td>5</td>
<td>25</td>
<td>anti-SLA/LP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>F</td>
<td>18</td>
<td>24</td>
<td>15.1</td>
<td>Azathioprine, prednisone</td>
<td>0.00%</td>
<td>2.11%</td>
<td>1042</td>
<td>0.00%</td>
</tr>
<tr>
<td>6</td>
<td>63</td>
<td>anti-SLA/LP&lt;sup&gt;+&lt;/sup&gt; anti-SMA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>F</td>
<td>53</td>
<td>28</td>
<td>22.3</td>
<td>None</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0</td>
<td>0.00%</td>
</tr>
<tr>
<td>7</td>
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<td>anti-SLA/LP&lt;sup&gt;+&lt;/sup&gt; anti-ANA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>F</td>
<td>61</td>
<td>48</td>
<td>NA</td>
<td>Azathioprine, prednisone</td>
<td>0.00%</td>
<td>0.99%</td>
<td>199</td>
<td>0.00%</td>
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<tr>
<td>8</td>
<td>46</td>
<td>anti-SLA/LP&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>20</td>
<td>25</td>
<td>NA</td>
<td>Azathioprine, budenoside</td>
<td>0.01%</td>
<td>0.00%</td>
<td>0</td>
<td>0.00%</td>
</tr>
<tr>
<td>9</td>
<td>46</td>
<td>anti-SLA/LP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>F</td>
<td>18</td>
<td>22</td>
<td>NA</td>
<td>Azathioprine</td>
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<td>0.17%</td>
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<td>NA</td>
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<td>Controls</td>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>65</td>
<td>anti-SLA/LP-negative, HBV&lt;sup&gt;+&lt;/sup&gt;, HLA-DR3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>F</td>
<td>10</td>
<td>17</td>
<td>NA</td>
<td>None</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0</td>
<td>0.00%</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>anti-SLA/LP-negative, HBV&lt;sup&gt;+&lt;/sup&gt;, HLA-DR3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>M</td>
<td>17</td>
<td>18</td>
<td>NA</td>
<td>None</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0</td>
<td>0.00%</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>anti-SLA/LP&lt;sup&gt;+&lt;/sup&gt; not HLA-DR3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>M</td>
<td>40</td>
<td>78</td>
<td>NA</td>
<td>None</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0</td>
<td>0.00%</td>
</tr>
</tbody>
</table>

NA, not applicable; ND, not done.

<sup>a</sup>Refers to the DRB1*0301-SLA/LP<sub>184-196</sub> tetramer.

<sup>b</sup>Refers to the DRB1*0301-SLA/LP<sub>373-383</sub> tetramer.

<sup>c</sup>Enrichment was performed with magnetic beads unless otherwise indicated. Positive values are shown in bold and underlined.

<sup>d</sup>Frequency of tetramer<sup>+</sup> cells after enrichment divided by ex vivo frequency.

<sup>e</sup>Peptide-stimulated T-cell line.
describe 2 CD4\(^+\) T-cell epitopes that are recognized in a DRB1*0301-restricted manner. CD4\(^+\) T cells specific for these SLA/LP epitopes were detected in HLA-DRB1*0301-transgenic mice and in PBMCs of HLA-DRB1*0301–positive patients with AIH. Only HLA-DRB1*0301-transgenic, but not nontransgenic, mice mounted SLA/LP-specific autoantibodies of the same specificity as in AIH in human beings, which suggests that HLA-DRB1*0301-restricted CD4\(^+\) T cells shape the antibody repertoire in the context of the genetic background. Interestingly, one of the HLA-DR\(^-\)-restricted CD4\(^+\) T-cell epitopes (Figure 4A and B) was located in the carboxy-terminal sequence of SLA/LP overlapping with the sequence that is recognized by antibodies.\(^4,6\) This close proximity of T- and B-cell epitopes within the SLA/LP autoantigen appears to be a characteristic of autoimmune diseases.\(^12,20,21\) HLA-DRB1*0301–restricted, SLA/LP–specific, CD4\(^+\) T cells may, for example, help autoreactive B cells to produce autoantibodies and to secrete proinflammatory cytokines and chemokines that recruit plasma cells and CD8\(^+\) T cells into the liver. Vice versa, autoantigen-specific B cells may bind and internalize SLA/LP via specific autoantibodies on their cell surface, process it into short peptides, and present these peptides on DRB1*0301 molecules to stimulate CD4\(^+\) T cells.\(^22\)

The absence of liver disease in the SLA/LP-immunized HLA-DRB1*0301–transgenic mice was not unexpected. Recent studies on autoimmune diabetes have suggested that the frequency of autoantigen-specific T cells needs to be much higher (>80% of all CD8\(^+\) T cells in that study) and that additional inflammatory cytokine- and toll-like receptor-mediated signals are required to initiate autoimmune disease.\(^23\) Furthermore, all published T-cell–mediated autoimmune models in HLA-transgenic mice involve organs other than the liver,\(^24,25\) which is characterized by a constitutive tolerogenic microenvironment.\(^26\) Thus, additional factors, such as tissue destruction by unrelated mechanisms (toxic or infectious), might be necessary to induce intrahepatic inflammation and to trigger AIH. Because SLA/LP\(^6\) and liver-kidney microsomes, the second cloned autoantigen in AIH, are cytotoxic
enzymes, it is possible that specific enzyme-substrate interactions initiate hepatocyte injury and death, followed by transport of cell-associated autoantigens into the regional lymph nodes and priming of autoantigen-specific T and B cells.

In the analyzed patients with anti-SLA/LP+ AIH, the frequency of antigen-specific CD4+ T cells detectable by ELISpot and tetramer analysis was in the same range as frequencies reported for other autoimmune diseases such as type I diabetes and chronic infectious diseases such as Mycobacterium tuberculosis, Borrelia burgdorferi, and HCV infection. On immune-suppressive treatment, as was the case for the patients in our study, the frequency of autoreactive T cells may decrease further. In this scenario, enrichment of tetramer+ T cells with magnetic beads may increase the sensitivity of detection and allow their functional characterization.

The tetramer-staining profiles displayed a relatively low mean fluorescence intensity. Similar profiles have been reported for other major histocompatibility complex class II tetramers in the literature. In this context, it recently was shown that CD4+ T cells in blood, spleen, and lymph nodes differ in their major histocompatibility complex class II tetramer binding profiles, with tetramer+ T cells of lymph nodes and spleen showing higher mean fluorescence intensities than tetramer+ T cells in the blood. The identified SLA/LP-specific T-cell epitopes and their corresponding tetramers should therefore be useful to monitor AIH-specific T cells during acute AIH and active disease progression and to study their role in the liver, the site of this disease.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.07.029.

References


