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Identification of Novel Human Aggrecan T Cell Epitopes in HLA-B27 Transgenic Mice Associated with Spondyloarthropathy

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The pathology of ankylosing spondylitis (AS) and reactive arthritis, and other spondyloarthropathies (SpA) is closely associated with the human leukocyte class I Ag HLA-B27. A characteristic finding in SpA is inflammation of cartilage structures of the joint, in particular at the site of ligament/tendon and bone junction (enthesitis). In this study, we investigated the role of CD8⁺ T cells in response to the cartilage proteoglycan aggrecan as a potential candidate autoantigen in BALB/c-B27 transgenic mice. We identified four new HLA-B27-restricted nonamer peptides, one of them (no. 67) with a particularly strong T cell immunogenicity. Peptide no. 67 immunization was capable of stimulating HLA-B27-restricted, CD8⁺ T cells in BALB/c-B27 transgenic animals, but not in wild-type BALB/c mice. The peptide was specifically recognized on P815-B27 transfectants by HLA-B27-restricted CTLs, which were also detectable by HLA tetramer staining ex vivo as well as in situ. Most importantly, analysis of the joints from peptide no. 67-immunized mice induced typical histological signs of SpA. Our data indicate that HLA-B27-restricted epitopes derived from human aggrecan are involved in the induction of inflammation (tenosynovitis), underlining the importance of HLA-B27 in the pathogenesis of SpA. The Journal of Immunology, 2004, 173: 4859–4866.

Spondyloarthropathies (SpA), such as ankylosing spondylitis (AS) and reactive arthritis, are frequently occurring inflammatory rheumatic diseases. Although the etiology of these diseases remains obscure, there is strong evidence that T cells play an important role. The strong association with HLA-B27 implies a role for MHC class I-restricted T cells in the pathogenesis (1–5). HLA-B27-restricted T cell responses to bacterial Ags from Chlamydia (6, 7) or Klebsiella (8) have been described in humans and animal models (7, 9–11). It has been suggested that potential arthritogenic peptides either from bacteria and/or from autologous Ag could activate B27-restricted CD8⁺ T cells (CTL), which then may contribute to the arthritic process of erosion and destruction of certain cartilage structures within the joint (12). In fact, a characteristic feature of SpA is inflammation of the joint, in particular at the site of ligament/tendon/bone (enthesitis) and other fibrocartilage structures (13). Therefore, in context with the strong HLA-B27 association, the putative identification of fibrocartilaginous epitopes presented by HLA-B27 to CD8⁺ T cells is of great interest. In humans, B27-restricted immunity to autoantigens derived from collagen structures has been described (14), and in the transgenic rat model, the importance of HLA-B27-restricted CTL has also been demonstrated (15).

Aggrecan is an important protein in fibrocartilaginous regions of the tendons that insert at the bone. It has been proposed that the cartilage, particularly the proteoglycan aggrecan, is the primary immunological target in SpA (12, 13, 16, 17). The aggrecan molecule consists of three globular domains (globular 1 domain (G1), G2, and G3) and the chondroitin sulfate and keratan sulfate side chains. The protein aggregates via the G1 to hyaluronan and link protein. This results in the formation of a macromolecular proteoglycan, aggrecan. In an animal model, it has been shown that systemic immunization of BALB/c mice with chondroitin sulfate-depleted proteoglycan aggrecan from fetal human cartilage resulted in spondylitis and erosive inflammatory polyarthritis (18, 19). More detailed experiments revealed that, in particular, CD4⁺ T cells recognize epitopes on the G1 domain of aggrecan (20, 21) and on the separate globular link protein (22). In a recent study, Zou et al. (23) examined the CD4⁺ immune response of synovial T cells to the G1 domain in AS and rheumatoid arthritis patients, indicating that the G1 domain of aggrecan might play a pathogenetic relevant role in the cellular autoimmune response in AS and rheumatoid arthritis. However, no data are available in mice (24) or patients (25, 26) about the role of CD8⁺ T cells recognizing epitopes on the G1 domain of aggrecan as an autoantigen.

In this study, we researched the role of HLA-B27 and human aggrecan as a potential Ag in SpA. We investigated the MHC class I response of HLA-B27-restricted CD8⁺ T cells in transgenic and wild-type BALB/c mice after immunization with the human and mouse nonamer sequences bearing the motif for HLA-B27 binding.

Materials and Methods

Mice and peptide immunization

BALB/c and BALB/c-B27hβ₃ transgenic mice (H-2b) (27, 28) were bred in the animal facilities of the Charité Universitätsmedizin (Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany). Mice were kept in a specific pathogen-free environment at the Department of Gastroenterology and Rheumatology, Charité Universitätsmedizin Berlin, Germany. The Animal Care Committee of the Charité approved the experiments.

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3 Abbreviations used in this paper: SpA, spondyloarthropathy; AS, ankylosing spondylitis; G1, globular 1 domain; pep, peptide.
aggrecan nonamer peptides by intracellular cytokine secretion

the early activation marker CD69 and intracellular IFN-\(\gamma\)

HLA-B27 transgenic mice, intracellular cytokine staining was performed before the pellet was resuspended for 10 min in permeabilization buffer.

were pooled, and used for cytokine secretion experiments, T cell culturing, cytotoxic T cell assays, or histology. All experiments with the wild-type and transgenic animals were performed under the guidelines of the animal health ethical committee.

**P815-B27** (H-2\(^d\)) transfectants

The P815 mouse mastocytoma line (H-2\(^d\)) was originally obtained from American Type Culture Collection (Manassas, VA) and stably transfected with the human gene for human \(\beta_2\)-microglobulin and HLA-B27 (29). The cells were cultured at 37 \(^\circ\)C and 5% CO\(_2\), in RPMI 1640 culture medium, supplemented with 10% FCS (Invitrogen Life Technologies, Karlsruhe, Germany), 2 mM l-glutamine, 100 U/ml penicillin and 100 \(\mu\)g/ml streptomycin sulfate, and 5 \(\times\) 10\(^{-7}\) M 2-ME (Sigma-Aldrich, St. Louis, MO).

**P815-B27 cell line and aggrecan peptide incubation**

For peptide loading, P815-B27 cells were preincubated in RPMI 1640 culture medium with 5% FCS overnight with an excess (100 \(\mu\)M) of selected aggrecan or control peptides. Subsequently, the cells were washed twice in culture medium, 5% FCS, and prepared as targets for a chromium release assay.

**Search for HLA-B27-binding peptides derived from human aggrecan**

A search for HLA-B27-binding nonamer peptides from the human proteoglycan aggrecan (www.ncbi.nlm.nih.gov/ accession no. P161112) was conducted with an epitope prediction program (www.syfpeithi.de), as described by Rammensee et al. (30). Peptides with a binding score of \(\geq\) 20 were chosen.

**Peptide synthesis**

Nonamer peptides were synthesized (Naturwissenschaftliches und Medizinisches Institut, Reutlingen, Germany) according to standard Fmoc solid-phase synthesis methods on a Syro-Synthesizer (MultisynTech, Witten, Germany), purified by HPLC (LC-10; Shimadzu, Columbia, MD), and identified by mass spectroscopy (LCQ-Thermospect, San Jose, CA). Purity of peptides was \(\geq\) 95\%. Peptides were dissolved in DMSO and further diluted with serum-free culture medium at a concentration of 10 mg/ml. The peptides were stored frozen at \(-20^\circ\)C.

**Determination of CD8\(^+\) T cell responses to B27-restricted aggrecan nonamer peptides by intracellular cytokine secretion and flow cytometry**

BALB/c-B27 transgenic mice were s.c. primed in vivo with pools of five to seven peptides that were emulsified in CFA/IFA. To determine which of the selected peptides may stimulate CD8\(^+\) splenocytes from immunized HLA-B27 transgenic mice, intracellular cytokine staining was performed after Ag-specific T cell stimulation (31). Briefly, 2 \(\times\) 10\(^6\) primed cells were stimulated for 6 h in 1 ml of culture medium in 10-ml tissue culture tubes (Greiner, Nütingen, Germany) with mouse anti-CD28 Ab (1 \(\mu\)g/ml) and individual peptides (10 \(\mu\)g/ml), with unrelated control peptide, or with anti-CD28 Ab alone (BD Biosciences, Heidelberg, Germany) (negative control). Stimulation was stopped with brefeldin A (5 \(\mu\)g/ml) after 2 h. CD8\(^+\) T cells were harvested and stained with anti-CD99-FITC (5 \(\mu\)g/ml) and anti-CD8-allophycocyanin (1 \(\mu\)g/ml) (BD Biosciences). Next, the cells were fixed in 2% formalin for 15 min at room temperature, and washed before the pellet was resuspended for 10 min in permeabilization buffer (BD Biosciences), followed by staining with PE-conjugated rat anti-mouse IFN-\(\gamma\) (1 \(\mu\)g/ml; BD Biosciences). CD8\(^+\) T cells, being double-positive for the early activation marker CD69 and intracellular IFN-\(\gamma\), were regarded as peptide (Ag) specific. Analysis was done on a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

**Determination of CD69 expression and IFN-\(\gamma\) secretion at different time points**

In the case of culturing CD8\(^+\) T cells over a longer period, we determined the CD69 expression and IFN-\(\gamma\) secretion at different time points with some modifications of the described stimulation assay and FACS analysis. To distinguish freshly added APC from cultured cells in a 6-h stimulation assay, fresh APC were labeled with CFSE (Molecular Probes, Leiden, The Netherlands). A total of 2 \(\times\) 10\(^5\) cultured cells was incubated with 1 \(\times\) 10\(^5\) CFSE-APC (10 \(\mu\)M) and CD28, the respective control alone and/or with stimulating Ag. Incubation was stopped after 2 h with brefeldin A, and the cells were treated as described. Cell staining was performed with anti-CD8-allophycocyanin-conjugated rat anti-mouse IFN-\(\gamma\) (1 \(\mu\)g/ml) Ab (BD Biosciences). The cells were analyzed using a FACSCalibur flow cytometer, and the analysis gate set on CD8\(^+\) T cells. Freshly added APC were distinguished by CFSE labeling.

**In vitro expansion of peptide-specific CTL for cytotoxicity assays**

To investigate CD8\(^+\) T cells for cytotoxicity against the selected aggrecan peptides, splenocytes of three mice primed in vivo with pools of peptides or single peptide were resuspended in RPMI 1640 culture medium. To increase the frequency of Ag-specific cytotoxic CD8\(^+\) T cells, 1 \(\times\) 10\(^5\) primed splenocytes were transferred in 7 ml of culture medium into 50-ml upright flasks each (Nunc, Wiesbaden, Germany) and restimulated with 10 \(\mu\)g/ml the respective single peptides or control peptide. After 6 days of incubation, 37\(^\circ\)C and 5% CO\(_2\), cells were harvested. Following Oli-Goli-Paque (Pharmacia, Freiburg, Germany) purification and resuspension in culture medium, the cells were distributed in 96-well V-plates and used as effector cells in cytotoxicity assays.

**Cytotoxicity assay against aggrecan-derived nonamer peptides**

Cytotoxic activity was measured on peptide-targeted P815-B27 (H-2\(^d\)) cells. To optimize MHC class I expression, the cell line was preincubated for 1 h with 10 \(\mu\)g/ml recombinant murine IFN-\(\gamma\) (BD Biosciences) before the cells were incubated overnight with an excess of peptides. Peptide-incubated cells were then washed, and the pellets were labeled with 100 \(\mu\)Ci of Na\(^{37}\)Cromat (Amersham Biosciences, Braunschweig, Germany) at 37 \(^\circ\)C for 1 h. After washing three times in PBS, Na\(^{37}\)Cromat-labeled target cells were resuspended in RPMI 1640, supplemented with 5% FCS, 2 mM l-glutamine, 100 U/ml penicillin and 100 \(\mu\)g/ml streptomycin sulfate, and 5 \(\times\) 10\(^{-8}\) M 2-ME (Sigma-Aldrich). For the assay, 5 \(\times\) 10\(^3\) targets were seeded in 96-well V-bottom plates (Nunc) along with different numbers of effector cells in a total volume of 200 \(\mu\)l. Spontaneous release was determined in wells with target cells and medium alone. Maximum release was determined by adding 100 \(\mu\)l of 2% Triton X-100 (Sigma-Aldrich) into the wells, containing 100 \(\mu\)l of target cells without CTL. Target cells were tested for lysis in triplicate in a standard 6-h chromium release assay. Specific lysis was calculated as follows: 100 \(\times\) ((cpm experimental release – cpm spontaneous release)/(cpm maximal release – cpm spontaneous release)). Data presented are means of triplicate determinations.

**B27-tetramer/pep67 construct and staining of peptide no. 67 epitope-specific CD8\(^+\) T cells**

Tetrameric HLA-B27 complexes were generated as described (32) with modifications. Purified HLA-B27 H chain and \(\beta_2\)-microglobulin were synthesized using the expression vector pET3a (Novagen, Darmstadt, Germany). The H chain was modified by deletion of the transmembrane and cytosolic tails and C-terminal addition of a sequence containing the BirA enzymatic biotinylation site. The expressed H chain and \(\beta_2\)-microglobulin were solubilized and refolded in the presence of peptide no. 67 (SRHHAF-CFR). The refolded 45-kDa product was isolated using fast protein liquid chromatography, biotinylated by BirA in the presence of biotin and ATP. Biotinylated products were separated from free biotin by gel filtration using fast protein liquid chromatography. streptavidin-PE conjugate (Molecular Probes) was added in a 1:1 molar ratio, and 1 mg/ml was concentrated to 2 mg/ml. As control, HLA-B27 tetramer complex with the recently described chlamydial peptide no. 138 (ARKLLLDNL) (7) was used. Functional analysis of the tetramer complexes was performed by staining 5 \(\times\) 10\(^5\) splenocytes from peptide-immunized mice for 25 min with the tetramer-PE. After washing two times in PBS, the cells were incubated for 20 min with anti-CD3-PerCP (1 \(\mu\)g/ml) and anti-CD8-allophycocyanin (1 \(\mu\)g/ml) (BD Biosciences). Tetramer staining with
tetramer HLA-B27/pep#138 (negative control) and HLA-B27/pep#67 was analyzed by FACS Calibur.

**Histopathological analysis with H&E and tetramer staining**

Spleen tissue sections were obtained from peptide injected BALB/c-B27 and wild-type BALB/c female mice. Spleen tissues were either stained by standard H&E dye or on frozen tissue sections by tetramers. For H&E staining, the tissues were fixed in 4% formalin before decaclification and embedding in paraffin and examined for tissue pathology. Frozen tissue (spleen) sections were embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands) and prepared by shock freezing in liquid nitrogen. Seven-micrometer microtome cryosections from frozen material were dehydrated in buffer and used (unfixed) for tetramer incubation. Tissue sections were blocked with 4% milk powder and streptavidin/biotin blocking kit (Vector Laboratories, Burlingame, CA). After 1-h staining with tetramer-PE and washing in PBS, the probe was incubated for further 30 min with rabbit anti-PE (Biomed, Foster City, CA) and washed in PBS. An incubation step for 30 min with anti-rabbit-Cy3 (Dianova, Hamburg, Germany) followed. Fixation of the tissue section was performed in 4% paraformaldehyde followed by a PBS washing step and a further 30-min staining with anti CD8-FITC (Immunotech, Krefeld, Germany). Visualization of the cell nucleus was done by standard methods using 4',6-diamidino-2-phenylindole (Roche, Penzberg, Germany).

**Results**

**Search for potential human cartilage derived HLA-B27 binding peptide motifs**

With the help of a computer binding algorithm (SYFPEITHI), the sequence of the human proteoglycan aggrecan (www.ncbi.nlm.nih.gov; accession no. P161122) was screened for HLA-B27 motifs (7). Of 79 potential candidate peptides with the HLA-B27 binding motif (R in position 2), 22 peptides with a binding score of 7 were selected and synthesized for further investigation.

**Intracellular cytokine staining of CD8+ BALB/c-B27 splenocytes primed with human aggrecan peptide**

To analyze the selected 22 human aggrecan peptides for their immunogenic potential, BALB/c-B27 transgenic mice were immunized in vivo with the 22 peptides (designated as peptides nos. 47–68) either as pools (pool 1 (nos. 47–51); pool 2 (nos. 52–56); pool 3 (nos. 57–61) consisted of five peptides; pool 4 (nos. 62–68) consisted of seven peptides) or with identified peptides alone, emulsified in CFA/IFA. Rechallenge in vitro was performed with single peptides from each pool. From these peptide pool experiments, four peptides were detected to be stimulatory for CD8+ T cells (Fig. 1). As described in Materials and Methods, CD69 and intracellular IFN-γ secretion of CD8+ T cells upon peptide pool stimulation were defined. All cells including the control vials (Fig. 1A, upper line) were stimulated with anti-CD28 (33). Four stimulatory peptides were found, one peptide (no. 47) in pool 1, none in pool 2, two peptides (nos. 57 and 59) in pool 3, and one peptide (no. 67) in pool 4 (Fig. 1B, bottom line). The other selected nonamers were not able to induce stimulation of CD8 T cells (data not shown).

In the following experiments, we were concentrating on further investigations of peptide no. 67. The other peptides will be reported elsewhere (W. Kuon, M. Hülsmeyer, D. H. Busch, E. H. Weiss, V. Krenn, and J. Sieper, manuscript in preparation). Peptide no. 67 was particularly potent when it was used as a single one for in vivo priming of BALB/c-B27 transgenic mice (Fig. 2).

**CTL lysis restricted by aggrecan epitopes is dependent on HLA-B27**

In the following experiments, we asked whether aggrecan peptide no. 67 could generate a CD8+ B27-restricted, aggrecan peptide no. 67-specific T cell response in BALB/c-B27 mice (Fig. 3). To analyze this question, CTL assays of in vitro-expanded T cell lines were performed. Besides an aliquot for FACS analysis, BALB/c-B27 transgenic splenocytes from in vivo immunization with peptide pool 4 (containing no. 67) were challenged in vitro with each peptide of pool 4. After 6 days of in vitro culture, CTL lysis for each individual peptide was determined by the lymphocytes’ ability to recognize and lyse P815-B27 cells loaded with the respective peptides (Fig. 3).

Only cells targeted with peptide no. 67 were lysed by peptide no. 67-restimulated effector cells, whereas the other peptides of peptide pool 4 did not generate a CTL response (Fig. 3A). To confirm the specificity of the peptide no. 67 CTL, a crisscoss experiment was performed. Splenocytes of mice that were primed in vivo and challenged in vitro with peptide no. 67 were cultured for 6 days. CTL activity was investigated on P815-B27 targets loaded with the different peptides (Fig. 3B). Again, only peptide no. 67 was recognized by CTL raised against no. 67, but by none of the other pool 4 peptides (Fig. 3B).

**FIGURE 1.** Identification of four stimulating peptides by cytokine secretion assay. Splenocytes from BALB/c-B27 transgenic mice that were immunized in vivo with four different pools of peptides were restimulated in vitro with the single peptides from each pool. Frequencies of CD69/IFN-γ double-positive lymphocytes are given as percentage of the CD8+ gated T cell population (upper right quadrant of each diagram). Background frequencies for CD8 T cells in the upper squares (no Ag but stimulation with anti-CD28 only) (A) are compared with the frequencies upon peptide-plus-anti-CD28 stimulation (B). Four stimulating peptides from three different pools were detected as indicated.

**FIGURE 2.** Stimulation of BALB/c-B27 splenocytes with peptide no. 67. Spleen cells from peptide no. 67-immunized mice were restimulated in vitro for 6 h. Readout for peptide stimulation of CD8+ T cells was the expression of the early activation marker CD69 and IFN-γ secretion, measured by flow cytometry (FACS). Frequencies of CD69/IFN-γ double-positive cells are given as percentage of CD8+ gated T cell populations (upper right quadrant of each diagram). A, Background is indicated by anti-CD28 stimulation alone. B, Stimulation by PMA/ionomycin (positive control). C, Specific stimulation with peptide no. 67.
Only the human but not the murine aggrecan sequence no. 67 is immunogenic in BALB/c-B27 mice

Next, we investigated whether both the human (SRHHAFCFR; accession no. P16112) and the respective mouse homologous (SKHHAFCFR) aggrecan sequence no. 67 (www.ncbi.nlm.nih.gov; accession no. NP_031450) were immunogenic (Fig. 4). Therefore, BALB/c-B27 transgenic and wild-type BALB/c mice were immunized with either the human (H)- or the mouse (M)-derived sequence. As shown in Fig. 4A, strong stimulation of CD8\(^+\) splenocytes of BALB/c-B27 transgenic mice primed with the human (H) peptide no. 67 was observed. In contrast, no stimulation was generated when the transgenic mice were immunized with the mouse (M)-derived sequence (Fig. 4B), which is substituted only at position 2 from arginine (R) to lysine (K). Moreover, no stimulation was observed in normal BALB/c mice after priming with the human no. 67 (H)- or the mouse no. 67 (M)-derived aggrecan peptide motifs (Fig. 4, C and D).

Besides the restimulation of in vivo-primed cells, we also used the same splenocytes to address the question whether CTL responses are detectable in these cultures (Fig. 5). Splenocytes from BALB/c-B27 and BALB/c wild-type strains that had been primed with the human or the murine peptide no. 67 sequences were restimulated with the respective peptides for 6 days in vitro. Target lysis by effector cells was measured on peptide-loaded P815-B27 and P815 targets and on P815-B27 targets alone. If the BALB/c-B27 transgenic mice were immunized with the human (H) aggrecan peptide sequence no. 67, only the P815-B27/human#67 (H)-loaded peptide was recognized and strongly lysed (Fig. 5A). No lysis was observed for P815 cells incubated with the human sequence no. 67 or for the unloaded P815-B27 cell line alone (Fig. 5A). In contrast, none of the peptide-loaded/unloaded targets was recognized by effector cells when the transgenic BALB/c-B27 mice were primed with the mouse (M)-derived aggrecan sequence (Fig. 5B). Also, no lysis of any target was observed on wild-type BALB/c mice, immunized with either the human aggrecan sequence (H) or the homologous mouse (M) sequence and assayed for target lysis (Fig. 5, C and D).

Human aggrecan peptide no. 67 can be used for the generation of HLA-B27 tetramer complexes

On the following approach, we analyzed whether peptide no. 67 could be used for tetramer complex formation and whether such complex could stain peptide no. 67-primed and in vitro-rechallenged splenocytes. As described in Materials and Methods, two tetrameric complexes were used, one tetramer complex with a recently described peptide from Chlamydia trachomatis (7) and the HLA-B27/aggrecan#67 construct. Therefore, peptide no. 67 in vivo-primed splenocytes were restimulated and cultured in vitro for a further 10 days, and then incubated with the control pep#138- and pep#67/B27-tetramer complexes (Fig. 6). Less to no staining was observed for the control pep#138/B27-tetramer (0.04%) (Fig. 6A), whereas in this experiment, a highly strong binding (34.97%) of the pep#67/B27 tetramer with CD8\(^+\) cultured splenocytes was achieved (B).

Determination of the frequencies of no. 67-specific CD8\(^+\) T cells upon immunization by cytokine secretion and tetramer staining

In the following set of experiments (Fig. 7), mice were immunized with peptide no. 67. After three injections, an aliquot of the spleen

![Image](http://www.jimmunol.org/download/4862_HUMANAgregarcan_EpitopeS_In_HLA-B27_Transgenic_Mice_Fig4A.jpg)

**FIGURE 4.** In vitro stimulation of peptide no. 67-primed murine splenocytes with mouse (SKHHAFCFR) or human (SRHHAFCFR) aggrecan peptide no. 67. A and B, Stimulation of BALB/c-B27 splenocytes primed and restimulated with either the human (H) (A)–or the mouse (M) (B)-derived nonamer peptide no. 67. C and D, The third (C) and fourth (D) lanes represent the same kind of peptide stimulation, but on splenocytes derived from BALB/c wild-type mice. In contrast to the mouse (M) sequence (B), only the human (H) sequence no. 67 strongly stimulated CD8\(^+\) T cells from transgenic BALB/c-B27 splenocytes (A). None of the human (H) and mouse (M) sequences stimulated CD8\(^+\) T cells of BALB/c mice (C and D). As controls, background stimulation with CD28 alone or unspecific PMA/ionomycin stimulation are shown.

![Image](http://www.jimmunol.org/download/4862_HUMANAgregarcan_EpitopeS_In_HLA-B27_Transgenic_Mice_Fig4B.jpg)

**FIGURE 3.** In vivo generation of human aggrecan peptide no. 67-specific CTLs. BALB/c-B27 transgenic mice were immunized with peptide pool 4. A, P815-B27 targets loaded with peptides of pool 4 were only recognized and lysed by peptide no. 67-specific CTL. The other peptides failed to induce a respective peptide-specific CTL response. B, Effector cells that were generated in vivo and in vitro only against peptide no. 67 revealed no reactivity in a crisscross experiment on P815-B27 targets loaded with other peptides of pool 4, except for peptide no. 67. Target lysis by murine CTL was measured on aggrecan peptide-incubated P815-B27 targets (H-2K\(^d\)) in a 6-h \(^{51}Cr\) release assay. Cytotoxicity values are shown as means of triplicates.
In Fig. 8, the percentages of tetramer-stained CD8 T cells were derived from the spleen of BALB/c-B27 transgenic mice and cultured for 10 days. A, Strong CTL response against human sequence no. 67 could be generated in transgenic mice, but no lysis was observed in P815-B27 cells without peptide or in P815 cells incubated with Pep#67 (H). B, No target kill was generated when BALB/c-B27 mice had been primed with the mouse (M) sequence and were analyzed on the indicated targets P815-B27/pep#67 (M), P815-B27, and P815/Pep#67 (M). C and D, The same experiments were performed on BALB/c wild-type mice immunized with either the human (H) (C) or the mouse (M) (D) sequence. No target lysis was detectable. Cytotoxicity values are shown as means of triplicates.

FIGURE 5. Determination of the specificity of CTLs for human (H)- and mouse (M)-derived peptide no. 67 sequences upon priming of BALB/c-B27 and wild-type BALB/c mice. CTL lysis was measured either on mouse or human aggrecan peptide (un)loaded P815 and P815-B27 (H-2b) targets. A, Strong CTL response against human sequence no. 67 could be generated in transgenic mice, but no lysis was observed in P815-B27 cells without peptide or in P815 cells incubated with Pep#67 (H). B, No target kill was generated when BALB/c-B27 mice had been primed with the mouse (M) sequence and were analyzed on the indicated targets P815-B27/pep#67 (M), P815-B27, and P815/Pep#67 (M). C and D, The same experiments were performed on BALB/c wild-type mice immunized with either the human (H) (C) or the mouse (M) (D) sequence. No target lysis was detectable. Cytotoxicity values are shown as means of triplicates.

FIGURE 6. In vitro staining of cultured peptide no. 67-specific CD8 T cells with B27-tetramer/pep#67 complex. Peptide no. 67-specific CD8 T cells were derived from the spleen of BALB/c-B27 transgenic mice and cultured for 10 days. A, Staining of CD8 T cells with the control B27-tetramer/chl.pep#138 complex with low background staining. B, Staining of epitope-specific CD8 T cells with the tetramer-PE/pep#67 complex. Very strong tetramer-PE/pep#67 CD8 T cell staining was observed in the experiment (upper right quadrant).

FIGURE 7. Frequency of peptide no. 67 epitope-specific CD8 T cells before and after 7 days of in vitro restimulation with peptide no. 67. A, Percentage of CD69 and IFN-γ-secreting CD8 T cells upon no Ag stimulation, PMA/ionomycin (unspecific control), epitope-specific challenge with peptide no. 67 and unrelated chlamydial (Chl)-derived OmpA peptide stimulation at day 0 of T cell culturing. B, At day 7 (bottom line), an increase (0.60%) of the frequency of CD8 T cells with specificity to human aggrecan peptide no. 67 was detected, but only a small increase if stimulated with an Ag or with the Chl-OmpA.
immunized with peptide no. 67 (0 of 26). To confirm the significance of the particular peptide in the induction of inflammation, we immunized 12 HLA-B27 transgenic BALB/c mice with pools of 15 unrelated peptides with a binding score of $\geq 20$ for HLA-B27 derived from human myelin basic protein and seven nonamer peptides derived from the human nerve growth factor (all with R in position 2) or with a human collagen-derived sequence (peptide no. C34). B27 transgenic mice were immunized with these peptides as described for the aggrecan peptides. None of the peptides was immunogenic or induced arthritis (data not shown).

Discussion
Cartilage proteins such as human aggrecan are known to play an important role in the inflammatory process, because they seem to be a major target in SpA and autoimmunity to aggrecan (19–21, 34) and to other proteoglycans (35) can lead to the development of arthritis, as was shown in mice. In this study, we present a new BALB/c-B27 transgenic mouse model for HLA-B27-restricted CD8$^+$ T cells with reactivity against human aggrecan peptide. Human aggrecan peptide no. 67 was selected by a binding program (SYFPEITHI), as recently described (7, 29). The peptide was identified as one of 22 peptide sequences chosen by the binding program.
algorithm with a binding score of ≥20. The “biological” reason for selecting such a binding score was that most of the identified and published natural ligands in HLA-B*2705 molecules show a binding score of ≥20.

Immunogenicity was demonstrated by stimulation of CD8\(^+\) splenocytes from BALB/c-B27 transgenic mice by measuring intracellular IFN-\(\gamma\) secretion (Figs. 1 and 2). The level of stimulation was higher when mice were immunized with peptide no. 67 alone (Fig. 1) compared with the peptide pool (peptides nos. 62–68) (Fig. 2). Because all of the peptides were selected as good B27 binders by the binding algorithm, in the case of pooled peptides, binding competition probably prevents a better presentation of ligand no. 67 to the immune system.

As indicated (Fig. 1), no stimulatory peptide was present in pool 2 (peptides nos. 52–57), even upon several stimulation experiments (data not shown). Interestingly, the sequence no. 56 (GRVRVNSAY) of this negative pool was recently reported as a consensus sequence in longer immunogenic peptide sequences recognized by aggrecan-specific CD4\(^+\) Th1 cell hybridomas derived from immunized BALB/c mice (36). However, from our experiments, the human aggrecan sequence GRVRVNSAY does not seem to evoke CD8\(^+\) T cell responses.

To confirm the significance of the detected sequence, we asked whether peptide no. 67 could generate CTLs upon immunization. A strong CTL response against the human aggrecan peptide no. 67 was observed only in BALB/c-B27 transgenic mice (Fig. 3A), whereas no CTL response was found in any of the control experiments. Interestingly, only the human sequence but not the autologous mouse sequence was recognized by CTL derived from BALB/c-B27 transgenic mice.

These experiments demonstrate that the mouse sequence (SKH-HAFCFR), which is different only in position 2 where an arginine (R)→lysine (K) substitution occurred, is tolerant in the transgenic and wild-type strains. Only the human sequence (SRHHACFCFR) strongly stimulated CD8\(^+\) T cells in the transgenic mice. However, the replacement of position 2 is crucial, because R is the anchor amino acid position for HLA-B27 binding. Therefore, in the transgenic BALB/c-B27 strain the mouse peptide should not be presented by HLA-B27. The detected response against the human sequence indicates that this epitope seems to be presented in BALB/c-B27 mice by the B27-molecule as a classical foreign Ag. When the mouse homolog is nonimmunogenic in mice, why should the human homolog, which is highly immunogenic in the B27 transgenes, be of interest in humans? There are reports about autoreactive T cells against aggrecan in arthritis patients (37) and even in healthy individuals (38). Therefore, it is suggested that tolerance could be broken in situations where, for example, synovial cells of a HLA-B27\(^+\) patient include “silent,” autoreactive T cells against particular self-aggrecan peptide epitopes. As reported, autoreactive T cells can be triggered and activated by a local infection, resulting in an increase of the amount of the antigenic epitope and induction of inflammation (39–42). Such a possibility is currently investigated by analyzing patients’ synovial fluids for self-reactive T cells against aggrecan peptide no. 67. We also found that another human aggrecan peptide (no. 57 in pool 3), which is characterized by identical amino acid sequences in mouse and man, raised an immune response upon several injections only in primed BALB/c-B27 transgenic mice (W. Kuon, M. Hülsmeyer, D. H. Busch, E. H. Weiss, V. Krenn, and J. Sieper, manuscript in preparation). Thus, the above-described scenario, that a silent, perhaps ignorant, CD8\(^+\) B27-restricted T cell response against aggrecan could exist and could locally be triggered by an increased level of local antigenic epitope(s) that induce an autoimmune process by breaking tolerance seems to be a possibility.

Tetramer constructs have been established as useful tools in experimental research (32, 43, 44). We successfully used peptide no. 67 for HLA-B27 tetramer generation, which, in contrast to unrelated tetramer, strongly bound peptide-primed splenocytes (Fig. 6). An increase of tetramer/peptide no. 67 binding of cells at different time points during cell culturing was accompanied by a similar increase of CD8\(^+\) T cells after short Ag-specific stimulation in vitro (Figs. 7 and 8) and reveals the usefulness of peptide no. 67 for establishing a CD8\(^+\) T cell line.

Frozen tissue sections of primed spleen were also successfully stained with tetramer/pep#67 complexes. From these experiments, we conclude that the tetramers are functionally fully correct. Such tetramers might be very useful for the investigation of frozen tissues from hip or joints of animals and patients suffering from SpA such as AS. The detection of particular proteoglycan epitopes such as aggrecan ligand no. 67 in the joint at the site of inflammation could be an issue for tetramer staining. However, we do not know at present whether and to what extend a CD8\(^+\) T cell response against human aggrecan plays a causative role in the pathogenesis.

Most importantly, the significance of the detected aggrecan peptide sequence no. 67 in the BALB/c-B27 strain was supported by the finding that immunized mice developed tenosynovitis of the knee joints. Inflammation was observed only in BALB/c-B27 transgenic animals but not upon immunization of wild-type BALB/c strain or of mice immunized with unrelated peptides. These findings argue in favor of the possibility that the aggrecan peptide no. 67 can indeed induce specifically an inflammation in the joint, especially because HLA-B27 potentially binding peptides derived from joint-unrelated proteins such as myelin basic protein or nerve growth factor did not induce arthritis in immunized mice. As discussed above, the foreign human aggrecan peptide no. 67 might induce a T cell response against a local mouse Ag. However, to finally prove a causal relation between peptide immunization, HLA-B27 restriction, and the occurrence of arthritis in the mouse model, two sorts of experiments should be performed: 1) detection of peptide no. 67-specific, HLA-B27-restricted T cells in the inflammatory joint lesions by HLA-B27/\#67 tetramers; 2) separation and expansion of aggrecan no. 67-specific CD8\(^+\) T cells using tetramer technology (43) and/or the IFN-\(\gamma\) secretion assay (31) and use of these cells for an adoptive transfer experiment trying to prevent the occurrence of arthritis in immunized mice. These two types of experiments are currently in progress in our laboratory.

The described mouse model may give the opportunity to investigate in more detail the pathomechanism of SpA in relationship to human disease by studying the role of cartilage structures such as aggrecan as candidate autoantigen for epitopes presented by particular HLA-B27 subtypes to CD8\(^+\) T cells. Just recently, strong arguments concerning the conformational structures of HLA-B27 subtypes and the ability to bind particular peptides (45, 46) underlined the arthriogenic peptide hypothesis (6, 47–50). Our findings could argue for the significance of potential arthriogenic peptides to HLA-B27 and a possible pathogenic role of HLA-B27-restricted CD8\(^+\) T cells in B27-subtype-dependent Ag presentation in SpA.

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