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Definition of a human suppressor T-cell epitope

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ABSTRACT **The quality of the response produced by regulatory or helper T (Th) cells presently receives much attention because of its possible implications for vaccine development and immunomodulation. Apart from cytokines and so-called costimulatory signals, antigens and the presenting major histocompatibility complex (MHC) molecules may play a role in determining the type of T-cell response generated toward antigens. To examine the role of antigen and/or HLA in control of T-cell subset activation, we have studied a special case, namely CD4$^+$ suppressor T (Ts) cells in leprosy. *Mycobacterium leprae*-induced Ts cell clones have been previously isolated from peripheral blood and skin lesions of lepromatous leprosy patients and were shown to specifically down-regulate mycobacterium-specific Th cell responses. Despite considerable effort, the antigens recognized by these Ts cells have thus far not been identified. Here we report that all HLA-DR2-restricted CD4$^+$ Ts cell clones derived from a lepromatous leprosy patient recognize an epitope that maps between the amino acid residues 439 and 448 of the mycobacterial hsp65. The peptide was presented to these Ts cells by HLA-DRB1*1503, a recently discovered HLA-DR2 variant. Non-suppressor T-cell clones derived from the same patient recognized antigens other than the hsp65 and were also stimulated by other HLA-DR2 variants. In independent cloning experiments peptide 435–449 and recombinant hsp65 induced exclusively Ts cells in this lepromatous leprosy patient. The Ts clones recognizing this particular epitope were derived from at least seven different progenitors, as they expressed different T-cell receptor α and β chains. Thus, our data indicate that a specific peptide–HLA class II combination may exclusively activate Ts cells.**

Over the past decade evidence has been accumulating for heterogeneity of CD4$^+$ helper T (Th) cells, the mouse paradigm of Th1 and Th2 cells being the clearest example (1). The factors that control which Th cell subsets will be preferentially activated during an immune response have recently also received much attention, because insight into these regulatory mechanisms during an immune response might be applied to the design of vaccines and lead to novel strategies for immunomodulation (2). Cytokines apparently play an important role—e.g., interferon γ and interleukin 12 have been shown to skew the immune response to a Th1 response (3), which is needed to kill intracellular pathogens such as listeria and mycobacteria. Presently most people do not attribute an important role to the peptide–major histocompatibility complex (MHC) combination that is recognized by the Th cells in this type of regulation. Indeed, in the leishmania model in rodents formal evidence against such a role has been presented (4). However there is also at least one good example arguing in favor of MHC control of CD4$^+$ T-cell subset activation (5).

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Differential activation of T-cell subsets by certain MHC–peptide combinations is a testable mechanism of certain MHC and disease associations, which would have clear implications for immunoprevention and immunotherapy (6). We have addressed this question in the HLA and disease model leprosy.

The clinical spectrum of leprosy reflects the striking differences between individuals in developing cell-mediated immunity to the causative agent, *Mycobacterium leprae* (7, 8). At the "high resistant" pole of this spectrum, tuberculoid leprosy (TT) patients develop strong cellular immune responses to *M. leprae* antigens and efficiently eliminate the bacilli, whereas at the "low resistant" pole, lepromatous leprosy (LL) patients fail to mount specific T-cell responses to *M. leprae* antigens and develop a multibacillary disseminated disease (9). Several lines of evidence suggest that the *M. leprae*-specific T-cell nonresponsiveness observed in LL patients is not due to an absence of *M. leprae*-reactive T cells or to defective antigen presentation as claimed in earlier reports (10, 11) but may be due to active down-regulation of *M. leprae*-specific T-cell responses, presumably by suppressor T (Ts) cells induced by *M. leprae* (12–14). Supporting this latter premise, we and others have previously isolated CD4$^+$ and CD8$^+$ T-cell clones from the peripheral blood and skin lesions of LL patients and shown that these T cells specifically suppress mycobacterium-specific T-cell responses *in vitro* (15–17). Previous analyses of these Ts clones revealed that they, like *M. leprae*-induced Th cells, express T-cell receptor (TCR) α and β chains and are restricted by HLA-DR or HLA-DQ molecules (15, 16, 18, 38). Unlike Th cells, Ts cells lack or poorly express CD28 (19) and display a low capacity to proliferate in response to *M. leprae*. The mechanism of antigen-specific T-cell nonresponsiveness in LL remains unresolved. One group has presented evidence in favor of a role for Th2 like cytokines in CD8$^+$ and CD4$^+$ Ts cell-mediated suppression (20). We have, however, found no such clear association of Th2 like cytokines and T-cell nonresponsiveness in LL patients (21).

Many antigens of *M. leprae* have been defined that stimulate Th cells, but the nature of the *M. leprae* determinants that activate Ts cells has remained elusive thus far. If the epitope(s) recognized by Ts cells were different from those recognized by Th cells, the definition of such so-called "suppressor epitopes" might have implications for the development of efficient leprosy vaccines and also shed light on the mechanisms that result in the down-regulation of anti-mycobacterial immune responses in LL patients. Moreover, the principles underlying these mechanisms might well be generalizable to other diseases such as tuberculosis, autoimmune diseases, and cancer. Previously it was suggested that the *M. leprae*-specific phenolic glycolipid-1 could sup-

Abbreviations: Th, helper T; Ts, suppressor T; MHC, major histocompatibility complex; TT, tuberculoid leprosy; LL, lepromatous leprosy; TCR, T-cell receptor; PBMC, peripheral blood mononuclear cells; APC, antigen-presenting cells; B-LCL, Epstein–Barr virus-transformed B-cell lines; r-, recombinant.

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press mitogen-driven responses of peripheral blood derived mononuclear cells of LL (but not TT) patients (22), but this finding has not been confirmed by others (23). Furthermore, it is still questionable whether T cells recognize nonprotein antigens. We have therefore started to search for the *M. leprae* antigens recognized by the Ts cell clones generated in our laboratory, using classical biochemical strategies which were successfully employed to define *M. leprae* antigens for Th cells (24). Here we present a peptide T-cell epitope that is exclusively recognized by Ts cells in an LL patient. This epitope resides on the mycobacterial hsp65 and it is presented to Ts cells by a recently described HLA-DR2 subtype, HLA-DRB1*1503.

MATERIALS AND METHODS

Antigens. Soluble *M. leprae* antigen (batch nos: CD135 and CD191) was kindly provided by R. J. W. Rees (Mill Hill, London). Truncated recombinant *Mycobacterium bovis* bacillus Calmette–Guérin hsp65 derivatives were provided by J. Thole (University Hospital, Leiden, The Netherlands). Peptides of hsp65 were synthesized by J.-W. Drijfhout, using solid-phase peptide synthesis. SDS/PAGE-separated electroblotted *M. leprae* antigenic fractions were prepared as described in ref. 24, with slight modifications.

T-Cell Clones. T-cell clones with established antigen-specific suppressor activity were generated from *M. leprae*-stimulated peripheral blood mononuclear cells (PBMC) of a borderline LL patient as described previously (15). The same protocol was used to generate T-cell clones of the same patient with hsp65 or peptide 435–449 (10 μg/ml) stimulation.

T-Cell Proliferation Assays. A total of 10^4 T cells were cocultured for 88 hr with irradiated antigen-presenting cells [APC; 3×10^4 cells of an Epstein-Barr virus-transformed B-cell line (B-LCL) irradiated with 5000 rads (1 rad = 0.01 Gy) or 5×10^4 PBMC irradiated with 3000 rads] in a total volume of 200 μl of Iscove's modified Dulbecco's medium (IMDM) supplemented with pooled 10% human serum (HS), penicillin (100 units/ml), and streptomycin (100 μg/ml) (GIBCO). Antigens were added in the assay mixture at concentrations indicated. Sixteen hours before termination 0.5 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine was added to the wells. The cells were then harvested on glass-fiber filters by using an automatic cell harvester, and the [^{3}H]thymidine incorporation into cell DNA was measured by liquid scintillation counting. The results are expressed as mean cpm of triplicate cultures. SEM of triplicate cultures never exceeded 15%.

T-Cell Suppression Assays. A total of 10^4 cloned responder T cells were cocultured with a total of 5×10^4 HLA class II-matched PBMC irradiated with 3000 rads and *M. leprae* antigen for 88 hr in the absence or in the presence of Ts cells that were added at 1:1 or 1:5 responder-to-suppressor T-cell ratios. [^{3}H]Thymidine incorporation was determined as described above. The results are expressed as mean cpm of duplicate cultures. SEM did not exceed 15%. Percentage suppression was calculated by using the following formula: % suppression = [1 − (cpm in the presence of Ts cells/cpm in the absence of Ts cells)] × 100%.

TCR Analysis. Total RNA was extracted from $5–10 \times 10^6$ cells by using RNAzol (Cinna/Biotecx Laboratories, Friendswood, TX) and converted into first strand cDNA by using oligo(dT) primers (Promega). cDNA was subjected to PCR amplification using 25 TCR V_β and 28 TCR V_α family-specific oligonucleotides as described previously (25–27). After 30–35 cycles of PCR, the products were analyzed and size fractionated in 1% agarose gel electrophoresis. The desired PCR products were captured in 1% low-melting-point agarose gel, and DNA was recovered by using the Magic PCR-Preps DNA purification system (Promega). The purified DNA was used in a PCR-based direct sequencing reaction (Circumvent Thermal Cycle Dideoxy DNA sequencing kit; New England Biolabs) as described previously (25).

RESULTS AND DISCUSSION

CD4$^+$, HLA-DR-restricted Ts cells that down-regulate mycobacterium-specific T-cell responses were previously isolated from the peripheral blood of a borderline LL patient (15). These Ts cells display low proliferation in response to *M. leprae* antigen and specifically suppress the proliferative activity of *M. leprae*-reactive T cells, whereas T-cell proliferation in response to control antigens such as herpes simplex virus (HSV) is not suppressed (15, 18, 19).

To probe the antigen(s) of *M. leprae* that trigger these Ts cells we first tested the activity of one Ts cell clone, SC2C8, against *M. leprae* fractions that had been separated by one-dimensional SDS/PAGE. This clone proliferated maximally in response to a fraction with a molecular mass between 43 and 67 kDa (data not shown). Prompted by these results, we next screened known individual mycobacterial antigens with molecular masses of around 60 kDa for recognition by Ts clone SC2C8. Ts SC2C8 responded to the mycobacterial recombinant hsp65 (Fig. 1*A*). No response was seen to the human hsp60 and to the recently discovered second *M. leprae* hsp65 (28) (Fig. 1*A*). Thus hsp65 triggers not only Th cells (29) but also Ts cells.

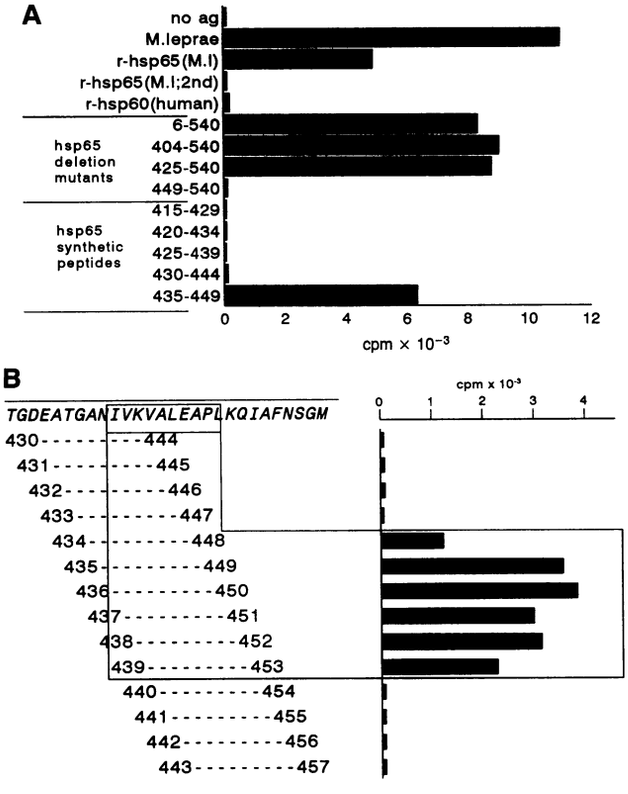

FIG. 1. Molecular mapping of the epitope recognized by Ts cell SC2C8. (*A*) The proliferative response of SC2C8 in response to intact recombinant (r-) antigens of 65 kDa [hsp65 (M.l) = 65-kDa heat shock protein of *M. leprae*; hsp65 (M.l; 2nd) = second 65-kDa heat shock protein of *M. leprae*; hsp60 (human) = human 60-kDa heat shock protein], deletion mutants of *M. bovis*/bacillus Calmette–Guérin hsp65, and to overlapping 15-mer peptides of r-hsp65 that have been synthesized according to the sequence of *M. leprae* hsp65. (*B*) The proliferative response of SC2C8 to 14-amino-acid-overlapping 15-mer synthetic peptides of hsp65. The amino acid sequence of the r-hsp65 spanning the region 430–457 is indicated, and the minimal sequence recognized by SC2C8 is boxed.

To identify the Ts cell epitope on the hsp65, N-terminal deletion mutants of the hsp65 protein were used (Fig. 1*A*). Ts SC2C8 significantly proliferated in response to the deletion mutants of hsp65 that lacked amino acids up to position 425, but when deletions were extended to amino acid 449 antigenic activity was completely lost, indicating that the epitope was located between amino acid residues 425 and 449. We analyzed this region in detail by using overlapping synthetic peptides (Fig. 1*A*). SC2C8 responded significantly to peptide 435–449, whereas other peptides spanning the region 415 to 444 were not stimulatory. The minimal sequence recognized by Ts clone SC2C8 mapped to residues 439–448 (Fig. 1*B*).

Surprisingly, however, this Ts cell epitope is not a new epitope but was previously reported to stimulate mycobacterium-reactive T cells in an HLA-DR1-restricted manner (30). Since the Ts cell clone SC2C8 recognizes *M. leprae* not in a DR1- but in a DR2-restricted fashion (18), we investigated the T-cell response to this particular epitope in more detail. We first tested the response of all available suppressor as well as nonsuppressor T-cell clones derived from patient SC to hsp65 and peptide 435–449 (Fig. 2*A*). All DR2-restricted suppressor T-cell clones of patient SC (n = 8) recognized both hsp65 and peptide 435–449 (Fig. 2*A*). T-cell receptor analysis (see below) revealed that these clones were derived from at least three different precursors. Experiments with 14-amino-acid-overlapping peptides indicated that the minimal core sequence of 439–448 was recognized by all Ts clones (data not shown). In contrast, none of the DR2-restricted nonsuppressor T-cell clones recognized hsp65 or peptide 435–449 (Fig. 2*A*). The response of Ts cell clones to peptide 435–449 could be blocked by antibodies directed to HLA-DR but not to HLA-DP or -DQ, confirming our previous observations that Ts cell clones were restricted by HLA-DR (data not shown). Peptide 435–449-reactive Ts cell clones recognized antigen only in context of APC that were genotyped as HLA-DRB1*1503 (DR2) (Fig. 2*B*) but not by APC that expressed some other HLA-DR2 variants such as HLA-DRB1*1501 or DRB1*1602. None of the other tested APC, including the HLA-DR1-positive APC (GER), could present peptide 435–449 to Ts clones (Fig. 2*B*). Substantiating these results, peptide 435–449 bound significantly to HLA-DRB1*1503 but not to HLA-DRB1*1501 (DR2) (Fig. 2*B*). In contrast to the Ts clones, the "nonsuppressor" T cells of patient SC recognized *M. leprae* antigens presented by both HLA-DRB1*1501- and HLA-DRB1*1503-expressing APC (Fig. 2*B*). Note that nonsuppressive T cells might recognize antigens also through HLA-DRB5*0101 (HLA-DRw51), which is always coexpressed with HLA-DRB1*1503 or HLA-DRB1*1501 on the cell surface. Thus, the data suggest that the immune response protein HLA-DRB1*1503, a recently discovered variant of HLA-DR2 (32–34), binds and presents peptide 435–449 to only Ts cells, whereas T-cell epitopes that stimulate nonsuppressive T cells can be presented by various HLA-DR2 alleles.

To confirm these results we have cloned peripheral blood T cells of patient SC after stimulation with hsp65 or with peptide 435–449 and tested their proliferative and suppressive capacities (Fig. 3). Several T-cell clones derived after stimulation with hsp65 (TLCαhsp65) or after stimulation with peptide 435–449 (TLCα435–449) proliferated initially weakly in response to *M. leprae* as well as to hsp65 but lost this capacity after restimulation (data not shown). All these T-cell clones, however, strongly inhibited *M. leprae*-triggered T-cell proliferation (Fig. 3), and they retained this suppressive activity consistently over time (data not shown). Note that control T-cell clones of patient SC that recognize antigens other than peptide 435–449 or hsp65-reactive clones from a healthy individual did not show suppressive activity (Fig. 3). Another set of T-cell clones that was generated by the subcloning of a bulk cell line stimulated with peptide 435–449 (designated as SC_{F2}) also proliferated in response to peptide 435–449 and suppressed *M. leprae*-induced T-cell proliferation (Fig. 3). These latter clones retained their proliferative and suppressive capacity after restimulation (data

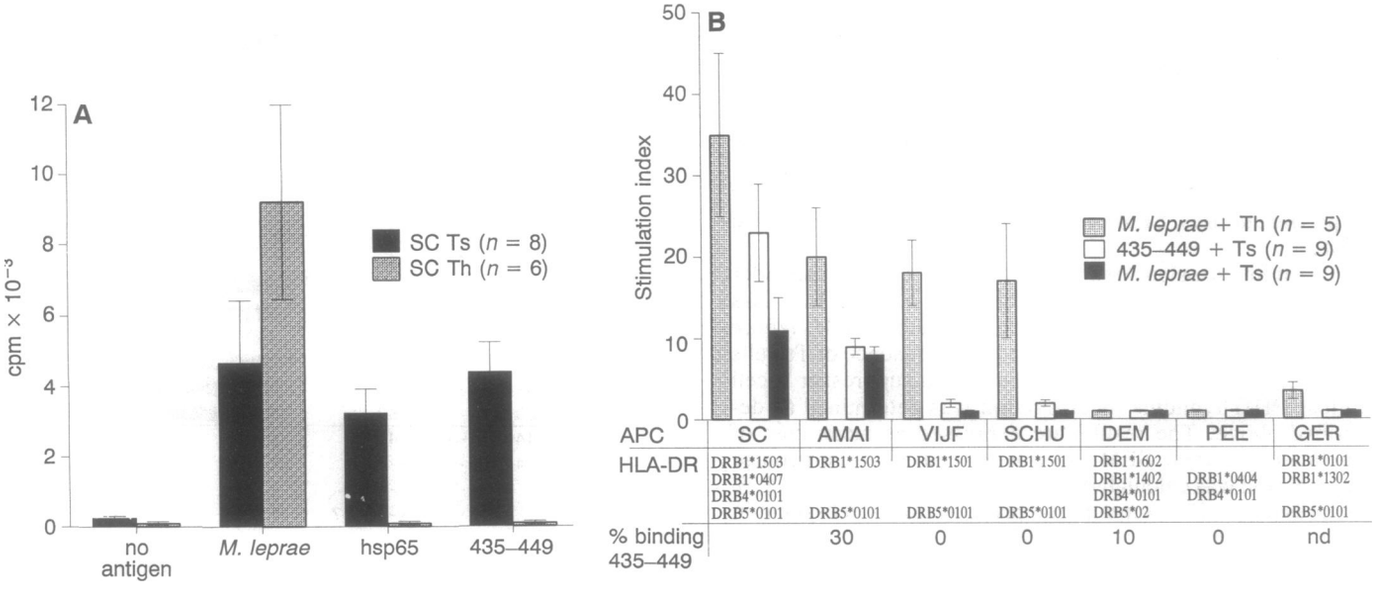

FIG. 2. (*A*) Proliferative response of patient SC's Ts cell clones and T-cell clones without suppressive activity to *M. leprae* r-hsp65 (2 mg/ml) and to peptide 435–449 (0.1 μg/ml). Results are expressed as the mean proliferative response (cpm) of T-cell clones to the antigens. Error bars indicate the SEM. (*B*) Proliferative response of suppressive (Ts) and nonsuppressive (Th) T-cell clones in response to antigens presented by various B-LCL with different HLA genotyping. The HLA-DR genotypes of the panel were determined by allele-specific oligonucleotide hybridization (oligotyping). The proliferative responses of T-cell clones are expressed as the mean of the stimulation indexes (SI = cpm in the presence of the antigen/cpm in the absence of the antigen) and the SEM are indicated as error bars. Proliferation of T-cell clones in the absence of relevant antigen did not exceed 500 cpm. The binding of biotinylated peptide 435–449 to the HLA-DR of intact B-LCL was measured by fluorescence-activated cell sorting analysis as described previously (31). The percentage binding of peptide to HLA-DR molecules was calculated as follows: % binding = [(relative fluorescein intensity channel number of B-LCL pulsed with peptide − channel number of untreated B-LCL)/(channel number of B-LCL incubated with anti-HLA-DR antibodies − channel number of untreated B-LCL)] × 100%. ND, not done.

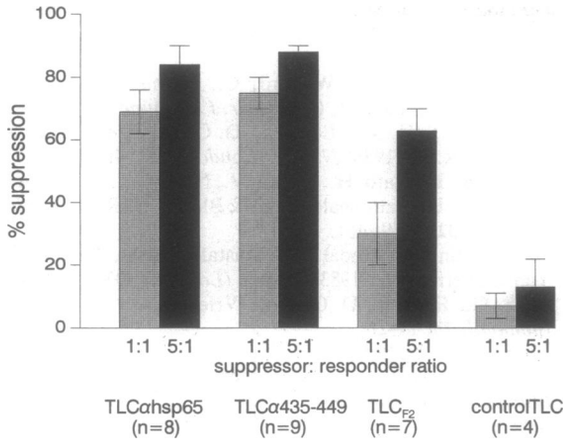

FIG. 3. Suppressive activity of newly generated Ts cell clones of patient SC derived from PBMC after stimulation with r-hsp65 (indicated as TLCαhsp65) and peptide 435–449 (indicated as TLCα-435–449, and TLC_{F2}) on the *M. leprae*-triggered proliferation of a nonsuppressive T-cell clone (SC1G6). Two *M. leprae*-specific non-suppressor T-cell clones of the same patient and two hsp65-reactive clones from a healthy individual were tested as controls (indicated as controlTLC). The *M. leprae* "responder" T-cell clone SC1G6 was added in the wells at 10^4 cells per well, and the "suppressor" cells were added at two different responder-to-suppressor ratios. The suppressive activity was calculated by using the following formula: % suppression = [1 − (cpm in the presence of the Ts/cpm in the absence of Ts)] × 100%. Results are expressed as the mean percent suppression induced by T-cell clones represented in each group. Error bars indicate the SEM. Response of the clone SC1G6 to phytohemagglutinin was not inhibited by the clones (data not shown).

not shown) and appeared to be restricted by HLA-DRB1*1503 as well. TCR analysis of some of these newly generated Ts clones revealed that they were derived from at least six different precursors (see below and Table 1). Together these results substantiate the idea that hsp65 peptide 435–449 induces HLA-DRB1*1503-restricted Ts cells in this particular patient.

To further characterize these Ts cells at the molecular level we analyzed the TCR α and β chains of peptide 435–449-reactive Ts cells by PCR-assisted amplification of Vα and Vβ variable region segments, followed by direct sequencing (Table 1). Ts cell clones that were derived after stimulation with *M. leprae* (shown in Fig. 2*A*) appeared to use Vβ-8.3 or Vβ-4. Sequencing data revealed that all Ts clones that were positive for Vβ-8.3 expressed identical β chains and identical Vα segments (Vα-2) (Table 1), suggesting clonal expansion of the same Ts precursor cell during antigenic challenge. Ts clones that expressed Vβ-4 appeared to originate from two different T-cell precursors that expressed very similar TCR β chains. Their cDNA sequences differed only at four positions, which were reproducibly found upon sequencing of different PCR products. These differences resulted in only one amino acid change, valine to alanine, in the N-D-N region. Other differences in the DNA sequences did not affect the amino acid compositions. Although the TCR β chains were similar, different α chains, namely Vα-19 and Vα-23, were expressed by these latter Ts clones. There was no similarity between the TCR of these latter clones and those that expressed Vβ-8.3.

Analysis of seven additional Ts clones that were obtained after stimulation of PBMC with peptide 435–449 revealed that they used Vβ segments other than Vβ-8.3 or Vβ-4. Four clones were positive for Vβ-5.1, one for Vβ-20, one for Vβ-21, and one for Vβ-23 (Table 1). Sequence analysis of Vβ-5.1-positive T-cell clones showed that they originated from a single Ts cell precursor, because they displayed identical Vβ N-D-N regions. Interestingly, both Vβ-5.1- and Vβ-8.3-expressing clones used the Jβ-2.7 joining region segment and both contained a serine residue at the same position in the hypervariable N-D-N region. These clones, however, showed no similarity with those expressing Vβ4 (Table 1). Some additional clones generated by stimulation with hsp65 were screened by pools of Vβ primers. Preliminary data revealed that at least two additional Vβ chains were used by the TCR of these clones. Thus, although some Ts cells showed similarities in the TCR-β chains, there is no absolute correlation between TCR usage and recognition of the peptide 435–449/HLA-DRB1*1503 specificity. We have found that peptide 435–449 challenge of PBMC gives rise to mature Ts cells developing from at least nine different TCR αβ cell precursors.

The fact that hsp65 peptide 435–449 exclusively induces Ts cells in patient SC confirms in a human context earlier work of Sercarz and Krzych (35), who showed in rodent models that suppressor determinants within a protein antigen are clearly distinct from Th cell determinants. However, since the recognition of hsp65 peptide 435–449 by Ts cells is

Table 1. TCR α- and β-chain usage of Ts cell clones directed to hsp65 peptide 435–449

Clone	Generated with	TCR Vβ	N-D-N	TCR Jβ	TCR Vα	TCR Jα
SC2C8	*M. leprae*	Vβ-8.3		Jβ-2.7	Vα-2	Jα-9.16
SC2G6		TTC TGT GCC AGC AGT	TTA GTT TCG TTT CGG GGC	GAG CAG TAC TTC GGG		
SC2G10		F C A S S	L V S F R G	E Q Y F G		
SC3G7						
SC2G5	*M. leprae*	Vβ-4		Jβ-2.2	Vα-19	Jα-17.1
		AGC ATA TAT CTC TGC AGC	GTT CAA CGC	ACC GGG GAG CTG		
		S I Y L C S	V Q R	T G E L		
SC2C5	*M. leprae*	Vβ-4		Jβ-2.2	Vα-23	Jα-1.9
SC2B4		AGC ATA TAT CTC TGC AGC	GCA CAG AGG	ACC GGG GAG CTG		
		S I Y L C S	A Q R	T G E L		
SC_{F2}.2A4	435–449	Vβ-5.1		Jβ-2.7		
SC_{F2}.3H3		CTT TGC GCC AGC AGC	TTG TTT TCC GGC GGG GAC	GAG CAG TAC TTC GGG		
SC_{F2}.3H11		L C A S S	L F S G G D	E Q Y F G		
SC_{F2}.4H4						
SC_{F2}.6.10	435–449	Vβ-23				
SC_{F2}.8.01		Vβ-21				
SC_{F2}.8.10		Vβ-20				

TCR α- and β-chain analysis was performed as indicated in the text. The amino acid sequences for TCR β sequences are shown in one-letter code under the DNA sequences. One sequence is given for T-cell clones that expressed the same TCR β chain. The breaks in the amino acid sequence indicate the junction sites between the known Vβ or Jβ sequences and D- and/or N-region sequences.

controlled by the immune response gene *HLA-DRB1*1503*, this peptide may induce Ts cells only in individuals that express this particular HLA-DR2 variant. In fact, this might be one of the reasons why we have not been able to generate hsp65 peptide 435–449-reactive T cells from other HLA-DR2-serotyped LL patients. On the other hand, previous observations indicate that peptide 439–448 can also induce T cells in the context of HLA-DR1 (31). The fact that these cells were derived from a *M. leprae* "high responder" tuberculoid leprosy patient whose polyclonal T cell lines responded strongly to *M. leprae* and to multiple HLA-DR1-restricted hsp65 epitopes (30) suggests that these T cells did not possess suppressive activity. Thus, although the idea is not formally proven, peptide 439–448 of hsp65 may not function as a universal suppressor epitope but instead induces distinct functional T-cell subsets depending on the presenting MHC molecule. Indeed, a similar phenomenon has been described in a murine model by Bottomly *et al.* (5, 36), where a single peptide induced different T-cell subsets (in this case Th1 or Th2 cells) depending on the MHC genetic background of the mice. In this model the ligand density (MHC–peptide complexes) on the APC may determine the induction of differential T-cell subsets by the same peptide (5, 36, 37).

In conclusion, our data provide the first, to our knowledge, evidence that a specific peptide–HLA class II combination may exclusively activate Ts cell clones in humans and suggest that the type of immune response to a peptide antigen can be controlled by HLA class II immune response genes.

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