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Examination of the highly diverse CD4+ T-cell repertoire directed against an influenza peptide: a step towards TCR proteomics

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Abstract We combined several recent technological advances in immunology and molecular biology to identify and sequence a large number of T-cell receptor (TCR) genes specific for a particular antigen. We utilized class II MHC tetramers and interferon-γ surface capture to isolate from samples of peripheral blood the population of $CD4^+$ T cells responding to a peptide derived from influenza hemagglutinin and restricted by HLA-DR1. Detailed analysis of hundreds of clones from three different patients revealed an extremely diverse repertoire, with little overlap between patients. We observed no dominant usage of particular Vβ segments nor any clear CDR3 sequence motif in the responding T cells, but most of the clones appear to utilize acidic residues in the CDR1 and CDR3 regions, presumably to interact with the exposed basic residues in the MHC-peptide complex. This methodology could be expanded to a large scale to identify the generalized rules governing TCR-MHC engagement and factors which shape the T-cell repertoire after vaccination and in autoimmune pathologies.

Keywords T lymphocytes · Antigens/peptides/epitopes · T-cell receptors · T-cell receptor repertoire · Proteomics

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Introduction

At the center of adaptive immunity is the vast repertoire of ligand specificities contained within the antigen-binding sites of immunoglobulins and T-cell receptors (TCR). In the last two decades immunologists have learned an enormous amount about how antigens are processed and presented to T cells, how T cells are triggered, and the consequences of T-cell activation, but relatively little is known about the rules governing which T cells will respond to which epitopes, how HLA haplotype affects the T-cell repertoire, why some epitopes are more immunodominant than others, why disease pathology can differ from person to person, and, ultimately, how we can manipulate these processes to build immunity or suppress autoimmunity.

The antigen binding region of TCR is formed by loops at the top of immunoglobulin-like domains of TCRα and TCRβ subunits. These loops, termed complementarity determining regions (CDR) -1, -2, -3, are the most diverse regions of the molecules. Although the gene fragments which combine to form these domains are known, and the mechanisms of their recombination have been studied for years, we still have little knowledge of the set of recombined TCR genes expressed by an individual, called the T-cell repertoire, and of the selective forces that shape the repertoire during immune function.

Several methods are currently available to describe the T-cell repertoire. The usage of $V\alpha$ or $V\beta$ genes in particular T-cell subpopulations, and the extent of skewing relative to "normal" reference distributions, can be evaluated using panels of monoclonal antibodies against particular families of TCR genes (Diu et al. 1993). Characterization of the distribution of CDR3 length variants ("spectratyping") can help to evaluate the extent of oligoclonality within the Vβ subpools of a responding population (Pannetier et al. 1993), as can PCR heteroduplex analysis (Wack et al. 1996). However, these methods cannot examine the polydispersity of the TCR repertoire on a clonal level. Moreover, immunodominant epitopes,

particularly those successfully countered by the immune system with a polyclonal response involving many clonal variants, may well elicit minimal skewing and thus be poorly characterized by these methods. Clonal analysis of antigen-specific T cells typically involves in vitro isolation and maintenance of individual T-cell clones. This process is labor intensive and technically difficult, and may not accurately reflect the in vivo repertoire of responding cells (Annels et al. 2000; Prevost-Blondel et al. 1995).

Two recent advances in the identification of antigen-specific T cells, MHC tetramer staining (Altman et al. 1996), and cytokine surface-capture (Brosterhus et al. 1999), have enabled immunologists to isolate T-cell clones with minimal manipulation ex vivo. We have utilized these technologies to isolate populations of $CD4^+$ T cells reactive for a peptide epitope from the hemagglutinin protein of A-strain influenza viruses. This epitope is known to be highly immunodominant in a variety of class II MHC backgrounds (Gelder et al. 1995; Lamb and Green 1983), including *HLA-DRB1*0101*, the common *DR1* allele investigated here. From sorted pools of responding cells, we prepared a TCRβ library using cDNA technology that enabled the simultaneous amplification of all TCRβ genes with minimal sequence-dependent bias. Direct sequencing yielded an extremely large and diverse set of TCRβ sequences, all specific for a single peptide, Ha, and restricted by a single class II MHC allele, *HLA-DR1*. We argue that this and other recent work (Bourcier et al. 2001; Bousso et al. 1999; Casrouge et al. 2000; Cohen et al. 2002; Douek et al. 2002; Hennecke and Wiley 2001; Lim et al. 2000; Naumov et al. 1998; Valmori et al. 2002) form the basis of an emerging field of TCR proteomics.

Materials and methods

Preparation of fluorescent class II MHC oligomers

Methodology for the production of fluorescent oligomeric DR1-peptide reagents has been described in detail previously (Cameron et al. 2001). Briefly, extracellular domains of the α and β subunits of *HLA-DRB1*0101* were expressed separately in insoluble form in *Escherichia coli* and refolded in vitro together with peptide, followed by biotinylation at a cysteine residue introduced at the C-terminus of the α subunit, and oligomerization using phycoerythrin-conjugated streptavidin. Peptides were synthesized using solid-phase Fmoc chemistry and purified by C18 reverse-phase chromatography.

T-cell culture

Freshly isolated peripheral blood mononuclear cells (PBMC) from three DR1+ volunteers were aliquoted into 24-well plates at 5×10^6 lymphocytes per well in RPMI-1640 media (Gibco) supplemented with 10% human serum, 50 units/ml penicillin G (Gibco), and 50 μg/ml streptomycin sulfate (Gibco), and stimulated by the addition of 5–20 μM peptide. Starting on day 7, interleukin (IL)-2 (40 units/ml, Aldesleukin, Chiron, Emeryville, Calif.) was added to the media every 3–4 days.

DR1 oligomer staining

T cells (~10^7/ml) were mixed with DR1-peptide oligomers for 3–5 h at 37°C, chilled for 5 min, supplemented with appropriate secondary antibodies for 30 min, and washed twice with cold wash buffer (PBS, 1% fetal bovine serum, 15 mM sodium azide). Secondary antibodies used were anti-CD4-APC (Diatec, Norway), and various anti-TCRVβ-FITC (Beckman-Coulter or Pierce Chemical).

Activation and gamma interferon surface-capture assay

The expanded T-cell populations were mixed at a 1:4 ratio with EBV-transformed DR1 homozygous B cells (EBV1.24) pulsed with 1 μM Ha peptide at a final density of 10^7 cells/ml. Antibodies against CD28 and CD49d (Becton-Dickinson) were added to 1 μg/ml to augment the stimulation. After 6–9 h at 37°C the gamma interferon (INF-γ) capture assay was performed as described (Cohen et al. 2002; Douek et al. 2002) essentially following the manufacturer's protocol (Miltenyi Biotec). The magnetically enriched IFN-γ secreting T cells were analyzed on a FACSCalibur and further purified on a FACSVantage SE cell sorter (Becton Dickinson).

PCR amplification, cloning and sequencing

Total RNA was extracted from the Ag-specific T-cell pools using Trizol reagent. First-strand DNA synthesis was accomplished using a downstream primer derived from near the end of the TCR *Cβ* gene (AATCCTTTCTCTTGACCATG) and Superscript II reverse transcription (Gibco). A hybrid RNA/DNA primer from the SMART cDNA kit (Clontech) was used to attach a common upstream anchor peptide to all first-strand DNA molecules in order to enable simultaneous and unbiased amplification of all *TCRβ* cDNA regardless of their Vβ sequence. PCR amplification was done using the Advantage cDNA PCR Kit (Clontech) following the manufacturer's SMART PCR protocol, and using a Clontech primer for the upstream primer, and a downstream primer derived from near the beginning of the TCR *Cβ* gene (TTGGGTGTGGGAGATCTCTGCTTCTGATGGC). PCR products were purified from agarose gels and cloned using the TOP TA cloning kit (Invitrogen) into TOP10 *E. coli* and hundreds of colonies were isolated. Plasmids were generated from individual colonies as needed and DNA sequencing was performed on the top strand sequence at the Massachusetts General Hospital DNA sequencing core facility. Errors relative to published TCR *Vβ* genes were observed at approximately 1 in 1,500 bases. This method is similar to one recently described elsewhere (Douek et al. 2002).

Statistical analysis

Statistical parameters (chi-square function, correlation coefficients) were calculated using standard formulas (Bevington and Robinson 1992), as described in the legend to Table 3.

Results

TCRβs can be analyzed in detail by sorting, cloning and sequencing

The methodology described here for the analysis of an antigen-specific T-cell repertoire was developed initially for $CD8^+$ T cells (Cohen et al. 2002; Douek et al. 2002), and is summarized in Fig. 1. Several key technological developments within the last few years have made this

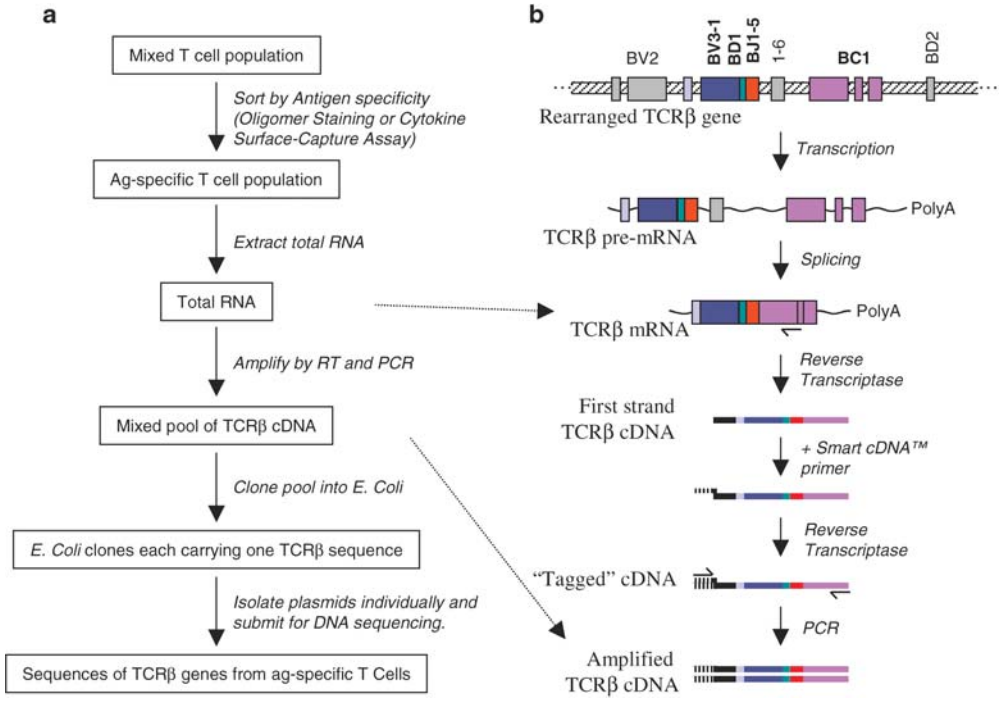

Fig. 1a, b Identification of TCRβ sequences specific for particular MHC-peptide. **a** Flow chart summarizes the experimental procedure for the identification, isolation, amplification and cloning of TCRβ sequences specific for particular peptide antigens. The procedure can be easily adapted for analysis of $CD8^+$ T cells or TCRα. **b** Schematic representation of the steps shown in a. This methodology has been made possible by several key technological advances in the last few years: MHC tetramer staining and cytokine surface-capture assay to identify and sort antigen-specific T cells, Smart cDNA technology to amplify sequences from only a few cells (10^4) regardless of TCR Vβ family, and the recent drastic reduction in the cost of large-scale DNA sequencing projects

methodology practical, including the use of soluble fluorescent oligomers of MHC-peptide complexes ("MHC-tetramers"; McMichael and O'Callaghan 1998) and bi-specific cytokine capture antibodies (Brosterhus et al. 1999) to identify antigen-specific $CD4^+$ T cells, a hybrid DNA/RNA primer technology commercially available from Clontech (SMART cDNA technology) that allows the simultaneous amplification all TCRβ mRNAs, and inexpensive automated DNA sequencing facilities. One limitation of class II MHC tetramer technology as currently practiced is that the sensitivity of detection generally is too low to allow analysis of antigen-specific $CD4^+$ T cells directly ex vivo in samples of peripheral blood (Cameron et al. 2002; Maini et al. 1998; Novak et al. 1999), even during acute infections (Kotzin et al. 2000), although inflammatory sites may prove to have T-cell frequencies sufficient for direct detection (Meyer et al. 2000).

For the influenza antigen under consideration in this study (Ha[306–318]), we were unable to identify specific $CD4^+$ T cells directly ex vivo from either healthy individuals (Cameron et al. 2002), or volunteers who had recently received influenza vaccinations (T.O.C., L.J.S., and E. Mellins, unpublished data), above our minimal detection frequency of about 0.1%. In previous studies, the frequency of HA-responding has been estimated at less than 0.005% (Novak et al. 1999). In order to boost frequencies of specific cells from known responders, we amplified the responding cells with a single antigenic stimulation ex vivo, as previously described (Novak et al. 1999). After this treatment, between 5% and 7% of the total T-cell population appeared to be Ha-specific, as judged by MHC tetramer staining (Fig. 2a,b) or by IFN-γ capture (Fig. 2e,f). These cells were isolated by flow cytometry and analyzed for their TCRβ gene sequences.

A large number of different TCRβ sequences are found in DR1-Ha specific $CD4^+$ T cells

From MHC oligomer and IFN-γ capture pools of Ha-specific T cells from three $DR1^+$ patients, 272 TCRβ clones were sequenced (Table 1). Very diverse populations were observed. Notably, only one TCRβ sequence appeared in more than one individual (WL2–21 and SL-8). In contrast, many of the sequences appeared in both the Tet and IFN-γ pools from a single individual (61 of 120 observations in WL1, 57 of 111 observations in SL). None of the newly identified TCRβ sequences match previously published data (Brawley and Concannon 1996; Hewitt et al. 1992; Ostrov et al. 1993; Prevost-Blondel et al. 1995; Snoke et al. 1993; Wedderburn et al. 1995; Yassine-Diab et al. 1999). Multiple observations of particular sequences reflects their frequency in the library of cloned receptor genes in *E. coli*, limited by the statistics of a relatively small sample size (for example, single observations are likely to be overestimates of infrequent clones). If the PCR reaction proceeded without skewing, then we expect this frequency to reflect the distribution of particular clones in the stimulated polyclonal line, which should be similar to the original polyclonal population in the volunteer depending on the differential growth of clones during the in vitro stimulation.A similar experiment in the literature, in which CFSE (carboxyfluorescein diacetate, succinimidyl ester) was used to track cell divisions, suggests that the responding population expands in parallel, all cells having undergone a

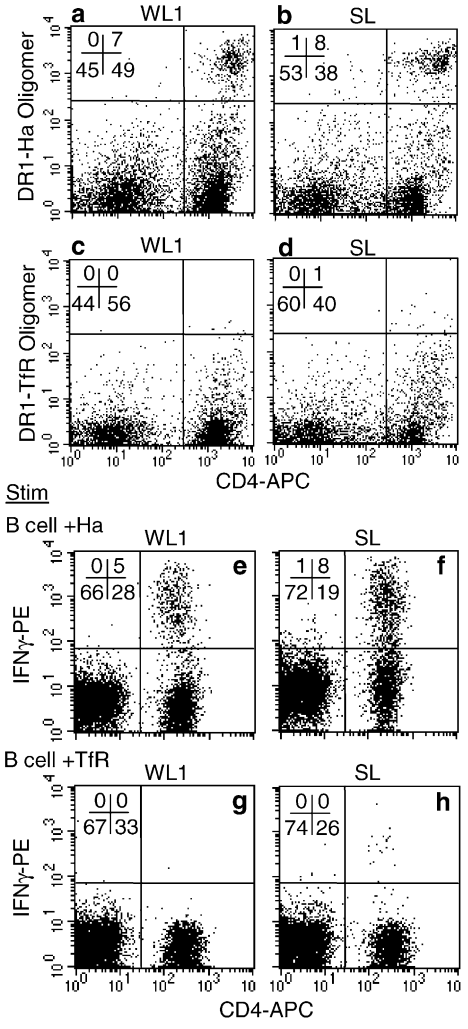

Fig. 2a–h Detection and sorting of Ha-specific DR1-restricted CD4+ T cells. Peripheral blood monocyte cells (PBMC) from volunteers WL1 (**a**, **c**, **e**, **g**), WL2 (not shown), and SL (**b**, **d**, **f**, **h)** were stimulated once in vitro with Ha peptide, cultured for 3 weeks and subsequently assayed for Ha specificity. Expanded PBMCs were stained with specific DR1-Ha oligomers (**a**,**b**) or control DR1-TfR oligomers (**c**,**d**). Percent of cells in each quadrant is indicated in the upper left quadrant of each dot plot. CD4+ T cells specific for Ha peptide are detected in the *upper right hand* quadrant of panels (**a**, **b**). Cells of this quadrant were isolated by FACS sorting and used to make total mRNA. (**e–h**) Expanded PBMCs were stimulated with DR1-homozygous B cells pulsed with either specific Ha peptide or control TfR peptide. Shortly after this stimulation, cells were stained for IFN-γ secretion using a surface-capture methodology. Dot plots of CD4 vs. IFN-γ are shown. Percent of cells in each quadrant is indicated in the *upper left quadrant* of each plot. CD4+ T cells specific for Ha peptide are detected in the *upper right hand* quadrant of panels (**e**, **f**). Cells of this quadrant were isolated by FACS sorting and used make total mRNA

similar number of divisions (Novak et al. 1999). Thus, we believe the frequency of particular sequences observed in our experiment to be similar to the frequency of these clones in the original PBMC sample. Skewing during either the in vitro stimulation or PCR reaction could lead to the over- or under-representation of particular clones, but nonetheless the total number of sequences observed provide a lower limit to the clonal diversity of the original population.

Validation of the method

The possibility of non-uniform PCR amplification leading to biases in the observed TCR repertoire has been addressed previously by analysis of a bulk, non-specific CD8+ T-cell population, for which an extremely high diversity was observed (Cohen et al. 2002; Douek et al. 2002) increasing our confidence that this methodology probes the TCRβ repertoire with minimal bias. However, we noted that clone WL1–19 appeared 10 times in the Tet pool of WL1, but not even once in the IFN-γ pool. Because all ten of the observations of WL1–19 occurred from the same sorting and PCR amplification experiment, and no WL1–19 sequences were observed in another similar reaction, it appears that some degree of skewing of the repertoire may occur during amplification. Therefore we analyzed the sequences without weighting by their occurrence frequency.

PCR amplification can introduce sequence errors into the analysis. To estimate the error rate, we compared *Vβ* sequence data with published *Vβ* sequences and estimated the error rate to be approximately 1 in 1,500 base-pairs. Clones WL1–33 and WL2–11 contain RAS instead of the conserved CAS sequences, each due to the substitution of a cytosine for a thymidine in the first position of the C/R codon. While these might represent errors introduced during PCR or during the sequencing reaction, we note that there are other published examples of *Vβ* genes with this same sequence (Arden et al. 1995).

In order to further confirm our results we compared the Vβ usage of our sequences with Vβ usage as determined by a conventional anti-TCR Vβ mAb analysis (Diu et al. 1993). A strong correlation was observed between the Vβ mAb analysis and the direct sequencing data for SL (r=0.94), and a moderate correlation for WL1 (r=0.58) (Fig. 3). Sources of error in the Vβ mAb analysis include variable brightness and incomplete specificity of different Vβ mAbs, difficulty staining T cells with Vβ mAbs after DR1-oligomer analysis due to CD3 down regulation, and possibly clonal distribution shifts during the time between direct sequencing analysis and Vβ mAb analysis (2 weeks). The direct sequencing approach is subject to possible skewing during PCR amplification. With these limitations in mind, we suggest that the correlations observed are within experimental error.

To further analyze the reliability of the new TCRβ sequences, we divided the sequences into two approximately equal-sized groups, one set of “validated” sequences considered to be more reliable, and the remaining non-validated sequences, and compared their Vβ usage. We assigned a sequence to the validated group if it appeared in both the Tet and the IFN-γ pools of the individual, or if the sequences exhibited significant homolo-

Table 1 Vβ TCRs specific for DR1-Ha identified by direct sequencing. Frequency of clones in either Tet or IFN-γ pools represent the number of times sequence was observed in independent sequencing reactions

WL1				Freq[c]	
ID	Vβ[a]	CDR3[b]	Jβ	Tet	IFN
WL1-1	1.1	CASSVGPGPGDTEAFF	1.1		1
WL1-2	3.1	CASSLISEPNTEAFF	1.1		2
WL1-3	3.1	CASTQGVLTYEQYF	2.7		2
WL1-4	3.1	CASSGVPEQTPSYEQYF	2.7		2
WL1-5	8.1	CASTPSWETEAFF	1.1		1
WL1-6	8.1	CASSPYRAQNTEAFF	1.1		1
WL1-7	1.1	CASSVVPEWEGELFF	2.2	2	1
WL1-8	1.1	CASSPLTGGQTDTQYF	2.3	3	1
WL1-9	3.1	CASSSPSLADNNEQFF	2.1	1	1
WL1-10	3.1	CASRTGTHTDTQYF	2.3	3	5
WL1-11	3.1	CASSLSPELETQYF	2.5	1	2
WL1-12	6.5	CASSVGQGKAFF	1.1	7	11
WL1-13	6.5	CASSLGQGEQFF	2.1	1	2
WL1-14	8.1	CASSLSQGDQPQHF	1.5	7	13
WL1-15	1.1	CASSVAPSPSETEAFF	1.1	1	
WL1-16	1.1	CASSVAPSGSESPLHF	1.6	1	
WL1-17	1.1	CASSTGFSEQFF	2.1	1	
WL1-18	1.1	CASSAGFSEQFF	2.1	4	
WL1-19	2.1	CSARGQAIYGYTF	1.2	10	
WL1-20	3.1	CASSPGQIYGYTF	1.2	2	
WL1-21	3.1	CASIKGGQDYSPLHF	1.6	1	
WL1-22	3.1	CASSPGGGDTYNEQFF	2.1	1	
WL1-23	3.1	CASSDSGTYNEQFF	2.1	2	
WL1-24	3.1	CASSTIGLDTGELFF	2.2	3	
WL1-25	3.1	CASSLAPELDTQYF	2.3	4	
WL1-26	3.1	CASSLVPELGEQYF	2.7	1	
WL1-27	6.6	CASSEAWTSGKNEQFF	2.1	1	
WL1-28	8.1	CASSSDGTGEKLFF	1.4	1	
WL1-29	11.1	CASSVLTDNGYTF	1.2	7	
WL1-30	13.1	CASSREGTVNHGGYTF	1.2	2	
WL1-31	13.1	CASTGSSYEQYF	2.7	5	
WL1-32	13.1	CASSSPWETQYF	2.7	1	
WL1-33[c]	20.1	RAWSRLVLGSGNIYF	1.3	1	
WL1-34	20.1	CAWDRLARFQETQYF	2.5	1	

WL2				Freq	
ID	Vβ	CDR3	Jβ	Tet	IFN
WL2-1	1.1	CASSVTPGGTEAFF	1.1	1	
WL2-2	1.1	CASSGTGEPTNEKLFF	1.4	1	
WL2-3	2.1	CSADSGTSTDTQYF	2.3	2	
WL2-4	3.1	CASSLNPELNYGYTF	1.2	1	
WL2-5	3.1	CASGATGHHNSPLHF	1.6	1	
WL2-6	3.1	CASRDSGTRNEQFF	2.1	1	
WL2-7	3.1	CASGFGSGSLTDTQYF	2.3	1	
WL2-8	3.1	CASSTTQGFYEQYF	2.7	4	
WL2-9	5.4	CASSLGLAGDQETQYF	2.5	1	
WL2-10	5.6	CASNNGLAHTPEYF	2.5	1	
WL2-11[c]	6.1	RASTTKGLRDSPLHF	1.6	1	
WL2-12	6.1	CASSQPAGPSTDTQYF	2.3	4	
WL2-13	6.4	CASSSYLWTGNLSPLHF	1.6	1	
WL2-14	6.5	CASSLGQNEQYF	2.7	1	
WL2-15	6.5	CASSLGQGEQYF	2.7	1	
WL2-16	6.5	CASSFLGQFSYEQYF	2.7	2	
WL2-17	6.5	CASSLDSDHEQYF	2.7	1	
WL2-18	8.1	CASSLGGEVSGQGNQPQHF	1.5	1	
WL2-19	11.1	CASSDARTGDIGQYF	2.3	1	
WL2-20	11.1	CASSDPTGTGANVLTF	2.6	1	
WL2-21[e]	11.1	CASSDAGTGDYEQYF	2.7	2	
WL2-22	13.1	CASGPLGGDNQPQHF	1.5	3	
WL2-23	13.3	CASSEIAGGLGFF	2.1	2	
WL2-24	15.1	CATSDTGTSVGGQYF	2.5	1	
WL2-25	21.1	CASSSSESGSYNEQFF	2.1	1	
WL2-26	22.1	CASVPGLSYEQYF	2.7	4	

SL				Freq	
ID	Vβ	CDR3	Jβ	Tet	IFN
SL-1	1.1	CASSVTPGHTEAFF	1.1		1
SL-2	1.1	CASSVAGGETEAFF	1.1		1
SL-3	1.1	CASSVAPSANTGELFF	2.2		4
SL-4	1.1	CASSFSPGPPEGELFF	2.2		1
SL-5	3.1	CASSLEEQRAFF	1.1		2
SL-6	3.1	CASNSGYMNFF	2.1		5
SL-7	11.1	CASSEDRRGSYEQYF	2.7		2
SL-8[e]	11.1	CASSDAGTGDYEQYF	2.7		2
SL-9	13.1	CASSPVQGASGNTIYF	1.3		1
SL-10	13.1	CASSYVDGSSYEQYF	2.7		1
SL-11	20.1	CAWSPLEIAGTDTQYF	2.3		2
SL-12	1.1	CASSVAPGSEGEAFF	1.1	6	8
SL-13	2.1	CSAGGWDRVNQPQHF	1.5	9	2
SL-14	11.1	CASSESQTGDYEQYF	2.7	16	5
SL-15	15.1	CATSDSTSGGTDTQYF	2.3	10	1
SL-16[f]	1.1	CASSFSAEATGELFF	2.2	4	
SL-17	3.1	CASSRLEARELFF	2.2	2	
SL-18	5.1	CASSLTNRGLNMNTEAFF	1.1	2	
SL-19	5.1	CASSLTGGSETQYF	2.5	1	
SL-20	6.1	CASSVPGEESGRGNTEAFF	1.1	1	
SL-21	6.5	CASSLGRGEQYF	2.7	1	
SL-22	7.2	CASSRGTENEKLFF	1.4	1	
SL-23	10.1	CASSKKLDSFSYEQYF	2.7	1	
SL-24	11.1	CASSGSGPLRGYTF	1.2	1	
SL-25	11.1	CASSPAGPGNTIYF	1.3	1	
SL-26	11.1	CASSDPGHQNSPLHF	1.6	3	
SL-27[f]	11.1	CASSFSAEATGELFF	2.2	4	
SL-28	13.1	CASSYEGTEAFF	1.1	1	
SL-29	13.1	CASSYDLEPTNEKLFF	1.4	1	
SL-30	13.1	CASSYSGSLGEKLFF	1.4	1	
SL-31	13.1	CASIEQGALTNEQFF	2.1	1	
SL-32	13.1	CASTLDRDGTQYF	2.5	2	
SL-33	13.1	CASSGSPGQGAEYF	2.7	1	
SL-34	15.1	CATSAGGAEPTDTQYF	2.3	1	
SL-35	23.1	CASSWRGGIQGVAGELFF	2.2	2	

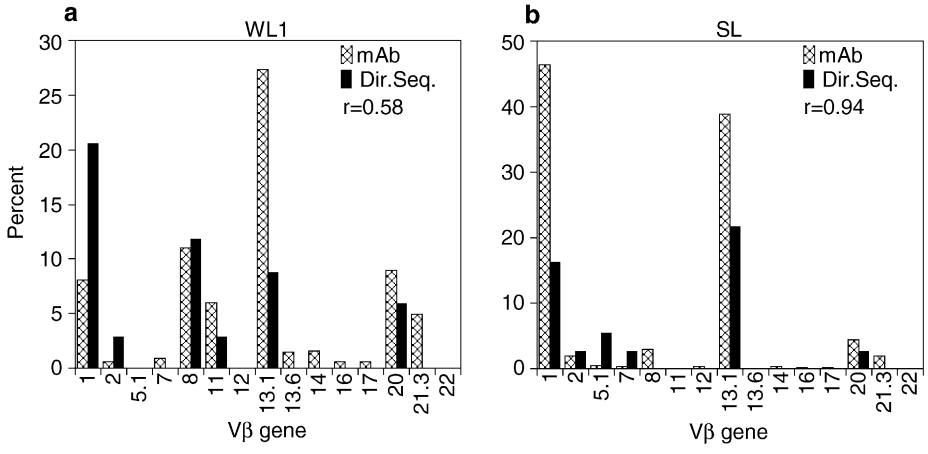

Fig. 3a, b Vβ usage assessed by mAb analysis and direct sequencing are similar. Percent of DR1-Ha oligomer staining cells which co-stained with particular anti-Vβ mAbs are plotted (*hatched bars*) next to percent of clones identified by the direct sequencing approach (*closed bars*) for individuals WL1 (**a**) and SL (**b**). The sequences were analyzed without weighting by number of occurrences. Pearson correlation coefficients (*r*) for the data sets are indicated, and were calculated using the formula, $r = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2 \sum (y_i - \bar{y})^2}}$

gy to another sequence within the data set or previously published sequences (Table 2). Using this logic, 45 of 95 unique sequences were "validated" (Table 3). Moderate to strong correlations were observed between the validated and non-validated groups within the Tet pool (r=0.61), IFN-γ pool (r=0.75), or both pools together (r=0.74). From this analysis we conclude that most of the non-validated sequences are genuine.

Particular *Vβ* genes are highly utilized by the DR1-Ha-binding repertoire

We analyzed the TCRβ sequences in order to identify any characteristics critical for interacting with DR1-Ha. In Fig. 4, Vβ usage is plotted for sequences from each individual and from the previously published

Table 2 Convergent evolution of TCRβ CDR3 region

ID/clone	Vβ	CDR3	Jβ	ID/clone	Vβ	CDR3	Jβ
HA1.7	3.1	CASSSTGLPYGYTF	1.2	WL1–28	8.1	CASSSDGT-GEKLFF	1.4
GS24	3.1	CASSSS–-YGYTF	1.2	SL-30	13.1	CASSYSGSLGEKLFF	1.4
SL-17	3.1	CASSRLEAR–ELFF	2.2	B103	5.2	CASSLGLAGGQDTQYF	2.3
AND.21	3.1	CASSYQEANTGELFF	2.2	WL2–9	5.4	CASSLGLAGDQETQYF	2.5
				AND.7	3.1	CASTP—GQETQYF	2.5
SL-23	1.1	CASSFSAEATGELFF	2.2				
SL-25	11.1	CASSFSAEATGELFF	2.2	WL1–3	3.1	CASTQGVL–TYEQYF	2.7
				WL2–26	22.1	CASVPGLS–-YEQYF	2.7
SL-25	11.1	CASSPAG–PGNTIYF	1.3	JS515	3.1	CASSPGTSGTTYEQYF	2.7
SL-9	13.1	CASSPVQGASGNTIYF	1.3				
				WL2–2	1.1	CASSGTGEPTNEKLFF	1.4
WL1–14	8.1	CASSLSQGDQPQHF	1.5	SL-29	13.1	CASSYDLEPTNEKLFF	1.4
14	3.1	CASSID-G–PQHF	1.5				
				WL1–1	1.1	CASSVGPGPGDTEAFF	1.1
SL-32	13.1	CASTLDRDG–TQYF	2.5	WL1–15	1.1	CASSVAPSPSETEAFF	1.1
SL-21	6.5	CASSLGR-G–EQYF	2.7	SL-2	1.1	CASSVAGG–ETEAFF	1.1
WL1–12	6.5	CASSVGQ-G–KAFF	1.1	WL2–1	1.1	CASSVTPG–GTEAFF	1.1
WL1–13	6.5	CASSLGQ-G–EQFF	2.1	SL-1	1.1	CASSVTPG–HTEAFF	1.1
WL2–14	6.5	CASSLGQ-N–EQYF	2.7				
WL2–15	6.5	CASSLGQ-G–EQYF	2.7	SL-14	11.1	CASSESQTGDYEQYF	2.7
AND.10	3.1	CASSL-Q-G-YEQYF	2.7	HC6	11	CASSESGTGDYEQYF	2.7
WL2–8	3.1	CASSTTQ-GFYEQYF	2.7	WL2–1	11.1	CASSDAGTGDYEQYF	2.7
				SL-11	11.1	CASSDAGTGDYEQYF	2.7
WL1–26	3.1	CASSLVPELGEQYF	2.7				
WL1–11	3.1	CASSLSPELETQYF	2.5	WL2–9	5.4	CASSLGLAGDQETQYF	2.5
WL1–25	3.1	CASSLAPELDTQYF	2.3	B103	5.2	CASSLGLAGGQDTQYF	2.3
WL2–6	3.1	CASRDSG–TRNEQFF	2.1	BC16	3.1	CASSFPRTGGKTLVHSYEQYF	2.7
WL1–23	3.1	CASSDSG–TYNEQFF	2.1	B08	3.1	CASSFPKTAG—-AYEQYF	2.7
WL1–22	3.1	CASSPGGGDTYNEQFF	2.1				
				WL2–2	1.1	CASSGTGEPTNEKLFF	1.4
B105	11	CASSDGTFTEAFF	1.1	SL-29	13.1	CASSYDLEPTNEKLFF	1.4
WL1–17	1.1	CASSTG-FSEQFF	2.1				
WL1–18	1.1	CASSAG-FSEQFF	2.1	WL1–1	1.1	CASSVGPGPGDTEAFF	1.1
				WL1–15	1.1	CASSVAPSPSETEAFF	1.1
WL1–30	13.1	CASSREGTVNHGGYTF	1.2				
3BC6.6	3.1	CASSLTGT—GYTF	1.2				

Table 3 Statistical comparison of Vβ usage in validated and non-validated TCRβ sequences

Pool	Number in both Tet and IFN-γ	Number related	Number validated[a]	Number non-validated	Vβ usage comparison[b]				
					r	*P*	χ^2	*df*	*n*
Tet	13	31	39	42	0.61	0.62	26.2	29	79
IFN-ZZZ;γ	13	11	19	11	0.75	1.00	7.0	29	30
Both	13	37	45	50	0.74	0.69	24.8	29	96

[a] TCRβ sequences were validated if they were either detected in both the IFN-γ and the Tet pools, or if they were related to another TCRβ sequence either newly identified (Table 1) or previously published in the literature (Table 2). Pearson correlation coefficient (*r*), chi-squared value (χ^2), one-tailed probability of the chi-squared distribution (*P*-value, *P*), degrees of freedom (*df*) and number of sequences analyzed (*n*) were calculated according to standard statistical formulas (Bevington et al. 1992). Correlation coefficients were calculated according to the formula in Fig. 3

[b] Statistical parameters were determined by comparing the Vβ usage distributions of the validated and suspect TCRβ sets

sequences (Brawley and Concannon 1996; Hewitt et al. 1992; Ostrov et al. 1993; Prevost-Blondel et al. 1995; Snoke et al. 1993; Wedderburn et al. 1995; Yassine-Diab et al. 1999). Although many different *Vβ* genes (20 of 48 total functional *Vβ* genes) were detected within the entire pool of sequences, most *Vβ* genes were entirely absent from both the new and previously published sequences. Statistical comparison of the Vβ usage for the three individuals suggests that there could be weak similarities in Vβ usage (pairwise correlation coefficients from 0.34 to 0.69, P=0.40), with significant differences as well. The differences in Vβ usage between individuals may be an imprint from lymphocyte development caused by negative selection against the other class II MHC alleles of the individuals' haplotype.

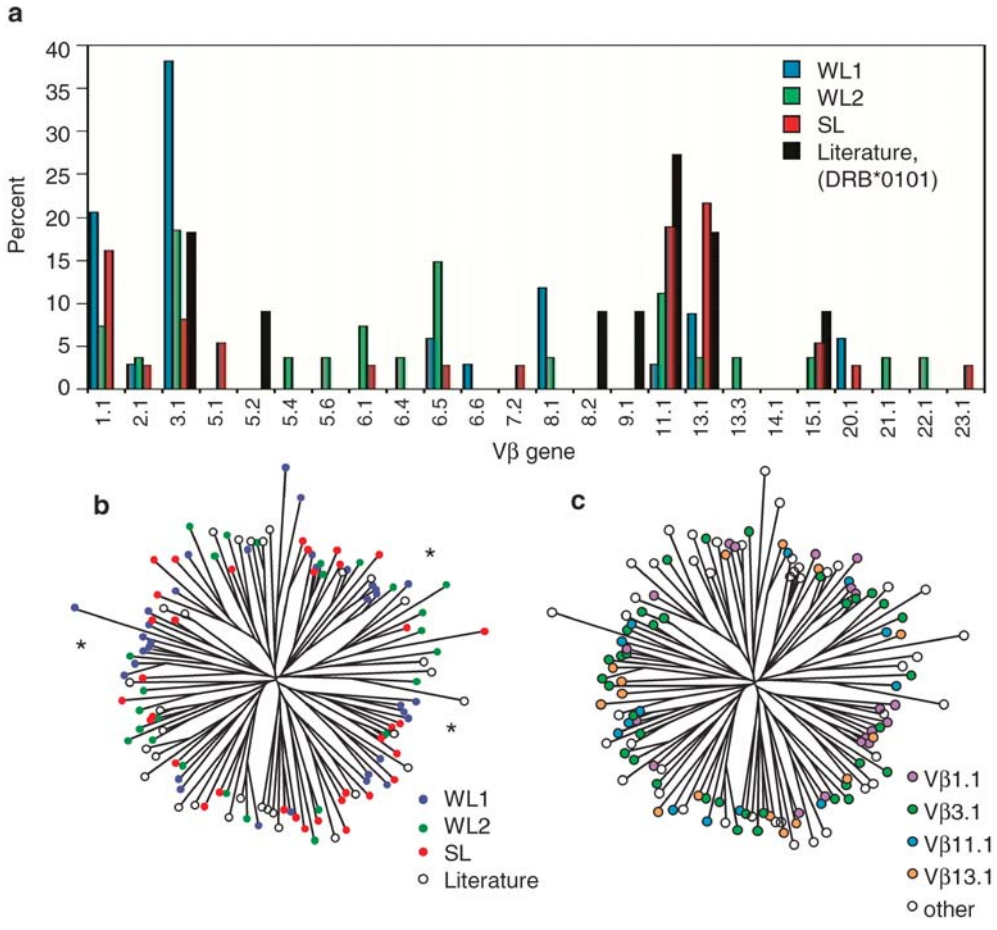

Fig. 4a–c Vβ and CDR3 sequence analysis of TCRβ genes from receptors specific for DR1-HA. **a** Percent of sequences with particular Vβs are plotted for individuals WL1 (*blue*), WL2 (*green*), SL (*red*), and for DRB1*0101-restricted clones from the literature (*black*). **b**, **c** The CDR3 regions from *TCRβ* sequences in Table 1 and ones previously published were scored for similarity using the ClustalW algorithm and mapped using TreeView software. Sequences were color coded for either sequence source (**b**) or associated Vβ (**c**)

Some TCRβ sequences contain similar CDR3 regions

We evaluated similarities in the CDR3 regions of our newly identified TCRβ sequences (Table 1) and previously published ones (Brawley and Concannon 1996; Hewitt et al. 1992; Ostrov et al. 1993; Prevost-Blondel et al. 1995; Snoke et al. 1993; Wedderburn et al. 1995; Yassine-Diab et al. 1999), using a ClustalW algorithm (Thompson et al. 1994) (presented graphically in Fig. 4b,c). When color-coded by patient, relatively little patchiness is observed on the tree (Fig. 4b), indicating no gross differences between individuals. However, small clusters of sequences can be observed within individuals and for WL1 in particular (see the clusters of blue dots marked by asterisks). This could reflect different influenza infection histories, or cross-reactivity with other antigens (Lin et al. 2000). When color-coded by Vβ usage, no patchiness is observed (Fig. 4c), indicating a lack of correlation between Vβ usage and CDR3 sequence. The top 0.7% of matches are listed in Table 2. Even in this set there are many unrelated families, and no clear motif can be observed in the CDR3 sequences.

DR1-Ha-binding TCRβ sequences tend to have negative charges in CDR1 and CDR3

The CDR3 sequence diversity together with the broad *Vβ* distribution leaves us with little understanding of why all of these divergent sequences bind the same antigen. The structure of the DR1-Ha peptide complex reveals three basic residues from the HA peptide accessible for TCR interaction (Stern et al. 1994), two of which are observed to interact with TCRβ residues in the structure of the HA1.7 TCR- DR1-Ha complex (Hennecke et al. 2000). We examined charge distributions in the CDR regions to evaluate the generality of such charge-charge interactions. In both IFN-γ and Tet pools, CDR1 and CDR3 regions were enriched for acidic residues (Fig. 5a). (The CDR2 region contacts MHC residues and not bound peptide in all known TCR-MHC crystal structures; Hennecke and Wiley 2001). Furthermore, 90% of the sequences in our TCRβ set have at least one negative charge, 73% have a net negative charge, and only 31% of them contain any positive charges, while 6% have no charges at all. To examine in more detail the charge characteristics of the CDR3 region of our TCRβ sequences we calculated the percent of negative and positive charges at ten positions starting at either end of the region (Fig. 5). Alignment at the N-terminus reveals no significant bias relative to the expected charge distribu-

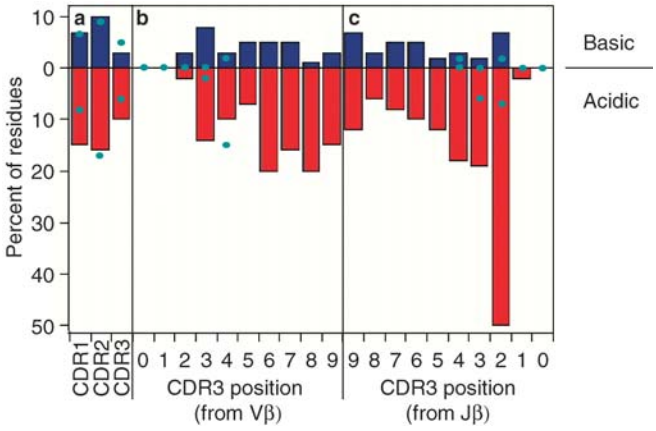

Fig. 5a–c Analysis of charged residues in CDR regions. **a** CDR regions from TCRβ sequences in Table 1 were analyzed for average number of basic or acidic charges in CDR1, CDR2 and CDR3 and plotted as *blue* (basic) and *red* (acidic) bars. Average number of charges expected for a non-specific unskewed TCR population are indicated by *green circles*. Sequences expected for the CDR3 regions of a non-skewed population were estimated from a combination of the last five residues of all 48 functional *Vβ* genes, the first five residues of all 13 functional *Jβ* genes, both *Dβ* genes in forward as well as inverted orientations, and random codon addition caused by nucleotide addition. **b**, **c** CDR3 regions aligned at either their N-terminal Cys residue (**b**) or C-terminal Phe residue (**c**). Percent of acidic (*blue*) and basic (*red*) residues at each of the subsequent ten positions relative to those starting points are plotted. Frequency of acidic and basic residues in all 48 Vβ (**b**) or in all 13 Jβ (**c**) segments are indicated with *green circles* for the CDR3 positions at which they could have potentially contributed

tion (Fig. 5b, green circles). However, alignment at the C-terminal end reveals that 50% of the CDR3 sequences have a negative charge at position 2 (Fig. 5c). This is caused by a bias towards Jβ segments 1.1, 2.1, 2.2 and 2.7, and in addition a preference for negative charge in the intervening region between the Vβ and Jβ segments. Because no *D* gene in either orientation has a net negative charge, these appear to have been introduced by N-region diversity during TCRβ recombination.

Discussion

The methodology (Cohen et al. 2002; Douek et al. 2002) utilized here overcomes some of the previous challenges in the field of TCR repertoire analysis: It analyzes the TCR repertoire at the clonal level with relative ease through direct sequencing of the TCR chains, and it can be used successfully for abundant mixed T-cell populations directly from a patient (Cohen et al. 2002; Douek et al. 2002) or for low-abundance populations after minimal in vitro expansion.

The influenza Ha antigen is of particular interest because it represents a promiscuous DR-binding epitope (Brawley and Concannon 1996; O'Sullivan et al. 1991; Zeliszewski et al. 1996) that stimulates a large repertoire of T cells, which is recognized by a broad spectrum of patients (Gelder et al. 1995). Further studies of the Ha-responding TCR repertoire, including analysis of the TCRα chain, larger TCRβ data sets, and studies on a variety of MHC haplotypes, will help us understand the formation of the memory T-cell compartment within the larger TCR repertoire and its reshaping after vaccination and infection.

Detailed examination of the Ha-specific TCRβ sequences described here provides insight into the recognition mechanism utilized by the immune system for this particular antigen. One clear conclusion is that many different TCRβ sequences, derived from many different *Vβ* and *Jβ* genes and with widely divergent CDR3 regions, are capable of interacting with DR1-Ha. Previous examinations of antigen-responding T-cell populations have identified only one or a few Vβ families, and/or particular CDR3 motifs (Acha-Orbea et al. 1988; Bourcier et al. 2001; Moss et al. 1991; Naumov et al. 1998; Utz et al. 1996). Despite the diversity of TCRβ sequences detected here, one common mechanism of binding can be gleaned from the data – the use of acidic residues in the CDR1 and CDR3 regions of the TCRβ chain. This is very likely for interaction with the three solvent-exposed basic residues of the Ha epitope. Such hydrophilic interactions may provide specificity to the MHC-TCR interaction, which does not require especially high bimolecular affinity ($K_d \sim 10^{-4}$–10^{-6}). Although this hypothesis is highly speculative, it suggests the potential value of a large-scale sequence-level TCR repertoire research program. Currently, we have a very limited understanding of the binding mode for MHC-peptide complexes and TCR molecules based on relatively few known structures (Hennecke and Wiley 2001). Additional insight will be gained by the analysis of multiple TCRs against the same antigen, either by direct structural analysis or by modeling. For example, the interactions between DR1-Ha and the TCRβ sequences described here may be distinct from that observed in the crystal structure of DR1-Ha and HA1.7 TCR, which has no negative charge in its CDR3 region (Hennecke et al. 2000).

Currently, most immunologists study a relatively limited set of human T-cell clones. Much research is performed on these clones, for example to develop peptidomimetic therapeutics or peptide antagonists of T-cell activity. However, there is very limited understanding of the TCR repertoire that will eventually encounter such antigens when they are used clinically. If, for example, one seeks to develop a peptido-mimetic compound to stimulate Ha-specific $CD4^+$ T cells, our work shows that the HA1.7 T-cell clone is likely to be an inadequate model to describe the response within the three volunteers examined in this study.

Within the last few years, technical advances have made the study of larger numbers of clones much easier. Two reports use direct sequencing approaches to estimate the diversity of T lymphocytes in the periphery (Arstila et al. 1999) or spleen (Casrouge et al. 2000). Other reports have examined fine details of the T-cell repertoire against single-antigens at a clonal level, simi-

lar to what we have presented here, using tools including MHC tetramers, DNA sequencing, and CDR3-length polymorphism analysis (Bourcier et al. 2001; Bousso et al. 1999; Casrouge et al. 2000; Douek et al. 2002; Lim et al. 2000; Naumov et al. 1998; Valmori et al. 2002). Given the full diversity of T cells, antigens, MHC haplotypes, and individual disease pathologies, these works are necessarily limited in scope. We specifically hesitate to extrapolate from our data anything beyond a knowledge of the Ha-specific T-cell repertoire. We believe that more general knowledge of the T-cell repertoire is likely to be gained only from large-scale approaches similar to the ones being developed in the fields of genomics, pro-teomics, and bioinformatics. This approach, which aims to characterize the total set of TCR in an individual responding to an immunological challenge, can be thought of as the proteomics of the TCR. For example, the examination of the Ha-specific response from a large panel of individuals of both matched and diverse haplotypes could help elucidate the relationship between haplotype and T-cell repertoire and determine the landscape of responses to be targeted by peptido-mimetic compounds, and it could provide insight into the differing pathologies of patients. The work presented here, in conjunction with other recent studies (Bourcier et al. 2001; Bousso et al. 1999; Casrouge et al. 2000; Cohen et al. 2002; Douek et al. 2002; Hennecke and Wiley 2001; Lim et al. 2000; Naumov et al. 1998; Valmori et al. 2002), represent a step towards this goal of detailed TCR repertoire analysis through large-scale analysis. Expansion of this methodology to more patients, other influenza epitopes, and to epitopes from other diseases will open the door to a new understanding of T-cell biology.

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