Longitudinal analysis of the T-cell receptor (TCR)-VA and -VB repertoire in CD8+ T cells from individuals immunized with recombinant hepatitis B surface antigen

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SUMMARY

Recent studies have suggested that vaccination induces alterations in the T cell receptor (TCR) repertoire. We investigate the diversity of the TCR repertoire after immunization with a recombinant hepatitis B surface vaccine in seven healthy subjects in CD8+ T cells in peripheral blood lymphocytes. Cellular immune responses were monitored over time by sorting CD8 T cells followed by TCR-VA and -VB complementarity determining region 3 (CDR3) analysis. Frequency of individual VB families was determined by flow cytometry. TCR-VA/VB repertoires obtained from CD8+ T cells drawn after vaccination were compared to the TCR repertoire determined prior to vaccination. Monoclonal TCR transcripts could be detected exclusively in CD8+, but not in CD4+ T cells. Such monoclonal TCR transcripts were either stable in some individuals, or could only be detected at certain time points after vaccination. Sorting of monoclonal TCR-VB3+ T cells, which constituted up to 5% of the CD8+ T cell population from one individual, revealed that this T cell clone recognizes an epitope provided by the recombinant hepatitis B vaccine presented by MHC-class I on autologous antigen-presenting cells. Examination of the structural anatomy, defined by the TCR, and the frequency of T cells responding to the immunizing antigen may be helpful to provide surrogate markers to monitor cellular immune responses induced by protein antigens utilized for vaccination.

Keywords   immunotherapy   T cell receptors   vaccination

INTRODUCTION

Cellular adaptive immune responses directed against viral pathogens rely on activation, expansion and generation of memory CD8+ or CD4+ T cells which recognize viral epitopes in the context of major histocompatibility complex (MHC) class I molecules [1]. Recently, with the advent of recombinant class I/peptide/beta-2 microglobulin complexes, i.e. 'tetramer-complexes', the magnitude of CD8+ T cells directed against defined peptide antigens could be addressed. Such MHC-class I and antigen-specific T cell responses may represent up to 50% of the entire CD8+ T cells in lymphocytic choriomeningitis infection in BALB/c mice [2,3]. Using a similar approach, a considerable number of T cells directed to EBV or HIV has been detected in human subjects [4,5]. Until now, only a limited number of appropriate hepatitis B surface peptide epitopes and alleles are available to probe for tetramer binding T cells. More recent studies

addressed the frequency of HBV-specific and MHC-class I restricted CD8+ T cell responses in patients with different stages of HBV infection [6–8].

Despite the enumeration of antigen-specific and MHC-restricted T cells, evaluation of the structural anatomy of T cells responses may also contribute to a better understanding of a cellular immune response: the amino acid sequence of a given TCR determines the specificity of individual T cell clones. Additionally, the final outcome of an immunization procedure or a natural infection may also depend whether the T cell response directed against an immunogen/pathogen is dominated by clonal or polyclonal T cell effector cells [9,10]. In general, clonal or oligoclonal T cells in the peripheral circulation in patients with viral infections appear to represent antigen-specific T cells [11]. The dominance of a few T cell clones directed against a defined set of epitopes appears to be beneficial in patients infected with HIV [12]. However, the focus of the cellular immune system by expanding a limited number of T cell clones may also be detrimental, particularly if permutation in the target-peptide epitope gives rise to variant ligands, which can be either not recognized

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by the T cell receptor (TCR) or lead to unproductive immune responses in T cells specific for the wild-type epitope [13,14]. Thus, the structural characterization of the T cell repertoire in response to an immunogen may provide a useful surrogate marker for the rational design of vaccines.

It appears unlikely that immunization with recombinant protein will yield a vigorous CD8+ T cell response; however, recent studies showed that a recombinant protein from the human cytomegalovirus (HCMV) is able to drive CD4+ and CD8+ T cell responses [15]. In addition, the antigen delivery system, e.g. HBsAg in the form of 'epidermal powder' coupled to gold beads [16], or the implementation of virus-like particles (VLPs) fed to dendritic cells (DCs), may aid activation of antigen-specific and MHC class I-restricted T cell responses [17]. Furthermore, analysis of TCR-VB usage after vaccination with recombinant HBsAg indicated alterations in the TCR-VAraible beta chain (VB) frequency in peripheral blood lymphocytes (PBL), associated possibly with vaccination [18]. In addition, the frequency of TCR-VB families in PBL has been described to undergo substantial alterations in patients with chronic hepatitis B [19]. In order to evaluate the CD4 and CD8+ T cell pools in PBL linked to vaccination with the recombinant hepatitis B vaccine, we determined the structural composition of the T cell response using a panel of 21 different MoAbs directed against the TCR-VB chains and the complementarity determining region (CDR3) length analysis of the TCR-variable alpha chain (-VA)/-VB chains by spectratyping.

MATERIALS AND METHODS

Individuals

Seven individuals who tested negative for anti-hepatitis B antibodies were immunized with recombinant hepatitis B surface antigen (Engerix-B, SmithKline Beecham). Injections were performed according to the manufacturer's instructions at day 0, 1 month and 6 months after the first vaccine administration. PBL were obtained from each individual by venipuncture, ficolled and separated into CD4 and CD8+ T cells using immunomagnetic beads obtained from Miltenyi Biotec, Bergisch Gladbach, Germany. PBL were obtained 4 weeks and 1 day prior to immunization and 2 weeks after each immunization, respectively. MHC class I and class II typing (Table 1) was performed in the local

blood bank. All subjects exhibited a serum titre >1000 IU anti-HBsAg 2 months after the last vaccination.

Cytokine release assays

Dendritic cells were prepared from peripheral blood mononuclear cells using IL-4/granulocyte macrophage colony-stimulating factor (GM-CSF) as described previously [20]. Dendritic cells were pulsed with 1 µg of the vaccine preparation 12 h before assay. VB3 or VA24-positive T cells were sorted by anti-CD8 immunomagnetic beads followed by a second purification step using the MoAbs listed in Table 2. Sorted T cells were cultured in AIM-V medium supplemented with 100 ng interleukin-7 (IL-7) and 100 IU IL-2 for 12 h and then tested for recognition of antigen(s) presented by autologous antigen-presenting cells. Dendritic cells were adjusted to 5×10^3/well and T cells 5×10^4/well. For blocking experiments, antigen-presenting cells were incubated at room temperature (RT) for 30 min with 5 µg of the anti MHC-class I (W6/32) and the anti-HLA-DR directed MoAb (L243), obtained from the American type culture collection, prepared from culture supernatants by FPLC using Protein-A sepharose. After incubation for 24 h, supernatants were harvested and stored at –20°C until assayed for IFN-γ by ELISA (R&D, Wiesbaden, Germany) according to the manufacturer's instructions.

Determination of the TCR repertoire

Aliquots of cDNA corresponding to 50 ng of total RNA were amplified in 20 µl reactions with 29 individual primers specific for the variable TCR alpha chain and with 24 individual primers specific for the TCR beta chain [21,22], as described [23]. Aliquots

Table 1. MHC alleles of individuals vacacinated with rHBsAg

	MHC-class I						MHC-class II			
Individual no.	A		B		C		DR		DQ	
1, 35y, m	1	3	8	62	3	7	3	13	2	6
2, 42y, f	2	26	49	62	3	7	1	13	1	
3, 52y, f	3	7	35	4	7	4	15	6	8	
4, 34y, f	2	30	13	44	5	6	7	11	2	7
5, 34y, f	2	30	13	44	5	6	7	11	2	7
6, 29y, m	3	32	8	13	6	7	3	11	2	7
7, 52y, f	24	29	7	44		7	7	15	2	6

Seven individuals were subjected to vaccination with a recombinant hepatitis B vaccine. Age (y, years) and gender (f, female; m, male) is indicated. Individuals no. 4 and 5 are twins.

Table 2. Monoclonal antibodies

Specificity	Designation	Labelled with
CD4	5FCI12T4D11(T4)	ECD
CD8	B9·11	PC5
VB1	BL37·2	P/F
VB2·1	MPB2D5	P/F
VB3	LE-89	F
VB5·1	Immu157	P/F
VB5·2	36213	P
VB5·3	3D11	P
VB7·1	20E	P/F
VB8·1/VB8·2	56C5·2	P/F
VB9·1	FIN9	P
VB11·1	C21	P
VB12·2	VER.32·1	F
VB13·1	Immuo222	P
VB13·6	JU74·3	F
VB14·1	CAS1·13	F
VB16·1	TAMAYA1·2	F
VB17·1	E17·5F3	P/F
VB18·1	BA626	P
VB20·1	ELL1·4	F
VB21·3	IG125	F
VB22·2	Immuno546	P/F
VB23·1	AF23	P
VA24	C15	Non-conjugated

ECD = energy coupled dye. PCS = R-phycoerythrin-cyanin 5. F = Fluoresceinisothicyanate. P = Phycoerythrin.

(2 μl) of the 24 unlabelled VB and the 29 VA amplicons were subjected further to 3–6 cycles of a run-off reaction using a fluorophore-labelled TCR C-alpha-specific (5′ ATACACATCAG AATCCTTACTTTG) or C-beta chain-specific primer (5′ GTG CACCTCCTTCCCATTCACC). The reaction volume was 10 μl, the final concentration of deoxynucleoside triphosphates was 0·2 mM, and 1·5 mM MgCl2 with 0·5 units taq-polymerase. Labelled products were analysed by DNA fragment analysis using a 310 ABI sequencer and Genescan software from ABI, Weiterstadt, Germany. Single peaks in individual TCR-VA families, suggesting clonality, were analysed further by direct sequencing of the polymerase chain reaction (PCR) products using the primer (5′-3′) GTCACTGGATTTAGAGAGTCT for the TCR alpha chain and the primer CACAGCGAC CTCGGGTGGG for the variable TCR beta chain on a 310-A DNA sequencer (ABI, Weiterstadt, Germany). Single peaks obtained by DNA fragment analysis may indicate a monoclonal TCR transcript or, alternatively, different TCRs with the identical TCR CDR3 length but with a different nucleotide sequence. TCR transcripts are termed 'oligoclonal' if one or two peaks are detected in the CDR length analysis and/or several individual TCR transcripts are present within a single peak. TCRs are termed monoclonal if direct sequencing of the PCR product, or if all individual clones after subcloning of the respective PCR amplicon revealed a single TCR transcript.

If the TCR-VA/-VB family is oligo- or polyclonal, a Gauss distribution occurs [24,25]. Each peak represents base pairs (bp) coding for one amino acid (aa) residue. The area under the curve represents the frequency of a distinct CDR3 length in an individual TCR-VA/-VB family. In order to condense the information from a single sample analysis, the individual TCR-VA or -VB families were grouped into a single figure with VA1-29 or VB1-VB24 along with the CDR3 length expressed as the number of amino acids. This TCR-CDR3 landscape provides the 'structural anatomy' as defined by the TCR-CDR3 length for each TCR family in a T cell subpopulation. The area under the curve of each CDR3 peak is expressed as the percentage of the entire CDR3 area (100%). For the sake of visibility, each 10% value is depicted in different colours. The CDR3 pattern obtained from CD8+ T cells harvested after vaccination can be compared with the sample harvested prior to vaccination. This TCR 'perturbation' within each CDR3 length is calculated by the areas between the CDR3 distribution in each sample and the control distribution (in the sample obtained prior to vaccination). Positive or negative perturbations may occur in each TCR-VA/-VB CDR3 peak and are depicted as differences as compared to the control sample. Each perturbation yielding a 10% difference is depicted in a different colour. Note that a 'flat' TCR landscape in this analysis implies that no perturbation exists, i.e. the TCR-VA/-VB landscape would yield the identical picture as compared to the control sample (i.e. CD8+ T cells prior to vaccination).

CDR3 analysis and TCR-VB staining: quantitative TCR analysis
TCR-spectratyping yields only the qualitative, but not the quantitative assessment of a T cell population. A panel of 21 individual MoAbs directed against the TCR-VB chain (Beckman Coulter, Krefeld, Germany) were grouped to three individual anti-VB MoAbs either labelled with FITC, PE or double-labelled with FITC/PE which can be gated either on ECD-CD4 or PC5-CD8+ T cells [26]. Thus, the frequency of 21 individual TCR-VB families

either in the CD4+ or CD8+ T cell populations can be analysed in seven different tubes, which yields the percentage (X% of a VB family) in CD3+ CD8+ T cells. This factor can now be used to correct the CDR3-VB landscape analysis. A MoAb panel directed exclusively against TCR-VB chains, but not TCR-VA chains, is available.

RESULTS

Detection of monoclonal TCR after hepatitis B vaccination
Individuals, which tested negative for anti-hepatitis B virus serum antibodies were immunized, T cells harvested at different time points and tested for the presence of monoclonal TCR mRNA transcripts in CD4+ and CD8+ T cells. Monoclonal TCRs could be detected exclusively in CD8, but not in CD4+ T cells (Table 3).

No monoclonal TCR in PBL prior to immunization and occurrence of monoclonal TCRs at different time points after vaccination
Monoclonal TCR-VA7 and -VA19 peaks could be detected prior to vaccination in CD8+ PBL from individual no. 1, and monoclonal VA17, VA19 and VB3 TCR transcripts could be detected 2 weeks after the second vaccination (Fig. 1). The dominant TCR-VB3 transcript could be detected exclusively at this time point as determined by CDR3 length analysis of the VB3 family (Fig. 1) and constituted up to 5% of the entire CD8+ T cell population (data not shown). Individual no. 5 exhibited a monoclonal TCR-VA17 transcript 2 weeks after the second vaccination (Table 3), as detected by directed sequencing of the VA17 specific amplicon (Table 4). However, this dominant TCR transcript could also be detected 2 weeks after the third vaccination, albeit with additional VA17 transcripts (Fig. 1). This phenomenon (i.e. dominance of a certain TCR transcript with additional TCR transcripts) may be termed 'clonal dominance'. Of note, the monoclonal TCR-VA17 transcripts were not identical in individuals no. 1 and no. 5, since they exhibited a different CDR3 size (data not shown).

Pre-existing monoclonal or oligoclonal TCR-VA/-VB transcripts, which may not be associated with vaccination
CD8 T cells obtained from individual no. 6 showed a prominent oligoclonal TCR-VB14 family (up to 5% of all CD8+ PBL) before vaccination, which could not be detected after vaccination (Fig. 2). Individual no. 7 exhibited monoclonal TCR-VA5 and VA24 transcripts 4 weeks prior to immunization (Fig. 2). One day before immunization, exclusively the VA24, but not the monoclonal VA5 transcript could be detected (Fig. 2). The same monoclonal TCR-VA5, -VA24 transcripts could be detected 2 weeks after the second vaccination. Additional monoclonal VA or VB transcripts (VA9, VA17, VB17) were readily detectable in PBL drawn 2 weeks after the third vaccination. If monoclonal TCR in PBL are indicative of antigen-specific T cells [11], only VA9, VA17 and VB17 would represent antigen-specific candidate T cell families, because VA5 and VA24 existed prior to the vaccination.

Clonal dominance of TCR transcripts shared between individuals
VB19 TCR transcripts were found to be polyclonal 2 weeks after the second vaccination in individual no. 1 (Fig. 1). However, 2 weeks after the third vaccination, the VB19 family was domi-

Table 3. Monoclonal TCR transcripts in PBL

	1		2		3		4		5		6		7	
–4 weeks	*VA7*	VA19	*Nil*	VA7 VB15	*VA14*	VA27	*VB15*	Nil	*VA17*	Nil	*VB14*	Nil	*Nil*	**VA5 VA24**
–1 day	*Nil*	Nil	*Nil*	Nil	*VA11 VA27 VB24*	VA17	*Nil*	Nil	*Nil*	Nil	*Nil*	Nil	*Nil*	**VA24**
2 weeks post 1.vacc	*n.d.*	n.d.	*VA7 VA24*	VA26	*Nil*	nil	*VB15*	Nil	*Nil*	Nil	*Nil (n.d.)*	(n.d.)	*Nil*	Nil
2 weeks post 2.vacc	*Nil*	VA17 VA19 **VB3**	*Nil*	VA22 VA26 VB7	*Nil*	VA9 VA19 VB17	*VA15 VB15*	Nil	*VA5 VA7 VB18*	**VA17**	*Nil*	Nil	*VA1 VA9*	**VA5 VA24**
2 weeks post 3.vacc	VA5 VA9 VA24 VB19	Nil	*Nil*	VA7	*VA11 VA17 VA27 VB9 VB24*	nil	*VB17*	Nil	*VB18*	**VA17**	*VB14*	Nil	*VA19 VB7 VB8 VB18 VB19*	VA9 VA17 **VB17 VA24**

PBL were obtained from individuals before and after vaccination with a recombinant hepatitis B surface vaccine by venipuncture, ficolled and segregated into CD4⁺ and CD8⁺ T cell population using immunomagnetic beads TCR-VA/-VB families were subjected to analysis of clonality, as described in Material and methods, in CD3⁺ CD8⁺ T cells. Single peaks in individual TCR-VA families, suggesting clonality, were analysed further by direct sequencing of the PCR products. Note that monoclonal TCR mRNA transcripts, particularly TCR-VA families, are present within the CD8⁺, but not in the CD4⁺ T cell population. nil = no detection of monoclonal TCR mRNA transcripts. Monoclonal TCR families: regular type; oligoclonal TCR families or 'clonal dominance': italic type. Amino acid sequences of selected TCRs (in bold and underlined) are given in Table 4. (n.d.) = TCR-VB, but not -VA analysis was determined at this point n.d. = TCR-VA and -VB analysis was not determined at this point.

Table 4. Monoclonal TCR in PBL

Individual	TCR-VA/-VB		CDR3	Joining region	
7	VA5	SQPADSATYLCA	LGN	TGGFKTIFGAG	AJ9
5	VA17	DSQPGDSATYFCA	AFRE	GKSTFGDGTT	AJ27
1	VA19	TASHPRDSAVYICA	VRP	SGTYKYIFGTG	AJ40
	VA24	SDSASYIC	VVSAFPHG	TGGFKTIFGAG	AJ9
7	VA24	SDSASYIC	VVSAFPHG	TGGFKTIFGAG	AJ9
1	VB3	NQTSMYLCA	SSLWTALG	TDTQYFGPG	BJ2-3
7	VB17	QKNPTAFLSCA	SSSPR	ERSGNMFFGQG	BJ2-2

T cell VA or VB families with a single peak in the CDR3 length analysis were subjected to DNA sequence analysis. Deduced amino acid sequences are indicated.

nated by a single TCR transcript in this individual (measuring 373 base pairs) and by two VB19 transcripts, measuring 373 and 373 bp, respectively, in individual no. 7 (Figs 2 and 3a). Similarly, the TCR-VA19 was found to be monoclonal 4 weeks prior to immunization and in CD8⁺ T cells obtained 2 weeks after the second immunization from individual no. 1 (Figs 1 and 3b). An mRNA transcript of a similar size (363 bp) could also be detected 2 weeks after the second immunization in individual no. 7 (Figs 2 and 3b). In contrast, 2 weeks after the third vaccination, a TCR-VA19 clone of a different size (345 bp) was found to be present in the CD8⁺ T cells in individual no. 7 (Figs 1, 2 and 3b).

TCR transcripts shared between individuals
The monoclonal TCR-VA24 was found to be present in PBL obtained from individual no. 7 (Fig. 2, Tables 3 and 4) and could also be identified 2 weeks after the third vaccination in CD8⁺ T

cells obtained from individual no. 1 (Figs 1 and 3c). Except for the detection of VB3 in individual no. 3, we have not been able to detect monoclonal TCR transcripts in the VB families. However, an antigen-specific T cell response may not necessarily be monoclonal or oligoclonal in nature. As the CDR3 length analysis is based on the PCR amplification of individual TCR-VB families, only the structural anatomy of a T cell response can be described. The frequency or the quantity of individual TCR families cannot be addressed by implementing the spectratype analysis as described in this report

Monoclonal TCR-VB3 cells are specific for the immunogen
In order to determine whether monoclonal TCRs may represent antigen-specific T cells recognizing the hepatitis B surface antigen, we isolated the VB3 T cell population from individual no. 1 and the monoclonal VA24 TCR family from individual no.
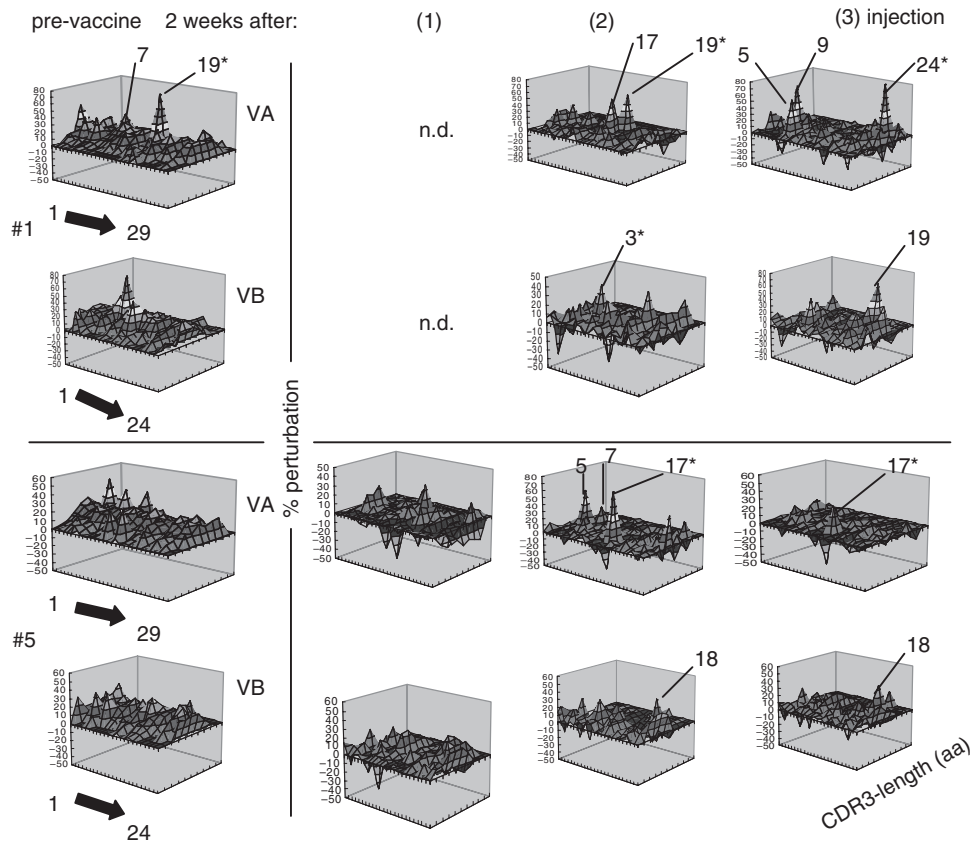
Fig. 1. Emergence of oligoclonal/monoclonal TCRs after vaccination in CD8+ T cells from individuals no. 1 or no. 5. TCR-VA/-VB 'landscape' 1 day prior to vaccination (left). The 'perturbations' within the CD8$^+$ T cells harvested 2 weeks after the first, second and third vaccination, respectively, depicts *not* the actual TCR-VA/-VB repertoire, but the difference of the TCR CDR3 profiles in respect to over- or under-representation of individual peaks in the TCR-VA or -VB families; 10% difference is depicted in a different colour. An 'unperturbed' repertoire (depicted here in blue) represents a smooth landscape surface, i.e. the TCR repertoire would show the identical pattern to the repertoire obtained prior to vaccination. *y*-axis: % difference in CDR3 distribution (perturbation); *z*-axis: CDR3 length (1–10 aa residues); *X*-axis: individual VA ($n = 29$) or VB ($n = 24$) families. Oligo- and monoclonal TCR families are indicated. * = TCR sequence is provided in Table 4. n.d. = not determined.

7 using immunomagnetic beads (Fig. 4). We could not isolate other monoclonal TCR-VA families due to the lack of appropriate anti-TCR-VA directed MoAbs. VB3- and VA24-sorted T cells were tested for recognition of autologous dendritic cells pulsed with the immunizing antigen as determined by IFN-γ secretion. Exclusively CD8$^+$ VB3$^+$ T cells secreted IFN-γ in response to antigen-presenting cells pulsed with the immunizing agent. This T cell response could be blocked successfully with a monoclonal antibody directed against MHC-class I antigens. In contrast, CD8$^+$ sorted PBL depleted of VB3 T cells did not yield significant IFN-γ production in response to the target antigen. CD8$^+$ VA24$^+$ T cells did not recognize antigen presented by autologous antigen-presenting cells. As a control, both VB3 or VA24 T cells secreted IFN-γ in response to cross-linking of the CD3 complex (Figure 4).

DISCUSSION

We characterized the cellular immune response induced by hepatitis B vaccination by DNA-fragment analysis of the TCR-VA and -VB chains in sorted CD4 and CD8 T cells.

Previous accurate measurements of the T cell receptor repertoire indicated that oligoclonal CD8$^+$ T cell populations can be identified in normal healthy individuals [27]. The number of oligoclonal or monoclonal TCR transcripts is identified predominantly in the TCR-VA chain and appears to be increased in patients suffering from cancer [23]. Some of the individuals examined in this report (e.g. no. 1 and 5) exhibited the occurrence of a single T cell clone in the peripheral circulation. However, the presence of this dominant TCR transcript was not stable over time. Two possibilities may account for this phenomenon: (i) the T cell clone may ultimately die and may therefore not be detectable or (ii) may not be present in the peripheral circulation. Noteworthy, individual prominent TCR-CDR3 peaks are also detectable in TCR-VA or -VB chains *prior* to vaccination (see Figs 1 and 2). This implies that clonal shifts after HbsAg vaccination may not be indicative of an HbsAg-specific cellular immune response. In fact, any exposure to infectious agents may give rise to oligoclonal/clonal T cell populations [28], and these infections may result in expansion of the memory T cell pool. The composition of this T cell pool may also contain 'cross-reactive' CD8 T cells, an event that appears to represent a common phenomenon [29–32]. T cell cross-reactivity may derive from recognition of unrelated peptides by a *bona fide*
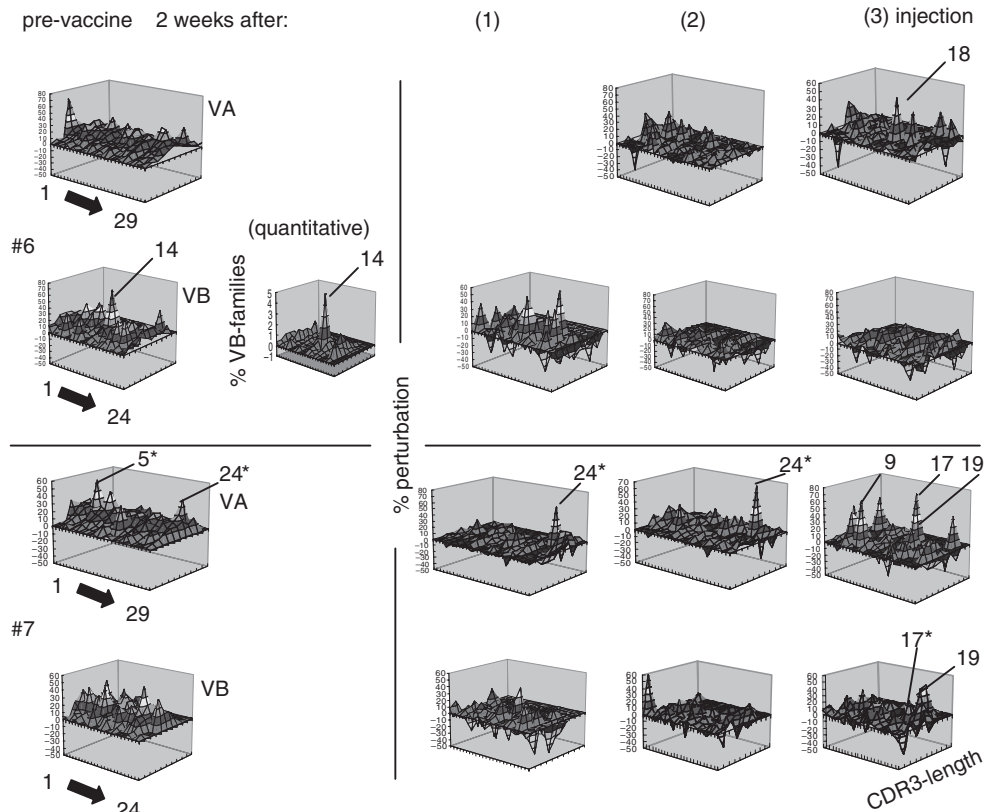
Fig. 2. Pre-existing oligo/monoclonal TCR-VA/-VB families prior to vaccination in individuals no. 6 and no. 7. TCR-VA/-VB CDR3 landscape analysis (left) prior to vaccination and qualitative TCR CDR3 differences ('perturbations') observed in CD8^{+} sorted T cells from PBL over time. Oligo- and monoclonal TCR families are indicated. * = TCR sequence is provided in Table 4. Note that the dominant TCR-VB14 peak in PBL from individual no. 6 as determined by TCR CDR3 length analysis was also prominent in the combination of qualitative (TCR CDR3 analysis) and quantitative TCR-VB family analysis determined by flow cytometry represented as '% VB families' within the CD3^{+} CD8^{+} population (see Materials and methods for details).

clonal $\alpha\beta$ TCR. Alternatively, expression of either dual alpha [33,34] or dual beta, or dual beta TCR chains [35] may be responsible for 'cross-reactive' T cells. Some memory T cells can be (re-)activated by heterologous viral infections [31,32]. Thus, pre-existing memory T cells recognizing unrelated reagents are able to alter the clinical outcome of infections [32]. Alternatively, such 'cross-reactive' T cells may also be responsible for recognizing MHC class I-presented peptides provided by the HBsAg antigen, such as the CD8^{+} VB3^{+} T cell responses detected in individual no. 1 (see Figs 1 and 4). We could not evaluate the fine specifity of the CD8^{+} VB3^{+} T cell response using HLA-A2 tetramer complexes because individual no. 1 tested HLA-A2 negative (Table 1).

Thus, one advantage of the TCR landscape analysis is that the entire 'structural' composition of the CD4^{+} or CD8^{+} T cell pool can be described. With the availability of different HLA-alleles and appropriate peptides, tetramer analysis, determining the frequency of antigen-specific T cells, can be complemented with determination of the molecular composition of the antigen-specific T cell response as described for the CD8^{+} T cell response directed against the HLA-A2 presented hepatitis B virus core$_{(18-27)}$ peptide antigen [8].

Most studies examining T cell responses directed against defined antigen used T cells from peripheral blood. However,

analysis of PBL is based on the presumption that the peripheral blood pool represents the entire T cell repertoire and a free exchange of T cells in solid organs, lymphatic tissues and the peripheral circulation takes place. Only a small fraction of the entire T cell population (about 2%) is present in the blood at any time [7,36,37]. This limits the use of PBL for immunomonitoring, although a significant increase in several TCR-VB families, particularly VB7, was observed in PBL from patients with chronic hepatitis B [38].

Of note, most of the clonal TCR transcripts could be detected in the TCR-VA chain (Table 3), which underlines that examination of T cell responses should include not only the TCR-VB chain, particularly since the TCR-VA chain appears to be critically responsible for interaction with MCH-class I molecules [39,40]. Lack of allelic exclusion at the TCR alpha chain may also contribute to the observation of prominent monoclonal TCR alpha transcripts in CD4^{+} or CD8^{+} T cells. In general, allelic exclusion appears to be more 'leaky' for the TCR alpha than for the TCR beta chain [41,42]. This impacts on TCR-CDR3 spectratyping: even if two potentially functional TCR-VA chains may be transcribed (and therefore be depicted in the TCR-VA CDR3 typing), they may still not be present on the cell surface owing to 'functional allelic exclusion' due to the failure to assemble the TCR heterodimer successfully [43]. Thus, there may be a dis-
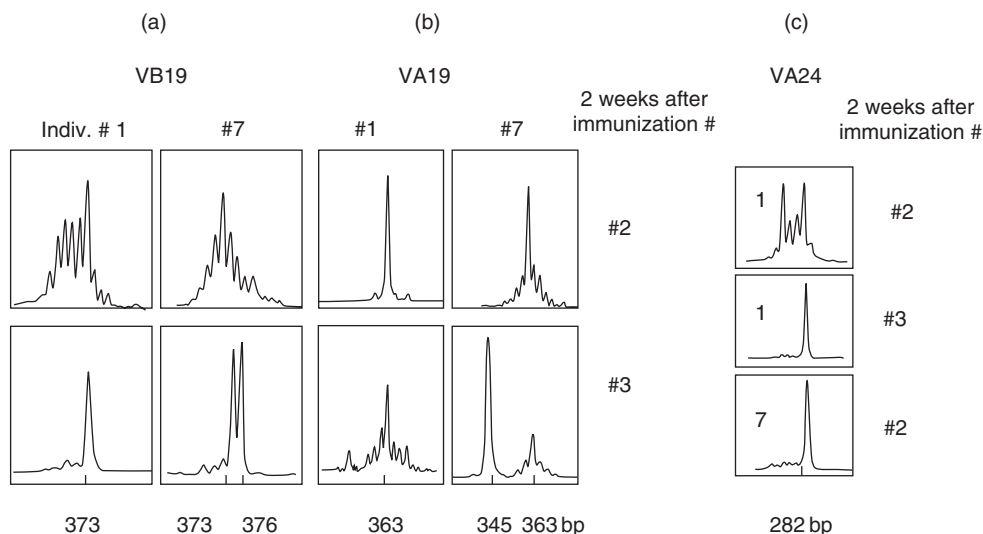
Fig. 3. (a–c) Similar pattern of clonal dominance in (a) VB19 + T cells and (b) different pattern of the TCR-VA19 transcripts over time, and (c) similar VA transcripts in different individuals. (a) Polyclonal TCR transcripts could be detected in the VB19 family. In individuals no. 1 (Fig. 1) and no. 7 (Fig. 2) a similar pattern of the TCR-VB19 transcripts (373 bp peak) could be observed 2 weeks after the third vaccination. Note the additional peak of 376 bp in individual no. 7. Bp, base pairs. (b) A monoclonal (individual no. 1) TCR-VA19 mRNA transcript was present in CD8+ T cells 2 weeks after the second vaccination. However, 2 weeks after the last vaccination, the dominant clonal TCR-VA19 transcript could not be detected. In contrast, a different (345 bp) monoclonal TCR emerged in the CD8+ PBL from individual no. 7. (c) Polyclonal VA24+ T cells in individual no. 1 2 weeks after the second immunization, which gave rise to a monoclonal VA24 transcript 2 weeks after the third vaccination. Note the identical TCR-VA24 transcript in CD8+ T cells from individual no. 7 2 weeks after the second vaccination.

crepancy between TCR-VA mRNA expression and TCR protein expression which cannot be easily addressed due to the lack of available antibodies.

Here, we show that, at least in a single individual, i.m. immunization with a recombinant HBs antigen vaccine leads to activation and expansion of a CD8+ T cell clone (Fig. 1 and Table 3) which recognized the recombinant HBs antigen displayed by autologous antigen-presenting cells. The fact that dendritic cells are capable of presenting exogenously added protein in the context of MHC class I molecules is not novel. Earlier studies showed that introduction of soluble protein into the MHC class I processing and presentation pathway takes place and is able to induce a strong and efficient CD8+ T cell response (reviewed in [44]). However, due to the limited number of clonal VB3+ T cells in the peripheral circulation at a single time point, we have not been able to determine which class I molecules serve as the restricting elements for the VB3+ T cell clone identified in individual no. 1. In addition, the fact that the CD8+ TCR-VB3− T cell population did not respond to HbsAg displayed by autologous DCs suggests that, at least at this time point, the MHC class I-restricted T cell response rests exclusively in the clonal VB+ T cell pool of individual no. 1. The existence of a stringent selection process, imposed by the antigen displayed by MHC class I molecules, may favour the expansion of T cell clones with conserved features in the TCR CDR3 region [45,46].

At first glance it appears surprising that similar or identical TCR transcripts may be present in CD8+ PBL obtained from MHC disparate individuals upon vaccination with protein antigens, e.g. the TCR-VA24 identified in individuals no. 1 and no. 7. Of note, the TCR-VA24 is not identical to the invariant VA24AJ18 TCR chain, which is expressed by CD4−CD8− 'human natural T cells' which define glycosylceramides – derivates pre-

sented by CD1d antigens [47–49]. It is also unlikely that the TCR-VA24 CD8+ T cell clone defines an antigen provided by the hepatitis B vaccine, since this monoclonal TCR transcript could also be identified in PBL prior to vaccination (Table 3). However, the TCR-VA24 CD8+ T cell subsets in individuals no. 1 and no. 7 may recognize closely related epitopes from common antigens (e.g. EBV), which may be presented by the shared MHC class C7 alleles of these individuals. Similar T cell receptor junction sequences have also been identified in patients with viral infections, suggesting a strong selective pressure and/or structural constraints imposed on the TCR interaction with the MHC/peptide complex [50]. Alternatively, other non-classical MHC alleles, may also serve as the restricting elements.

We combined analysis of TCR-VB frequency, determined by flow cytometry and the TCR-VA/VB CDR3 length measurement in individuals undergoing vaccination with recombinant protein. Our results clearly demonstrated that the structural analysis of the TCR repertoire should not exclusively rely on the TCR-VB analysis, but include examination of the TCR-VA chain. Additionally, monoclonal TCR expansion of antigen-specific and MHC-class I restricted CD8+ T cells may be induced by protein vaccination. Future studies are needed to address the functional role of CD8+ T cells induced by a recombinant hepatitis B vaccine. Lastly, the TCR landscape analysis suggests that the TCR-VA/VB repertoire is relatively stable over time in healthy individuals (e.g. see Fig. 1), although a few monoclonal TCR-VA transcripts may be present.
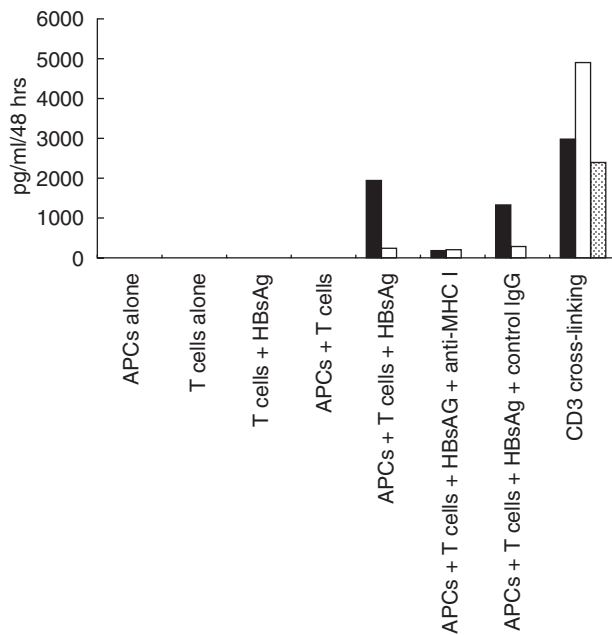
Fig. 4. CD8+ VB3+ T cells (black bars) were isolated from individual no. 1 (see Fig. 1) and tested for recognition of autologous APCs pulsed with recombinant HBsAg 12 h before assay. APCs and effector T cells were incubated for 48 h, supernatants were harvested and tested for IFN-γ production. T cell reactivity directed against target antigens could be reduced significantly using the anti-MHC class I w6/32 (10 μg/well). Note that CD8+ PBL, depleted of VB3+ T cells, were not capable of effectively producing IFN-γ in response to the target antigen (grey bars). We performed a cytokine release assay, as only a limited number of TCR-VB3+ T cells could be isolated. These were split into two aliquots: one for the functional assay and the other for the molecular analysis of the TCR composition (see Table 3), We did not resort to intracellular cytokine staining after T cell exposure to antigen, as different peptide concentrations displayed by APCs may impact on the intracellular cytokine level(s) and therefore underestimate the number of antigen-specific T cells [51]. The monoclonal VA24+ T cells isolated from individual no. 7 did not recognize HBsAg presented by autologous DCs. Cross-linking of the TCR by an anti-CD3 MoAb attached to plastic served as the positive control. Note that the anti-HBsAg reactivity rests exclusively in VB3+ T cells, as CD8+ PBL, depleted of VB3 T cells, did not produce IFN-γ in response to HBsAg. ■, VB3+; □, VB3−; ▨, VA24+.

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