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A Novel Approach of Direct Ex Vivo Epitope Mapping Identifies Dominant and Subdominant CD4 and CD8 T Cell Epitopes from *Listeria monocytogenes*[1]

Gernot Geginat,[2]* Simone Schenk,* Mojca Skoberne,* Werner Goebel,† and Herbert Hof*

We used a novel approach for the direct ex vivo identification and characterization of T cell epitopes based on the screening of peptide spot libraries with freshly isolated splenocytes in a sensitive enzyme-linked immunospot (ELISPOT) assay. This technique was applied for the analysis of splenocytes from *Listeria monocytogenes*-infected BALB/c and C57BL/6 mice. The screening of peptide spot libraries covering the whole listeriolysin O and p60 of *L. monocytogenes* confirmed all known CD4 and CD8 T cell epitopes of these proteins and additionally revealed six new H-2^d and six new H-2^b-restricted T cell epitopes. New epitopes were categorized into CD4 and CD8 T cell epitopes by ex vivo ELISPOT analysis with separated T cell populations. The quantitative analysis of cells reactive with these CD4 and CD8 T cell epitopes revealed the existence of dominant and subdominant CD4 and CD8 T cell populations during *L. monocytogenes* infection. As a consequence of these data we suggest that ELISPOT-based screening of peptide spot libraries could be a general approach for the rapid identification and characterization of pathogen-specific T cell populations during various infectious diseases. *The Journal of Immunology,* **2001, 166: 1877–1884.**

The identification of T cell epitopes is crucial for the understanding of the host response during autoimmune diseases and infections with pathogenic microorganisms. MHC molecules on the surface of APC present peptide fragments derived from foreign protein Ag and display them to T lymphocytes (1, 2). The identification of relevant T cell epitopes is generally based on the specificity analysis of T cell lines propagated in vitro (3). Once a T cell target protein is defined, the antigenic epitope can be mapped with synthetic peptide libraries synthesized according to the known sequence of the target protein. Alternative approaches are the biochemical purification and sequencing of MHC-bound peptides or the T cell-screening of expression libraries prepared from the organism of interest. A more recent method is the identification of unknown T cell epitopes by the screening of combinatorial peptide libraries made up of millions of different peptides (4). Epitope screening with synthetic peptides is expedited by the use of peptide spot libraries, which allow the automated and economic synthesis of multiple peptides (5, 6). The identification of MHC class I-restricted T cell epitopes is further facilitated by the existence of relatively strict peptide binding motifs of individual MHC class I molecules (7). If the CD8 T cell target protein is already identified, the knowledge of MHC class I binding motifs allows the educated guessing of epitopes and thus greatly reduces the number of synthetic peptides required for epitope identification, but epitopes that do not exhibit the typical binding motif may be overlooked. Regarding the identification of CD4 T cell epitopes the less strict binding requirements and thus the limited predictive value of MHC class II motifs (8, 9) makes this approach less suitable.

The murine *Listeria monocytogenes* infection is one standard model system for the analysis of the mechanisms governing specificity, induction, and expansion of T cells during microbial infection (reviewed in Ref. 10). The murine *L. monocytogenes* infection is characterized by the development of a protective T cell-dependent immunity mediated by both CD4 and CD8 T cells. Most of the previous work on the antilisterial T cell response focused on the specificity and function of *L. monocytogenes*-specific CD8 T cells. The identification of the K^d-restricted CD8 T cell epitopes listeriolysin O (LLO)[3] 91–99 (11), p60 217–225 (12), and p60 449–457 (13) from *L. monocytogenes*-infected BALB/c mice was catalytic for the further analysis of the antilisterial CD8 T cell response and revealed a distinct hierarchy of peptide-specific CD8 T cells (14). Significantly less information is available concerning the function and specificity of *L. monocytogenes*-specific CD4 T cells. Both the LLO and the secreted p60 protein of *L. monocytogenes* are targets for CD4 T cells, but until now only the H-2E^k-restricted peptide LLO 215–234 (15, 16) and the H-2A^d-restricted peptide p60 301–312 have been studied in some detail (17).

We used a novel approach for the direct ex vivo identification and characterization of T cell epitopes based on the screening of peptide spot libraries with ex vivo isolated lymphocytes in a highly sensitive enzyme-linked immunospot (ELISPOT) assay. This approach was applied for the analysis of LLO- and p60-specific T cells during *L. monocytogenes* infection. The data presented in the current report show that this approach rapidly identified *L. monocytogenes*-specific CD4 and CD8 T cell epitopes.

Making use of sequence data available for a variety of pathogenic microorganisms and also target structures relevant in tumor or autoimmune diseases ELISPOT-based screening of peptide spot

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[3] Abbreviations used in this paper: LLO, listeriolysin O; ELISPOT, enzyme-linked immunospot; p.i., postinfection.

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libraries could represent a general approach for the rapid identification and characterization of T cells during various disease states.

Materials and Methods

Mice and infection of mice

Female C57BL/6 (H-2^b) and BALB/cOlaHsd (H-2^d) mice were purchased (Harlan-Winkelmann, Borchen, Germany), kept under conventional conditions, and used at 8–10 wk of age. Mice were immunized by i.p. injection of 1×10^3 CFU *L. monocytogenes* serovar 1/2a EGD in 0.2 ml PBS and boosted i.p. with 1×10^6 CFU 2 wk later. Generally, spleens were removed and tested in the ELISPOT assay 10–14 days postinfection (p.i.). Bacteria used for infection were in the logarithmic growth phase. The bacterial concentration was estimated by determination of the OD at 600 nm.

Cell lines

Transfected cell lines expressing specific MHC class I molecules were used for the restriction analysis of CD8 T cell epitopes. Transfected L fibroblasts (H-2^k) expressing the MHC class I molecules K^d (18), D^d, or L^d (19) and nontransfected parental L-*tk*$^-$ cells were obtained from M. Reddehase (University of Mainz, Mainz, Germany). Nontransfected 1T22-6 cells (H-2^q) and transfected 1T-K^b and 1T-D^b cells expressing the MHC class I molecules K^b and D^b, respectively, were kindly provided by B. Arnold (German Cancer Research Center, Heidelberg, Germany) (20).

Peptides and peptide spot libraries

Cellulose-adsorbed peptide spot libraries were prepared commercially by automated spot synthesis on cellulose membranes (Jerini Biotools, Berlin, Germany) using a modified standard protocol (5, 21). The amino acid sequences of LLO (22) and p60 (23) were represented by peptide spot libraries with 247 and 229 dodecamer peptides, respectively. The offset between consecutive peptides was two residues. Fine mapping of regions antigenic for CD4 or CD8 T cells was performed with dodecamer or nonamer sublibraries, respectively. Sublibraries were synthesized with a single residue offset between consecutive peptides. After the final dry state cleavage by ammonia vapor peptides remain adsorbed to the cellulose membrane and carry an additional glycineamid at the carboxyl terminus. The peptide spots with adsorbed peptides were punched out, transferred to 96-well microtiter plates, and were eluted with 200 μl PBS for 30 min at room temperature to yield a 2.5×10^{-4} M peptide stock solution, which was stored at −80°C. Bulk peptides were commercially synthesized using standard Fmoc chemistry (Jerini Biotools) and purified by HPLC. Bulk peptides were synthesized with a free carboxyl terminus and thus did not contain an additional carboxyl-terminal glycineamid. Bulk peptides were diluted to a concentration of 1×10^{-3} M in 30% acetonitril in distilled water and stored at −80°C.

T cell separation

CD4 and CD8 T cells were purified by immunomagnetic separation using Minimacs (Miltenyi Biotec, Bergisch Gladbach, Germany) equipment. Spleen cells were stained either with FITC anti-CD4 (clone RM4-5; PharMingen, San Diego, CA) or FITC anti-CD8 (clone 53-6.7; PharMingen) mAbs and separated after secondary labeling with paramagnetic microbeads coupled with monoclonal mouse anti-FITC isomer 1 Ab (Miltenyi Biotec) applying the standard positive selection protocol provided by the manufacturer. Purity of separated T cell populations was generally between 80 and 90%.

ELISPOT

The frequency of T lymphocytes was determined with an IFN-γ-specific ELISPOT assay 10–14 days post booster infection (14, 24). In some experiments splenocytes were tested 6 wk after booster infection when most *L. monocytogenes*-specific T cells show a memory T cell phenotype (25). Assays were performed in nitrocellulose-backed 96-well microtiter plates (Nunc, Wiesbaden, Germany) coated with rat anti-mouse IFN-γ mAb (RMMG-1; Biosource, Camarilla, CA). For the testing of peptide spot libraries, the 2.5×10^{-4} M peptide stock solution was further diluted 25-fold with PBS to yield a 1×10^{-5} M diluted peptide solution. In round-bottom 96-well microtiter plates per well 6×10^5 unseparated splenocytes in 135 μl culture medium (α modification of Eagle's medium (Life Technologies, Eggenstein, Germany) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1×10^{-5} M 2-ME, and 2 mM glutamine) were mixed with 15 μl prediluted peptide to yield a final peptide concentration of 1×10^{-6} M. After 6 h of incubation at 37°C, cells were resuspended by vigorous pipetting, and 100 μl or 10 μl of cell suspension (4×10^5/well or 4×10^4/well, respectively) was transferred to Ab-coated ELISPOT plates and incubated overnight at 37°C. In the ELISPOT plates, the final volume was adjusted to 150 μl to ensure homogenous distribution of cells. Bulk peptides were tested similarly at a final concentration of 10^{-6} M peptide. Purified $CD4^+$ or $CD8^+$ T cells were tested in a modified assay as follows: 15 μl prediluted peptide (1×10^{-5} M) was directly added to Ab-coated ELISPOT plates and mixed with 4×10^5 splenocytes from nonimmune animals as APC to yield a final volume of 100 μl. After 4 h of preincubation of APC at 37°C, 1×10^5 $CD4^+$ or $CD8^+$ cells purified from *L. monocytogenes*-immune mice were added per well in a volume of 50 μl and plates were incubated overnight at 37°C. The ELISPOT-based ex vivo MHC restriction analysis was performed after loading of cell lines expressing specific MHC class I molecules with 1×10^{-6} M peptide for 2 h at 37°C. Subsequently, unbound peptides were washed off (four times) to prevent binding of peptides to responder splenocytes. Per well of the ELISPOT plate, 1×10^5 peptide-loaded APC were mixed with 4×10^5 or 4×10^4 responder splenocytes in a final volume of 150 μl. After overnight incubation at 37°C ELISPOT plates were developed with biotin-labeled rat anti-mouse IFN-γ mAb (clone XMG1.2; PharMingen), HRP streptavidin conjugate (Dianova, Hamburg, Germany), and aminoethylcarbazole dye solution. The frequency of Ag-specific cells was calculated as the number of spots per splenocytes seeded. The specificity and sensitivity of the ELISPOT assay was controlled with IFN-γ secreting CD8 T cell lines specific for p60 217–225, p60 449–457, and LLO 91–99. Recovery of seeded CD8 T cells was higher than 90% for all cell lines.

Results

Ex vivo T cell epitope screening with peptide spot libraries

The identification of antigenic peptides generally depends on the analysis of T cell lines selected by repeated rounds of in vitro restimulation. We used a different approach for the direct ex vivo identification of T cell epitopes based on the screening of peptide spot libraries with ex vivo isolated lymphocytes in a highly sensitive ELISPOT assay.

Peptide spot libraries with dodecamer peptides and two residues offset between neighboring peptides were synthesized according to the known sequences of LLO (22) and p60 (23) of *L. monocytogenes*. These proteins were chosen as representative model Ag for the study of this experimental approach because both are secreted abundantly by *L. monocytogenes*, both are known targets for CD4 and CD8 T cells, and for both proteins a number of T cell epitopes has been defined (11–13, 15, 17). These peptide libraries were screened with splenocytes from *L. monocytogenes*-infected BALB/c and C57BL/6 mice in an IFN-γ-specific ELISPOT assay. Fig. 1 shows the frequency of IFN-γ secreting cells among splenocytes incubated with individual peptides from the LLO (Fig. 1*A*) and p60 (Fig. 1*B*) peptide spot libraries, respectively. Peptides that induced significant activity are indicated with the peptide number and the corresponding sequences are listed in Table I. Antigenicity of peptides was considered significant if the induced activity was more than three SDs above the mean background activity in the absence of peptides, which was 1.98 ± 1.2, and 3.3 ± 1.6 per 4×10^5 spleen cells for BALB/c and C57BL/6 splenocytes, respectively. Splenocytes from nonimmune mice were not significantly activated by any peptide tested (data not shown). Peptides that contain one of the known CD8 T cell epitopes of *L. monocytogenes* (i.e., LLO 91–99, p60 217–225, and p60 449–457) were clearly positive and also peptide p60 no. 135, which contains most of the sequence of the p60 epitope p60 301–312, was antigenic in the ELISPOT assay (Fig. 1). In addition to these known T cell epitopes, 12 new antigenic regions of LLO and p60 were identified (Table I). Six of these new antigenic regions were defined in BALB/c and six in C57BL/6 mice.

Discrimination between CD4 and CD8 T cell epitopes

To determine the MHC class restriction of the newly identified epitopes from LLO and p60, reactive peptides from the peptide spot libraries were tested in an ELISPOT assay with separated $CD4^+$ and $CD8^+$ cells from *L. monocytogenes*-infected BALB/c

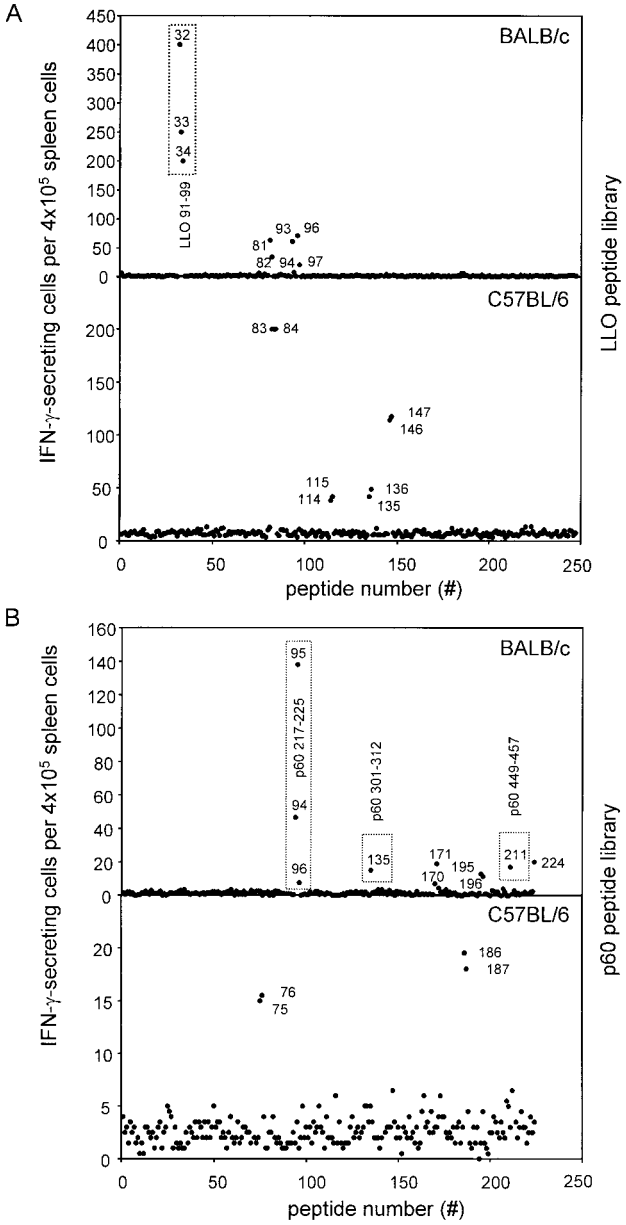

FIGURE 1. Ex vivo identification of LLO and p60 T cell epitopes. Overlapping dodecamer peptide spot libraries covering the whole LLO (*A*) and p60 (*B*) proteins of *L. monocytogenes* were tested in an IFN-γ-specific ELISPOT assay with spleen cells from *L. monocytogenes*-infected BALB/c or C57BL/6 mice as indicated. The offset between subsequent peptides was two residues. Results are shown as number of spots per 4×10^5 splenocytes and represent the mean of three experiments. The library peptide number is indicated for positive peptides, and sequences of positive peptides are listed in Table I. Positive peptides containing one of the known CD4 (p60 301–312) or CD8 T cell epitopes (LLO 91–99, p60 217–225, p60 449–457) of p60 or LLO are marked (dotted rectangles). A peptide was considered positive if it yielded an activity more than three SDs above the mean background activity in the absence of peptide, which was 1.98 ± 1.2, and 3.3 ± 1.6 per 4×10^5 spleen cells for BALB/c and C57BL/6 splenocytes, respectively.

(Fig. 2, *A* and *B*) and C57BL/6 mice (Fig. 2, *C* and *D*). Because the absolute number of T cells reactive with different peptides varies over a wide range, the frequencies of cells reactive with a specific peptide are presented as the percentage of $CD4^+$ or $CD8^+$ cells in relation to the indicated total number of cells reactive with an individual peptide per 1×10^5 $CD4^+$ and $CD8^+$ cells, which was defined as 100%. This ELISPOT analysis clearly separated putatively MHC class II-restricted peptides recognized by CD4 T cells from putatively MHC class I-restricted peptides recognized by CD8 T cells. The assay confirmed the restriction of peptides containing the known CD8 T cell epitopes LLO 91–99, p60 217–225, and p60 449–457 and the known CD4 T cell epitope p60 301–312 (summarized in Table I). In the absence of synthetic peptide, the spontaneous activity of sorted T cells was less than one spot per 1×10^5 cells (not shown). Among the 12 newly identified antigenic regions, peptides from 10 regions stimulated CD4 T cells and peptides from 2 regions stimulated CD8 T cells (Table I).

Fine mapping of new T cell epitopes

Screening of LLO and p60 peptide libraries revealed 12 antigenic regions that could not be attributed to any known T cell epitope of *L. monocytogenes*. For the fine mapping of the newly identified antigenic regions, the putative MHC class restriction of these peptides as summarized in Table I was taken into consideration. Antigenic regions recognized by CD4 T cells were mapped with dodecamer sublibraries with a single residue offset between consecutive peptides. Regions antigenic for CD8 T cells were mapped similarly with nonamer sublibraries with a single residue offset between consecutive peptides. These sublibraries of the antigenic regions of LLO (Fig. 3, *A* and *C*) and p60 (Fig. 3, *B* and *D*) were screened with freshly isolated splenocytes from *L. monocytogenes*-immune BALB/c (Fig. 3, *A* and *B*) or C57BL/6 mice (Fig. 3, *C* and *D*) as appropriate. As an internal control, also a sublibrary of the antigenic region p60 no. 135, which covers the A^d-restricted CD4 T cell epitope p60 301–312 was included. The fine mapping of the two CD8 T cell epitopes revealed single peptides with an antigenicity significantly above the antigenicity of related peptides. Peptides LLO 296–304 (VAYGRQVYL, Fig. 3*C*) and p60 476–484 (KYLVGFGRV, Fig. 3*B*) were identified as CD8 T cell epitopes from the antigenic regions LLO no. 135/no. 136 and p60 no. 224, respectively. The mapping of CD4 T cell epitopes generally revealed broader antigenic plateaus as evident for the LLO antigenic regions LLO no. 81/no. 82, LLO no. 93/no. 94, LLO no. 96/no. 97 (Fig. 3*A*), LLO no. 83/no. 84, LLO no. 114/no. 115, and LLO no. 146/no. 147 (Fig. 3*C*), and also the p60 antigenic regions p60 no. 135, p60 no. 170/no. 171, p60 no. 195/no. 196 (Fig. 3*B*), p60 no. 75/no. 76, and p60 no. 186/no. 187 (Fig. 3*D*). Peptides with a central position in the sublibrary and also a strong antigenic activity were considered as core CD4 T cell epitopes and are marked in Fig. 3. These peptides were synthesized as bulk peptides with free acid at the carboxyl terminus and used for further confirmatory experiments.

MHC restriction analysis of CD8 T cell epitopes

BALB/c and C57BL/6 mice express multiple MHC class I and class II molecules. To define the MHC restriction of the two new CD8 T cell epitopes, the recognition by freshly isolated splenocytes of peptide-loaded transfected cells that express specific MHC molecules was tested in an IFN-γ-specific ELISPOT assay. Due to the limited availability of MHC class II-transfected cell lines this approach was only possible for the analysis of CD8 T cell epitopes. The restriction analysis of the new H-2^d-restricted CD8 T cell epitope p60 476–484 was performed with K^d-, D^d-, and L^d-expressing transfected L cells. The known K^d-restricted CD8 T cell

Table I. *List of antigenic peptides identified from LLO and p60 peptide spot libraries*[a]

Mouse Strain	LLO Peptides				p60 Peptides			
	No.	Sequence	Position	Type	No.	Sequence	Position	Type
BALB/C	32	PRKGYKDGNEYI	88–99	CD8	94	LSVKYGVSVQDI	214–225	CD8
	33	KGYKDGNEYIVV	90–101	CD8	95	VKYGVSVQDIMS	216–227	CD8
	34	YKGDNEYIVVEK	92–103	CD8	96	YGVSVQDIMSWN	218–229	CD8
	81	VERWNEKYAQAY	186–197	CD4	135	PTEAAKPAPAPS	299–310	CD4
	82	RWNEKYAQAYPN	188–199	CD4	170	NSSASAIIAEAQ	366–377	CD4
	93	MAYSESQLIAKF	210–221	CD4	171	SASAIIAEAQKH	368–379	CD4
	94	YSESQLIAKFGT	212–223	CD4	195	AQYASTTRISES	417–428	CD4
	96	QLIAKFGTAFKA	216–227	CD4	196	YASTTRISESQA	419–430	CD4
	97	IAKFGTAFKAVN	218–229	CD4	211	IYVGNGQMINAQ	449–460	CD8
					224	GWGKYLVGFGRV	473–484	CD8
C57BL/6								
	83	NEKYAQAYPNVS	190–201	CD4	75	QTTQATTPAPKV	176–187	CD4
	84	KYAQAYPNVSAK	192–203	CD4	76	TQATTPAPKVAE	178–189	CD4
	114	KQIYYNVNVNEP	252–263	CD4	186	YTKYVFAKAGIS	399–410	CD4
	115	IYYNVNVNRPTR	254–265	CD4	187	KYVFAKAGISLP	401–412	CD4
	135	SSVAYGRQVYLK	294–305	CD8				
	136	VAYGRQVYLKLS	296–307	CD8				
	146	KAAFDAAVSGKS	316–327	CD4				
	147	AFDAAVSGKSVS	318–329	CD4				

[a] Peptide number, sequence, and position of spot-synthesized peptides which activated ex vivo isolated lymphocytes are listed. For each peptide the cell type ($CD4^+$ or $CD8^+$) stimulated is indicated.

epitope p60 217–225 was also included as a control. The restriction of LLO 296–304 was tested similarly with K^b- and D^b-expressing transfected 1T cells. As shown in Fig. 4*A*, the majority of p60 217–225- and p60 476–484-specific cells reacted in the context of the K^d molecule. The majority of LLO 296–304-specific cells responded in the context of the K^b molecule (Fig. 4*B*). Nontransfected cells and cells expressing the wrong MHC class I molecule also induced some T cell activation. However, this activity was not enhanced by the addition of peptide, which indicates that it was triggered by the allogeneic MHC of the parental cell line.

Hierarchy of CD4 and CD8 T cell epitopes

To confirm the T cell epitopes identified by the screening of peptide spot libraries and to determine the hierarchy of responding T cells, peptides were newly synthesized with free acid at the carboxyl terminus. This was necessary to exclude any influence of the

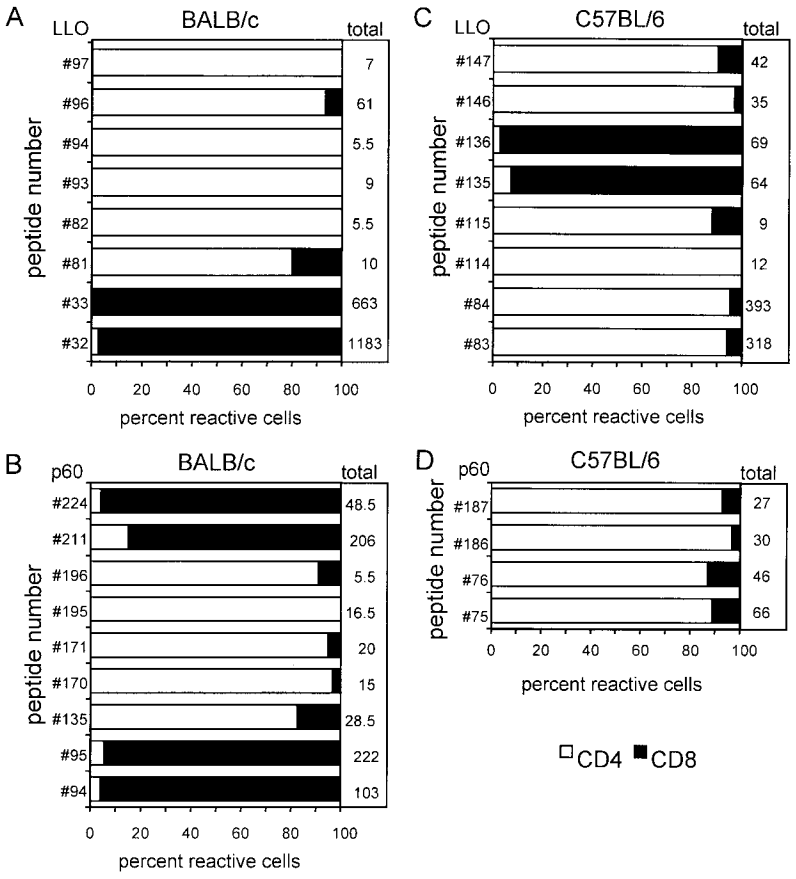

FIGURE 2. Ex vivo discrimination between CD4 and CD8 T cell epitopes. Previously identified peptides from peptide spot libraries representing T cell antigenic regions of LLO (*A* and *C*) and p60 (*B* and *D*) were tested with separated $CD4^+$ and $CD8^+$ cells from BALB/c (*A* and *B*) and C57BL/6 mice (*C* and *D*) as appropriate. Individual peptides are indicated by the peptide number (e.g., LLO no. 97) and the corresponding sequences are listed in Table I. Results are shown as the percentage of reactive $CD4^+$ (□) and $CD8^+$ (■) cells in relation to the indicated total number of reactive cells per 1×10^5 $CD4^+$ and $CD8^+$ cells (total), which was defined as 100%.

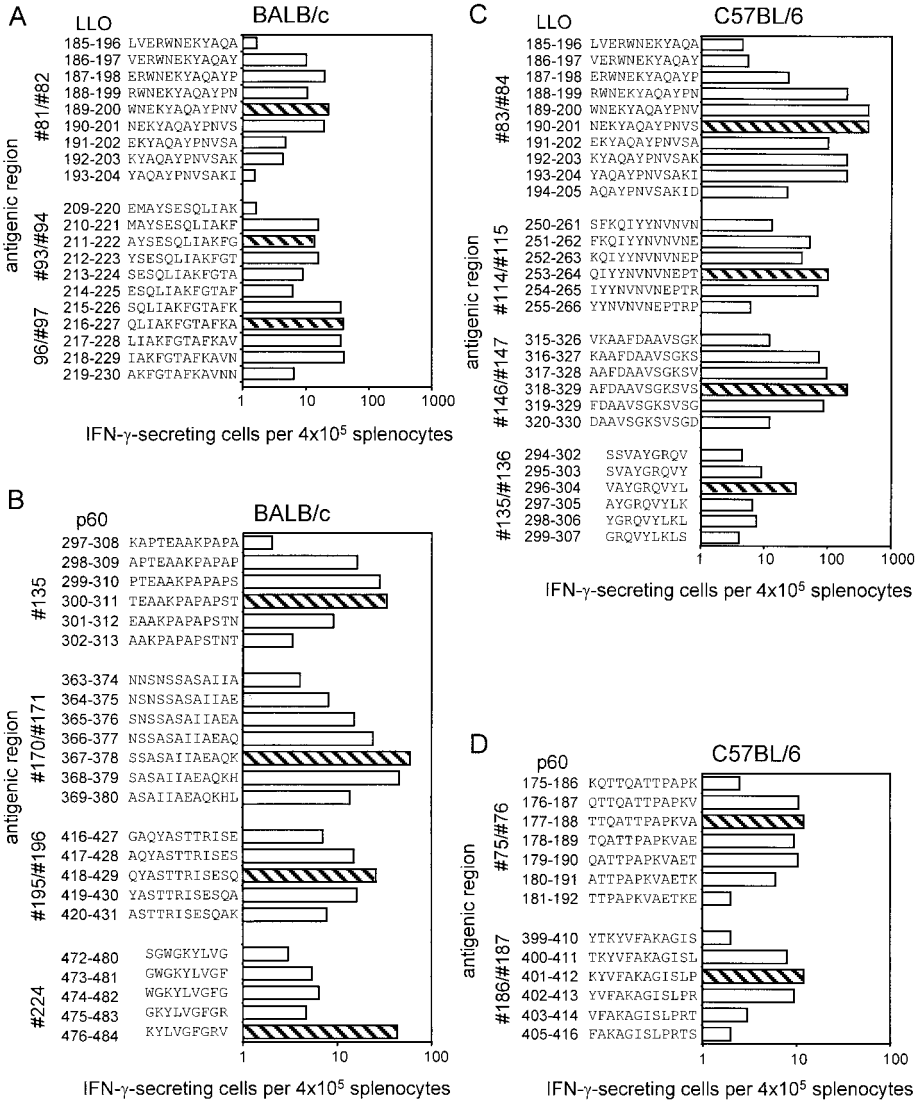

FIGURE 3. Fine mapping of T cell epitopes. Fine mapping of previously identified antigenic regions of LLO (*A* and *C*) and p60 (*B* and *D*) was performed with sets of spot-synthesized peptides with only a single residue offset between consecutive peptides. Spot-synthesized peptides were tested with splenocytes from *L. monocytogenes*-infected BALB/c (*A* and *B*) or C57BL/6 mice (*C* and *D*). According to the T cell type stimulated (Table I), CD4 and CD8 T cell epitopes were mapped with dodecamer and nonamer peptides, respectively. Data are indicated as the number of spots per 4×10^5 splenocytes and represent the mean of three experiments. The central epitope of each antigenic region is marked by hatched bars. These peptides were synthesized using standard Fmoc chemistry and used for additional experiments.

glycineamid, which remained at the carboxyl terminus after cleavage of spot-synthesized peptides. Peptides were tested in an IFN-γ-specific ELISPOT assay with splenocytes from *L. monocytogenes*-infected BALB/c and C57BL/6 mice (Table II). Splenocytes were tested 10–14 days and 6 wk p.i. when most responding T cells exhibit an effector and memory T cell phenotype, respectively (25). In BALB/c mice 10–14 days p.i. the dominant T cell populations were CD8 T cells specific for the well known K^d-restricted CD8 T cell epitopes LLO 91–99 and p60 217–225. The new CD8 T cell epitope p60 476–484 and the new CD4 T cell epitope LLO 189–200 showed significantly lower frequencies between 20 and 15 positive cells per 1×10^5 splenocytes. The remainder of the H-2^d-restricted epitopes were recognized with frequencies between 10 and 1 positive cells per 1×10^5 splenocytes. Interestingly, in C57BL/6 mice 10–14 days p.i. the most dominant cell population with 125 positive cells per 1×10^5 spleen cells reacted with the CD4 T cell epitope LLO 190–201. The CD4 T cell epitopes LLO 318–329, LLO 253–264, p60 401–412, and p60 177–188, and the CD8 T cell epitope LLO 296–304 showed a weaker reaction in the range between 25 and 5 positive cells per 1×10^5 splenocytes. We tested also the octamer peptide LLO 297–304 because it is known that K^b molecules preferentially bind octamer peptides (7). Interestingly, compared with LLO 297–304, significantly more cells reacted against the nonamer peptide LLO 296–304. In the absence of peptide, the background activity was less than one IFN-γ-positive cell per 1×10^5 splenocytes for both BALB/c and C57BL/6 mice. The general hierarchy of strong and weak epitopes did not change significantly if splenocytes were tested in the memory phase 6 wk p.i. (Table II). Minor changes in the hierarchy occurred only within the group of weak epitopes, which induced <10 spots per 1×10^5 splenocytes (p60 367–378 and LLO 211–222; p60 401–412 and p60 177–188). These data obtained with bulk synthesized peptides confirmed the T cell epitopes identified by screening of spot-synthesized peptide libraries. Furthermore, a distinct hierarchy between immunodominant and subdominant CD4 and CD8 T cell epitopes is shown.

Discussion

We used a novel approach for the direct ex vivo identification and characterization of T cell epitopes. The analysis was based on the screening of spot-synthesized peptide libraries with freshly isolated lymphocytes in a sensitive ELISPOT assay. This approach was applied for the analysis of LLO and p60-specific T cells during *L. monocytogenes* infection. The model system of the murine *L. monocytogenes* infection and particularly the T cell response against LLO and p60 was studied, because these bacterial proteins contain several known T cell epitopes (11–13, 15, 17), which allows direct comparison with the results of the ELISPOT-based

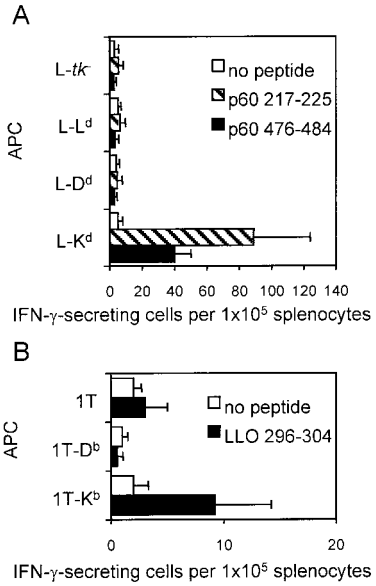

FIGURE 4. Ex vivo MHC restriction analysis of CD8 T cell epitopes. The MHC class I restriction of CD8 T cell epitopes was tested with splenocytes form *L. monocytogenes*-infected mice in an IFN-γ-specific ELISPOT assay. Transfected cell lines expressing specific MHC class I molecules were loaded with the indicated peptide and used as APC in the ELISPOT assay. *A,* The restriction analysis of the new H-2^d-restricted epitope p60 476–484 was performed with untransfected L-*tk*$^-$ cells and transfected L cells expressing K^d, D^d, or L^d MHC class I molecules. APC were tested in the absence of peptide (□), after loading with the K^d-restricted control epitope p60 217–225 (▨), and after loading with p60 476–484 (■) *B,* The restriction of the H-2^b-restricted CD8 T cell epitope LLO 296–304 was tested with untransfected 1T cells and transfected 1T cells expressing K^b or D^b MHC class I molecules. APC were tested in the absence of peptide (□) and after external loading with LLO 296–304 (■). Data are shown as the number of spots per 1 × 10^5 splenocytes and represent the mean of triplicate determinations ± SD.

approach. The ELISPOT-based screening of dodecamer peptide spot libraries confirmed all three previously described H-2^d-restricted CD8 T cell epitopes LLO 91–99, p60 217–225, and p60 449–457. Identification of these epitopes was successful despite the fact that peptides synthesized by the spot method had an additional glycineamid at the carboxyl terminus. As dodecamer peptides were used for primary screening, additional residues were present at the amino or carboxyl terminus of spot-synthesized peptides. However, these additional residues did not prevent CD8 T cell recognition, indicating that either peptides were spontaneously cleaved by serum proteases (26–28) or binding of extended peptides to class I molecules occurred because of the high peptide concentration (29–31). The ex vivo epitope screening of peptide spot libraries confirmed the known A^d-restricted CD4 T cell epitope p60 301–312 (17). As CD4 T cell epitopes revealed broad antigenic regions the difference between p60 300–311 and the previously reported epitope p60 301–312 is not significant. The H-2^d-restricted CD4 T cell epitope LLO 216–227, further delimits the antigenic peptide LLO 215–234, a E^k-restricted epitope that has been reported to cross-react with LLO-specific T cells from BALB/c mice (15). Antigenic peptides related to LLO 354–371, another E^k-restricted epitope that also should cross-react with H-2^d-restricted LLO-specific CD4 T cells (15), were not detected.

The ex vivo analysis identified a number of new CD4 T cell epitopes from LLO and p60 in BALB/c and C57BL/6 mice. As C67BL/6 mice solely express the MHC class II molecule A^b, it can be assumed that CD4 T cell epitopes identified in this mouse strain are A^b-restricted. An ex vivo MHC restriction analysis for the H-2^d-restricted CD4 T cell epitopes in BALB/c mice, could not be performed because appropriate MHC class II-transfected cell lines were not available.

The T lymphocyte response to pathogenic microorganisms focuses on a limited number of epitopes. Immunodominant epitopes generate a strong T cell response, while subdominant epitopes induce a comparatively weak response (32). According to this definition the epitopes LLO 91–99 and p60 217–225 of *L. monocytogenes* are considered immunodominant and p60 449–457 and mpl 84–92 are considered subdominant (14, 25). Similarly, comparison of the frequencies of H-2^b-restricted CD4 T cells revealed a distinct hierarchy of dominant and subdominant epitopes. This hierarchy of CD4 T cell epitopes was principally conserved in the early effector and late memory phase of the antilisterial T cell response confirming previous studies with CD8 T cell epitopes (14, 25). Interestingly, in C57BL/6 mice the dominant T cell epitope, LLO 190–201, which reacted with 5-fold more T cells than the second strong peptide, is a CD4 T cell epitope. This antigenic region was conserved in BALB/c mice, where LLO 189–200 was also the strongest CD4 T cell epitope. The existence of a dominant LLO-specific CD4 T cell response is remarkable in the light of the immunoevasive function of LLO (33). A number of detailed in vitro studies show that LLO inhibits the MHC class II-restricted recognition of LLO itself (16), of p60 (24) and also of a model Ag as OVA (34). A recent study that monitored the expansion of adoptively transferred lymphocytes from *Listeria*-immune mice shows that during *L. monocytogenes* infection, in addition to a strong CD8 T cell response, also a significant CD4 T cell response occurs (35). The identification of an immunodominant MHC class II-restricted T cell epitope directly demonstrates the existence of a strong *L. monocytogenes*-specific CD4 T cell response in vivo and allows the direct ex vivo analysis of these CD4 T cells.

The ex vivo ELISPOT analysis identified two new CD8 T cell epitopes of *L. monocytogenes*. The MHC class I-restricted epitopes p60 476–484 and LLO 296–304 were identified from BALB/c and C57BL/6 mice, respectively. As the other known CD8 T cell epitopes from p60, p60 217–225, and p60 449–457, also the new epitope p60 476–484 is K^d restricted. This antigenic peptide was not detected in previous detailed studies of possible epitopes recognized by p60-specific CD8 T cells (12, 13). The p60 476–484 epitope has a high antigenicity score and ranks at position five among the possible K^d-binding nonamer peptides (36). It exhibits the typical K^d-peptide binding motif with a tyrosine at position two and valine at the carboxyl terminus (9). Compared with the frequency of CD8 T cells specific for the immunodominant epitope p60 217–225 and the subdominant epitope p60 449–457, the third CD8 T cell epitope of p60 revealed an intermediate strength. An interesting feature of p60 476–484 is that it represents the carboxyl-terminal nine amino acids of the intact p60 protein.

The peptide LLO 296–304 is the first H-2^b-restricted CD8 T cell epitope identified of *L. monocytogenes*. It is known that K^b generally binds octamer peptides (7) suggesting that the octamer LLO 297–304 could be the naturally processed epitope in vivo. However, it was consistently found that more splenocytes react against the nonamer LLO 296–304 than against the octamer LLO 297–304. Recent studies that tested the peptide length preference of K^b show that K^b can also accommodate longer peptides suggesting that both antigenic peptides are possible candidates for this epitope. The antigenic peptide LLO 296–304 is difficult to predict from the K^b-binding motif and the known LLO sequence. The peptide exhibits the hydrophobic anchor at the carboxyl terminus but misses the typical octamer K^b-binding motif with a tyrosine or

Table II. *Hierarchy of LLO- and p60-specific T cells in BALB/c and C57BL/6 mice*

Mouse Strain	Peptide	Sequence	Type	Frequency[a] ($\times 10^{-5}$) ± SD	
				10–14 days p.i.	6 wk p.i.
BALB/c	LLO 91–99	GYKDGNEYI	CD8	75.0 ± 8.3	47 ± 9.7
	p60 217–225	KYGVSVQDI	CD8	41.4 ± 11.8	25 ± 5.3
	p60 476–484	KYLVGFGRV	CD8	18.1 ± 8.0	17 ± 5.1
	LLO 189–200	WNEKYAQAYPNV	CD4	15.4 ± 8.2	10 ± 8.2
	LLO 216–227	QLIAKFGTAFKA	CD4	10.4 ± 1.7	7.1 ± 2.3
	p60 449–457	IYVGNGQMI	CD8	9.7 ± 4.2	6.5 ± 2.3
	p60 367–378	SSASAIIAEAQK	CD4	7.6 ± 0.9	2.0 ± 1.0
	LLO 211–222	AYSESQLIAKFG	CD4	5.4 ± 4.4	2.5 ± 1.6
	p60 418–429	QYASTTRISESQ	CD4	2.1 ± 0.7	0.4 ± 0.2
	p60 300–311	TEAAKPAPAPST	CD4	1.9 ± 0.4	0.6 ± 0.3
	None			0.6 ± 0.4	0.5 ± 0.2
C57BL/6	LLO 190–201	NEKYAQAYPNVS	CD4	125.0 ± 10.3	78.3 ± 13.3
	LLO 318–329	AFDAAVSGKSVS	CD4	25.0 ± 3.0	16.7 ± 4.3
	LLO 296–304	VAYGRQVYL	CD8	16.1 ± 5.3	9.2 ± 4.2
	LLO 297–304	AYGRQVYL	CD8	12.7 ± 2.0	6.6 ± 2.6
	LLO 253–264	QIYYNVNVNEPT	CD4	12.6 ± 1.0	5.1 ± 2.9
	p60 401–412	KYVFAKAGISLP	CD4	5.1 ± 1.1	1.5 ± 0.5
	p60 177–188	TTQATTPAPKVA	CD4	4.9 ± 3.9	1.8 ± 1.0
	None			0.4 ± 0.2	0.6 ± 0.2

[a] The frequency of IFN-γ-secreting cells per 1×10^5 spleen cells is indicated as mean ± SD of three mice tested. ELISPOT assays were performed 10–14 days p.i. and 6 wk p.i. as indicated. Peptides are sorted according to the T cell frequency obtained when tested between day 10 and day 14 p.i. Bulk peptides with free acid at the carboxyl terminus were tested at a final concentration of 1×10^{-6} M.

phenylalanine residue at position five (7), which results in the low K^b-binding score of this peptide (36). A previous study has already shown that LLO is a target for protective H-2^b-restricted CD8 T cells (37). LLO 296–304 and LLO 297–304 are possible targets of these CD8 T cells for which an epitope has not yet been defined.

Compared with the analysis of T cell lines or clones the identification of T cell epitopes with freshly isolated splenocytes is not prone to any selective pressure exerted during extended in vitro selection and expansion of T cell lines and allows the direct identification of strong T cell activities. A previous study of p60-specific CD4 T cell clones identified p60 301–312 as a CD4 T cell epitope (17). The ex vivo ELISPOT analysis with *Listeria*-immune BALB/c mice showed that this epitope is just the weakest of four p60 CD4 T cell epitopes. The different results obtained from the ex vivo analysis are likely due to the selection of specific T cell clones during in vitro restimulation. CD8 T cells have specific demands for the concentration of antigenic peptide necessary for successful in vitro expansion. Although the requirement of a minimal peptide concentration for T cell stimulation seems obvious, detailed studies of the in vitro expansion of CD8 T cell lines specific for *L. monocytogenes* and the murine CMV showed that, remarkably, also concentrations above a defined optimum can prevent the expansion of peptide specific CD8 T cells (38, 39). This would explain the loss of some T cell specificities during in vitro expansion of T cell lines.

Recent genome sequencing programs generated a wealth of sequence information of the human genome and a number of microorganisms including pathogenic viruses, bacteria and protozoa (40). The current report demonstrates that ELISPOT-based screening of peptide spot libraries with freshly isolated lymphocytes can rapidly identify target structures of murine antilisterial T cells. Thus it can be anticipated, that with this approach also the T cell response in other pathologic immune responses could be diagnosed as well. Importantly, the use of peptide libraries as an Ag source also overcomes MHC restriction of CD4 and CD8 T cell epitopes, which means that a single peptide library could be used for the study of the T cell response in an outbred population. Thus, ELISPOT-based screening of peptide spot libraries representing single or multiple proteins or even whole genomes of small viruses could be a general approach for the rapid identification and monitoring of relevant T cell populations during various disease states.

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