Bi-specific MHC Heterodimers for Characterization of Cross-reactive T Cells

Zu T. Shen, Michael A. Brehm, Keith A. Daniels, Alexander B. Sigalov, Liisa K. Selin, Raymond M. Welsh, and Lawrence J. Stern

From the Department of Pathology, Division of Diabetes, and Department of Biochemistry & Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

T cell cross-reactivity describes the phenomenon whereby a single T cell can recognize two or more different peptide antigens presented in complex with MHC proteins. Cross-reactive T cells have previously been characterized at the population level by cytokine secretion and MHC tetramer staining assays, but single-cell analysis is difficult or impossible using these methods. In this study, we describe development of a novel peptide-MHC heterodimer specific for cross-reactive T cells. MHC-peptide monomers were independently conjugated to hydrazide or aldehyde-containing cross-linkers using thiol-maleimide coupling at cysteine residues introduced into recombinant MHC heavy chain proteins. Hydrazone formation provided bi-specific MHC heterodimers carrying two different peptides. Using this approach we prepared heterodimers of the murine class I MHC protein H-2Kb carrying peptides from lymphocytic choriomeningitis virus and vaccinia virus, and used these to identify cross-reactive CD8+ T cells recognizing both lymphocytic choriomeningitis virus and vaccinia virus antigens. A similar strategy could be used to develop reagents to analyze cross-reactive T cell responses in humans.

The cellular immune response to foreign antigens depends on T cell receptor (TCR) recognition of short peptides bound to cell-surface major histocompatibility complex (MHC) proteins. For CD8+ T cells, antigen specificity is determined by the interaction of TCR with 8–10-mer peptides presented by class I MHC proteins (1). During T cell development, TCR genes are assembled by somatic recombination of V, D, J, and C gene segments, with additional diversity introduced by nucleotide addition at the junctions, resulting in a vast repertoire of TCR specificities. In this context, the diversity of foreign antigens is equally large or larger, and individual TCRs are able to recognize multiple peptide sequences. During the negative selection phase of T cell development, autoreactive T cells capable of binding self-MHC-peptide complexes at high affinity are deleted, and it has been suggested that negative selection removes so many TCR sequences that a high level of TCR cross-reactivity is required for the immune system to be able to recognize a sufficiently large set of foreign peptides (2). T cell cross-reactivity has been observed in many systems (3–6) and the structural basis for the phenomenon is beginning to be clarified (7, 8). T cell cross-reactivity appears to provide a molecular basis for T cell heterologous immunity, in which exposure to one pathogen provides protection against another (9). For example, prior infection by lymphocytic choriomeningitis virus (LCMV) protects mice from a lethal dose of vaccinia virus (Vv), with LCMV immune mice showing alterations in their T cell response to Vv infection due to LCMV-specific T cells cross-reacting with Vv (10, 11). Similar patterns of T cell cross-reactivity have been suggested to underlie protective heterologous immunity between influenza A virus, Epstein-Barr virus (12), and hepatitis C virus (13), and also immunopathology following sequential infection with Dengue virus subtypes (14). T cell cross-reactivity in these systems has been difficult to study, in part because reagents are not available for isolation and characterization of the cross-reactive T cells.

MHC tetramer staining is a very effective method for isolating and characterizing antigen-specific T cell populations (15). In this technique, biotin-labeled recombinant MHC molecules loaded with specific antigenic peptides are oligomerized using fluorescent streptavidin to form highly specific reagents for analysis of antigen-specific T cells in mixed populations using flow cytometry. The use of oligomeric species is necessary because the MHC-TCR interaction is characterized by low affinity and rapid dissociation kinetics (16), which precludes use of labeled MHC monomers as specific staining reagents (17). MHC tetramers conventionally are used in T cell staining protocols, mostly because of the ease of introducing biotin labels into recombinant proteins and the availability of a wide variety of fluorescent streptavidin conjugates (15). In addition to MHC tetramers, other oligomeric forms of MHC proteins including dimers, trimers, and higher order oligomers are available and also have been used as effective staining reagents (18, 19). Antigen-specific analysis and isolation of T cells using MHC tetramers and similar reagents is a mainstay of current immunological practice, and for example, has been used to characterize the fine specificity of the T cell response to VV and...
LCMV (5, 10, 20). However, MHC-based staining reagents currently are available only as homo-oligomers with identical MHC-peptide components, and as such have not been used extensively to characterize T cell cross-reactivity. In principle two MHC tetramers could be used in co-staining experiments to evaluate the ability of a particular T cell to cross-react with different MHC-peptide complexes, but in practice competition between the two tetramers severely limits this approach (5). As an alternate approach to the specific detection and analysis of cross-reactive T cells, we describe here the development of novel bi-specific MHC-peptide dimers, and their use in characterization of cross-reactive T cells by flow cytometry. We employed thiol-maleimide and hydrazine-carbonyl chemistries (21, 22) to functionalize and then cross-link specific peptide complexes of the murine class I MHC molecule H-2Kb, and used fluorescent versions of these MHC heterodimers to specifically stain CD8+ T cells cross-reactive toward LCMV and VV. A previous strategy for production of class II MHC heterodimers has been reported (23), but that strategy relies on sequential differential affinity purification for isolation of heterodimers after non-specific cross-linking, and was not used for T cell staining or characterization of T cells in mixed populations.

**EXPERIMENTAL PROCEDURES**

*Production of Class I H-2Kb Complexes—Extracellular domains of the murine MHC class I H-2Kb heavy chain carrying a C121R mutation and a non-native C-terminal cysteine introduced at position 282 (17), and full-length human light chain β2-microglobulin, were expressed separately as inclusion bodies in *Escherichia coli* and were folded *in vitro* by dilution in the presence of excess peptide, as previously described for human class I MHCs (1). Synthetic peptides purified by reverse phase-HPLC were purchased from 21st Century Biochemicals. Folded H-2Kb monomers were purified by anion exchange chromatography on Poros HQ columns (Roche Applied Science) using a gradient of NaCl from 0–0.5 M in 20 mM Tris buffer, pH 8.0. The concentration of each H-2Kb monomer was calculated by absorbance spectroscopy after anion exchange chromatography using ε280 = 74,955 cm−1 M−1 for H-2Kb heavy chain, ε280 = 20,003 cm−1 M−1 for β2-microglobulin light chain, and varied ε280 for peptides depending on sequence. Purified H-2Kb monomers were adjusted to a concentration of 10–20 mg/ml using regenerated cellulose filters (Amicon) and stored in 5 mM DTT to prevent the disulfide-linked species.

*Cross-linking of Soluble MHC Class I H-2Kb Complexes—Reduced H-2Kb monomers were purified by size exclusion chromatography using NAP-5 columns (GE Healthcare) to remove DTT prior to thiol modification. Reduced H-2Kb monomers were reacted with heterobifunctional cross-linkers MTFB (maleimidostrioxaformyl benzaldehyde) or MHPH (3-N-maleimido-6-hydraziniumpyridine hydrochloride) in a 6-fold molar excess for 3 h in 100 mM NaH2PO4, 150 mM NaCl, pH 6.0, buffer (Solulink). Cross-linker-modified H-2Kb monomers were purified from free cross-linker by size exclusion chromatography using a Superdex 200 column (GE Healthcare). MHPH-modified H-2Kb monomers and MTFB-modified H-2Kb monomers were mixed together in a 1:1 molar ratio. The concentration of the reaction mixture (containing MHPH modified H-2Kb monomer and MTFB modified H-2Kb monomer) was adjusted to 1–2 mg/ml using a regenerated cellulose filter (Amicon) and incubated for 20 h at room temperature in the presence of 5 mM aniline (Acros Organics), which was found to catalyze hydrazide formation (24). Chemically cross-linked H-2Kb dimers were purified from H-2Kb monomers by size exclusion chromatography using a Superdex 200 column (GE Healthcare). Purified, unlabeled H-2Kb dimers were stored at 4 °C at a concentration of ~1 μM for up to 1 month.

*Alexa 647 Labeling of H-2Kb Monomers, Cross-linked MHC Dimers, and Tetramers—H-2Kb monomers were purified by size exclusion chromatography using NAP-5 columns (GE Healthcare) to remove DTT prior to thiol modification. Reduced H-2Kb monomers were reacted with a 5-fold molar excess of Alexa 647 maleimide (Invitrogen) for 2 h at room temperature. Alexa 647-labeled H-2Kb monomers were purified by size exclusion chromatography using a Superdex 200 column (GE Healthcare). H-2Kb homodimers and heterodimers, prepared as described above, were adjusted to a concentration greater than 10 μM and labeled with a 20-fold molar excess of Alexa 647 succinimidyl ester (Invitrogen). The labeling reaction was quenched using a 50-fold molar excess of ethanolamine (Sigma), and H-2Kb dimers were re-purified by size exclusion chromatography using a Superdex 200 column (GE Healthcare). Reaction yield and labeling efficiency were determined by absorbance spectroscopy after gel filtration purification, using ε280 = 265,000 cm−1 M−1 for Alexa 647 succinimidyl ester and the above mentioned ε280 for H-2Kb heavy chain, ε280 = 20,003 cm−1 M−1 for β2-microglobulin light chain, and peptides. All of the H-2Kb dimers were labeled with approximately two Alexa 647 per H-2Kb dimer with the exception of the A11R-A11R-H-2Kb homodimer, which was labeled with approximately four Alexa 647 per dimer. For preparation of Alexa 647-labeled streptavidin-linked H-2Kb tetramers, H-2Kb monomers were first purified by size exclusion chromatography using NAP-5 columns (GE Healthcare) to remove DTT prior to thiol modification, and then biotinylated by reacting with a 5-fold molar excess of biotin maleimide (Pierce) for 2 h at room temperature and subsequently re-purified by size exclusion chromatography using a Superdex 200 column (GE Healthcare). Biotinylation yield was determined by adding a 2-fold molar excess of streptavidin to the biotinylated H-2Kb monomers with analysis by 12% SDS-PAGE. The biotinylated H-2Kb monomers were subsequently oligomerized by stepwise addition of Alexa 647-streptavidin (Invitrogen) to the biotinylated H-2Kb to a final molar concentration of 1:6.

*Mass Spectroscopy—Alexa 647-labeled H-2Kb tetramers/dimers/monomers were checked for the presence of intact peptide by matrix-assisted laser desorption ionization (MALDI). Alexa 647-labeled H-2Kb complexes were purified by size exclusion chromatography using a Superdex 200 column (GE Healthcare), and purified fractions were mixed with UV absorbing matrix (α-cyano-4-hydroxy-cinnamic acid) prior to laser desorption, ionization, and detection of the ionized species. The sample was analyzed on a Waters MALDI LR spectrometer.
Bi-specific MHC Heterodimers

Isolation of Antigen-specific CTL—LCMV (Armstrong strain), an RNA virus in the Old World arenavirus family, was propagated in BHK-21 baby hamster kidney cells as previously described (25). BL/6 mice were infected intraperitoneally with a non-lethal dose of \( 5 \times 10^4 \) plaque-forming units of LCMV as previously described (25). Mice were considered immune at greater than 6 weeks after infection (9). Splenocytes from LCMV immune mice were co-cultured with mouse RMA cells that were pulsed with \( 1 \mu M \) GP34 peptide, washed, and then \( \gamma \)-irradiated (3000 rads) as previously described (9). RMA is a \( \text{K}^b \)-positive, Rauscher virus-induced, T-lymphoma cell line of BL/6 origin. Briefly, the co-culture of splenocytes and GP34-pulsed RMA cells were grown in RPMI supplemented with 100 units/ml of penicillin G, 100 \( \mu g/ml \) of streptomycin sulfate, \( 2 \text{mM L-glutamine}, 10 \text{mM HEPES}, 1 \text{mM sodium pyruvate}, 0.1 \text{mM minimal essential medium nonessential amino acids, 0.05 mM } \beta \text{-mercaptoethanol, and 10% FBS for 5 days at } 37^\circ \text{C at 5% CO}_2 \). Following this initial culture period, cells were harvested and stimulated with GP34 peptide-pulsed RMA cells in the presence of 10% BD T-Stim (BD Biosciences), an IL-2 culture supplement. LCMV-GP34 peptide-pulsed RMA stimulation was repeated every 4 to 5 days. After 20 to 25 days of stimulation (four or five stimulations), T-cell lines were characterized by intracellular cytokine staining or MHC staining.

Cell Surface and MHC Staining by Flow Cytometry—Cell suspensions were incubated in staining buffer (phosphate-buffered saline containing 1% FBS and 0.2% sodium azide) containing anti-mouse CD16/CD32 (Fc-block, clone 2.4G2). Cells were washed once with staining buffer and then stained with H-2K\(^b\) monomers/dimers/tetramers for 90 min. Thereafter, cells were washed twice with staining buffer and fixed in Cytofix (BD Pharmingen). Samples were analyzed using a BD Biosciences LSRII flow cytometer and FlowJo software (Tree Star).

Intracellular Cytokine Staining—A suspension of \( 10^6 \) cells was stimulated with \( 1 \mu M \) synthetic peptide or a medium only control. Stimulation were performed for 5 h at \( 37^\circ \text{C} \) in a total volume of 200 \mu l of RPMI medium supplemented with 10% FBS, 10 units/ml of human recombinant interleukin-2 (IL-2), and 0.2 \mu M brefeldin A (GolgiPlug; BD Pharmingen). After incubation, cell-surface antibody staining with anti-CD8\(\alpha\) (clone 53–6.7) and anti-CD44 (clone IM7) was performed. Thereafter, cells were washed twice with staining buffer, and then fixed and permeabilized (Cytofix/Cytoperm; BD Pharmingen). Intracellular cytokine-producing cells were detected with phycoerythrin (PE)-labeled anti-mouse interferon-\(\gamma\) (IFN-\(\gamma\), clone XMG1.2) and APC-labeled anti-mouse tumor necrosis factor \(\alpha\) (TNF-\(\alpha\), clone MP6-XT22) monoclonal antibodies. Antibodies were purchased from BD Pharmingen. The samples were analyzed as described above for cell surface staining.

RESULTS

Isolation and Characterization of a Cross-reactive CD8+ T Cell Line—To evaluate the utility of bispecific MHC heterodimers in analysis of T cell cross-reactivity, we made use of a previously described system of heterologous immunity, in which infection of mice with the old world arenavirus LCMV confers partial T cell immunity to infection with the poxvirus VV (20). Some T cells responding to the LCMV peptide GP34 (\(\text{AVYNFATM}\)) can cross-react with VV peptide A11R (\(\text{AIVNYANL}\)) (5). The GP34 and A11R peptides have side chains that are identical at three of the eight positions (underlined in sequences above), and are conservatively substituted at three other positions. A crystal structure is available for the H-2K\(^b\)-GP34 complex (26), and shows that the bound GP34 peptide places peptide side chains at the P2(V), P5(F), and P8(M) positions into pockets in the K\(^b\) binding site, with the intervening peptide side chains available for TCR interaction (26). T cells recognizing this peptide form a substantial component of the overall K\(^b\)-restricted response to LCMV (27). The A11R peptide conforms to the K\(^b\)-binding motif (26), and would be expected to bind similarly. No structural information is available for the K\(^b\)-A11R complex, but the peptide is known to bind to K\(^b\) (9), forming a subdominant epitope that persists in the K\(^b\)-restricted response to VV (5). These peptides, together with two unrelated K\(^b\)-binding LCMV-derived peptides NP205 and GP118, and control tight-binding designed peptide SIY (4) (Table 1), were used in the experiments reported below.

T cell cross-reactivity in this system was addressed first by intracellular cytokine staining (Fig. 1A). BL/6 mice were infected with LCMV and allowed to recover for 6 weeks. Splenocytes from the LCMV immune mice were stimulated in vitro with K\(^b\)-expressing RMA cells pre-pulsed with the LCMV-GP34 peptide, to expand the population of T cells that respond to this peptide, and were rested for 3–4 days prior to experimentation. After expansion, intracellular cytokine staining experiments (Fig. 1A) showed that the majority (56.3%) of the CD8\(\alpha\), CD44+ T cells secreted both TNF\(\alpha\) (x axis) and IFN\(\gamma\) (y axis) in response to the GP34 peptide. The same cells were tested for their response to other peptides. In response to the VV-A11R peptide, 31.5% of T cells secreted both IFN\(\gamma\) and TNF\(\alpha\). Thus, at least a portion of the LCMV-GP34-specific T cells must be cross-reactive for VV-A11R. Similarly, if only the cells secreting TNF\(\alpha\) are considered, 34.6% were positive in response to VV-A11R. Because nearly all (96.3%) were positive in response to LCMV-GP34, a majority of the 34.6% positive to VV-A11R must also cross-react with LCMV-GP34. The T cell population showed no reactivity against two other peptide epitopes from LCMV, GP118, and NP205 (Fig. 1A).

MHC tetramer staining similarly demonstrates that a portion of the T cell population can engage both VV-A11R and LCMV-GP34 peptide epitopes (Fig. 1B). T cells were stained with fluorescently labeled streptavidin-linked tetramers carrying VV or LCMV peptides bound to biotinylated H-2K\(^b\), or with control tetramers carrying an unrelated SIY peptide (SIYRYGYGL). The SIY peptide is a self-antigen not expected to

### TABLE 1

| Source        | Protein Description | Abbreviation | Sequence |
|---------------|---------------------|--------------|----------|
| VV            | Nonstructural protein | A11R         | AVYNYANL |
| LCMV Glycoprotein | GP34             | AVYNFATM    |          |
| LCMV Nucleoprotein | NP205         | YTVKYIPNL   |          |
| LCMV Glycoprotein | GP118          | ISHFSNL     |          |
| Control       | Designed sequence | SIY         | SIYRYGYGL |

\(^a\) This peptide carries a C-terminal Cys → Met mutation relative to the native LCMV sequence. The substitution has been used in previous studies (26, 29) and does not have a significant impact on interaction with MHC or T cell receptors.
induce T cell responses in K\(^b\) + mice (4). In Fig. 1B, cognate tetramer staining (blue traces) is overlaid with control-SIY tetramer staining (red traces). Staining with A11R tetramer and GP34 tetramer clearly identifies both in cases positive and negative populations, with the negative staining population overlapping with the control tetramer stain. The fraction of cells staining positive for the VV-A11R tetramer is 50.9% and the fraction staining positive for LCMV-GP34 is 63.7%, again indicating that at least some of the T cells cross-react with both VV-A11R and LCMV-GP34 peptide complexes. The specificity of the MHc tetramers is further confirmed by the lack of staining for the related LCMV-GP118 tetramer, which completely overlaps with the control-SIY tetramer staining (Fig. 1B).

**Competition between MHc Tetramers in Conventional Staining Experiments**—In principle, cross-reactive T cell populations could be identified by co-staining with two differently labeled tetramers, but in practice competition between the two MHc tetramers greatly complicates this approach. We evaluated such tetramer cross-competition using the VV-A11R and LCMV-GP34 cross-reactive cell line. Double MHc tetramer staining experiments were performed using various mixtures of cognate MHc tetramers (VV-A11R and LCMV-GP34) and noncognate MHc tetramers (LCMV-GP118, control-SIY). In each experiment one MHc-tetramer was prepared using streptavidin coupled to PE, with another prepared using streptavidin coupled to allophycocyanin (APC), so that binding of both tetramers could be monitored simultaneously (Fig. 2). Prior to coupling to streptavidin, each MHc-peptide complex was evaluated by an SDS-PAGE mobility shift assay (supplemental Fig. S1) to ensure essentially complete biotinylation. When cognate VV-A11R-PE tetramer was mixed with non-cognate LCMV-GP118-APC tetramer or with the control-SIY-APC tetramer, the T cell staining percentages and intensities were similar to staining experiments performed with VV-A11R-PE tetramer alone. For example, the VV-A11R-PE tetramer in the presence of non-cognate LCMV-GP118-APC stained 52.8% of the cells (Fig. 2A), as compared with 50.9% for the VV-A11R-PE tetramer alone (Fig. 1B), or 50.5% for VV-A11R-PE in the presence of control SIY-APC tetramer (Fig. 2B). Similarly, staining of the cross-reactive cell population by the cognate LCMV-GP34-APC tetramer, which stained 63.7% of the cells (Fig. 1B), was not greatly altered in the presence of the non-cognate LCMV-GP118-PE tetramer, which stained 59.9% (Fig. 2C). However, staining of the LCMV-GP34-APC tetramer was dramatically reduced in the presence of the cognate VV-A11R-PE tetramer, with only 15.5% of the cells staining positive (Fig. 2E). For the VV-A11R-PE tetramer, the presence of the LCMV-GP34-APC tetramer had a much smaller effect, with the reduction in staining barely discernable (48.7% positive, Fig. 2E). The fluorescence intensity changes followed the same pattern, with the VV-A11R-PE tetramer exhibiting mean fluorescence intensity (MFI) of 843 in a single tetramer stain, 825 and 793 in a double tetramer stain with non-cognate LCMV-GP118, 825 and 793 in a double tetramer stain with LCMV-GP34. LCMV-GP34-APC MFI was 1268 as a single tetramer, 1082 in the presence of non-cognate SIY-PE, and decreased substantially to 551 in the presence of cognate VV-A11R-PE. Overall, the presence of cognate tetramer causes a shift in the LCMV-GP34-APC staining intensity, so that the double positive population (upper right quadrant) now significantly overlaps with the single positive VV-A11R-PE single positive population (upper left quadrant), with only 12.5% of the cells clearly identified as positive for both tetramers (Fig. 2E). Presumably the stronger competition by VV-A11R-PE tetramers as compared with LCMV-GP34-APC tetramers is due to stronger binding of K\(^b\)-VV-A11R as compared with K\(^b\)-LCMV-GP34 to TCR on most of the cells in the cross-reactive population. In summary, both the LCMV-GP34-APC tetramer and VV-A11R-PE tetramer can engage TCR to stain cells. However, in double stain-
ing experiments with LCMV-GP34 and VV-A11R, the tetramer co-staining experiments due to competition between the tetramers, we investigated the possibility of developing reagents that would be individually specific for the cross-reactive population of interest. One such reagent would be a bi-specific, MHC heterodimer carrying two different MHC-peptide complexes. MHC homodimers, prepared as fusion proteins using immunoglobulin Fc domains (18) or by cysteine-mediated coupling (19), have already been developed for use as staining reagents, and can be used similarly to MHC (homo)tetramers, although with somewhat lowered binding avidity (17). If MHC heterodimers were available, they would be expected to exhibit specific binding only to T cells carrying receptors able to bind to both component MHC-peptide complexes, as the affinity of monomeric MHC-peptide complexes, as the affinity of monomeric MHC-peptide engagement by T cell receptors (~10 µM (28)) generally is too low to survive typical washing protocols.3

Our strategy for production of a novel, bi-specific H-2Kb heterodimer is shown in Fig. 3. This strategy takes advantage of heterobifunctional cross-linkers, MTFB and MHPH, and previously developed H-2Kb proteins carrying C-terminal cysteine residues (17). MTFB and MHPH each contain a maleimide moiety, which can be used to couple the cross-linkers onto thiols, such as unpaired cysteine residues introduced into the membrane-proximal domains of MHC proteins (19). Opposite the maleimide group is an aldehyde or hydrazine, which can be used in cross-linking to form the desired hydrazone-linked dimers (22, 24).

Both maleimide-thiol coupling (21) and aldehyde-hydrazine cross-linking reactions can be carried out under relatively mild conditions appropriate for maintaining MHC peptide binding and native structure (Fig. 3). In independent tubes, H-2Kb monomers containing a C-terminal cysteine (P282C) were conjugated with MHPH and MTFB heterobifunctional linkers via their respective maleimides. The MHPH and MTFB H-2Kb modified monomers (R1,

3 Z. T. Shen and L. J. Stern, unpublished data.

**Figure 2.** Cross-reactive T cells engage cognate pMHC tetramers in a manner that reveals that there exists cross-reactive TCR on these cells. Visualization of VV-A11R- and LCMV-GP34-specific T cells by double MHC tetramer staining at 300 nM. Double MHC tetramer staining experiments were set up using a PE-labeled peptide-Kb tetramer and a APC-labeled peptide-Kb tetramer as follows. A, A11R tetramer (PE) and GP34 tetramer (APC); B, A11R tetramer (PE) and SIY tetramer (APC); C, GP34 tetramer (APC) and SIY tetramer (PE); and D, SIY tetramer (PE) and A11R tetramer (APC).

**Figure 3.** Chemical reaction scheme of heterobifunctional cross-linkers MHPH and MTFB. Peptide-Kb monomers (R1 and R2) containing a free thiol at the C terminus of the class I heavy chain (position 282) were reacted with heterobifunctional linkers, MTFB or MHPH through their maleimide moieties. The hydrazine on the MHPH-modified peptide-Kb monomer subsequently cross-links with the benzaldehyde on the MTFB-modified peptide-Kb monomer (when mixed in a 1:1 molar ratio) to generate a hydrazone cross-linked peptide-Kb dimer.
MHC-peptide complexes migrate with the dye front and are not visible by this analysis (Fig. 4B). Analysis of the A11R-GP34-H-2Kb heterodimer reaction mixture shows that slightly less than half of the total monomers react to form the desired heterodimer, confirming the results from size exclusion chromatography. Reducing SDS-PAGE also reveals that the A11R-GP34-H-2Kb heterodimer is covalently linked and not formed as a consequence of disulfide bonding or non-covalent interaction, because no appearance of a monomeric species (around 37,000 daltons) is observed for the Alexa 647-labeled, A11R-GP34-H-2Kb heterodimer after boiling and reduction (Fig. 4B). Finally, Alexa 647-labeled MHC monomers also exhibited no tendency to form disulfide-linked dimers (supplemental Fig. S2).

All of the Alexa 647-labeled, H-2Kb dimers used in this study were analyzed by MALDI mass spectroscopy to confirm that the peptide antigens are present in unmodified form in the MHC complexes (supplemental Fig. S3). In the A11R-H-2Kb monomer and A11R-H-2Kb homodimer, a peak at 899 daltons, corresponding to the molecular mass of A11R peptide, was observed, and in the GP34-H-2Kb monomer and GP34-H-2Kb homodimer, a peak at 938 daltons, corresponding to the molecular mass of GP34 peptide, was observed. For the A11R-GP34-H-2Kb heterodimer, peaks at both 899 (A11R peptide) and 938 daltons (GP34 peptide) were observed.

Identification of Cross-reactive TCR on T Cells Using MHC Heterodimers—The previously characterized VV-A11R and LCMV-GP34-H-2Kb cross-reactive T cells (Fig. 2) were re-stimulated with the LCMV-GP34 peptide-pulsed targets prior to use to maintain the cells during the in vitro culture. To ensure that the T cells did not change specificity from VV-A11R and LCMV-GP34 after re-stimulation, MHC tetramer staining was performed on the re-stimulated line (Fig. 5A). The cells exhibited strong staining with the A11R (88.5%) and GP34 (80.9%) tetramers, and little staining with the LCMV-GP118 tetramer (2.0%), both of which overlap almost completely with the unstained population (Fig. 5A, red curves). This confirms that the specificity of the T cells did not change upon re-stimulation with peptide-pulsed targets, although the percentage of VV-A11R and LCMV-GP34 tetramer-positive cells increased as expected because of preferential expansion of the specific population.

To attribute H-2Kb heterodimer binding to cross-reactive T cell receptors, it was necessary to ensure that H-2Kb monomers do not exhibit appreciable binding under our experimental...
conditions. For this reason, binding of all H-2Kb monomers was tested on VV-A11R and LCMV-GP34 specific T cells (Fig. 5B). No binding of any of the H-2Kb monomers (VV-A11R, LCMV-GP34, LCMV-GP118, control-SIY) was observed above the unstained background. Thus, as expected, MHC-peptide binding to T cells requires more than a single cognate MHC-TCR and MHC-CD8 interaction.

Our MHPH-MTFB cross-linking strategy generates a novel MHC-MHC linkage, and we wanted to ensure that the hydrazone-linked H-2Kb dimers could engage T cells with sufficient affinity and specificity for use in conventional staining protocols. For this purpose cognate (VV-A11R and LCMV-GP34) and noncognate (control-SIY) H-2Kb homodimers were made using the same MHPH-MTFB chemistry described above, and binding was tested on the re-stimulated VV-A11R, LCMV-GP34 cross-reactive T cells. As shown in Fig. 6A, both cognate A11R-A11R-H-2Kb homodimer and GP34-GP34-H-2Kb homodimer stain the T cells, with MFI = 1093 and 395, respectively. (The increased staining intensity for the A11R-A11R-H-2Kb homodimer reflects the greater degree of Alexa 647 labeling, approximately twice that of the other H-2Kb dimers; see
“Experimental Procedures” for details.) In contrast, the non-cognate SIY-SIY-H-2Kb homodimer control staining (MFI = 199) is only slightly higher than the unstained background (MFI = 132). These results parallel the tetramer staining results (Fig. 5A) in terms of specificity and demonstrate that heterobifunctional cross-linking does not alter the ability of H-2Kb homodimers to engage TCRs.

Finally, we wanted to evaluate whether T cells that express cross-reactive TCR could be identified using the bi-specific, cognate H-2Kb heterodimer. As shown in Fig. 6B, the bi-specific, A11R-GP34-H-2Kb heterodimer is able to stain the cross-reactive T cell population (MFI = 377), with 32% positive as compared with 4.9% positive for the SIY-SIY homodimer (MFI = 199). The staining signal is clearly distinguishable from the unstained background, and the staining intensity is similar to that observed for the specific GP34-GP34-H-2Kb homodimer (MFI = 395) with 49% positive (Fig. 6B). To confirm that MHC heterodimer binding depends on MHC-TCR contacts with both cognate peptide epitopes, we generated “control” H-2Kb heterodimers where one H-2Kb monomer is folded with a self-peptide (control-SIY) and the other with a cognate peptide (VV-A11R or LCMV-GP34), and compared the staining of these reagents to the A11R-GP34-H-2Kb heterodimer of interest (Fig. 6B). These controls are necessary for two reasons. First, if either the VV-A11R monomer or LCMV-GP34 monomer undergoes unexpected dimerization, an artificial H-2Kb “homodimer” would result and binding would be observed. Second, relative to the non-binding MHC monomers, the non-cognate MHC heterodimers carry an additional CD8-MHC contact as well as an additional nonspecific pMHC-TCR contact, either or both of which could potentially provide sufficient binding affinity to allow significant staining under typical experimental conditions. However, significant staining by the non-cognate A11R-SIY-H-2Kb (MFI = 162) and SIY-GP34-H-2Kb (MFI = 215) control heterodimers was not observed, with 2.8 and 4.6% positive, respectively, values similar to those for the control SIY-SIY homodimer. Thus, staining of the cross-reactive T cell line by the A11R-GP34-H-2Kb heterodimer requires bivalent engagement involving both MHC-peptide components, and reflects specific detection of cross-reactive T cells within the population.

**DISCUSSION**

We developed a specific heterodimerization strategy to prepare hydrazene-linked MHC dimers carrying two different MHC-peptide complexes. As expected from previous work using other disulfide or Ig-linked MHC dimers, the avidity of such complexes was sufficient to allow for specific staining and flow cytometric analysis of T cell cultures. Unlike previous work, the heterodimers were specific for cross-reactive T cells, i.e. T cells able to bind to two different MHC-peptide complexes. Unlike dual-tetramer staining, the MHC heterodimer experiments do not suffer from cross-competition between their component MHC-peptide complexes. A cross-reactive T cell with significantly greater affinity for one of the two component MHC-peptide complexes was easily detected. We expect that such MHC heterodimers will find application in analysis of heterologous T cell responses induced by vaccination, infection, or autoimmune stimuli.

One potential pitfall in development of the heterobifunctional cross-linking strategy was the high reactivity of the hydrazine moiety in the MHPH cross-linker, such that the MHPH-modified H-2Kb monomers (and particularly Alexa 546-labeled MHPH-modified H-2Kb monomers) exhibited a propensity to self-react upon extended storage in higher concentrations at 4 °C (data not shown). For this reason, MHPH-modified H-2Kb monomers were kept at low concentrations after modification, and were promptly mixed with previously prepared MTFB-modified H-2Kb monomers, so that once conjugated the hydrazine moieties (MHPH) could preferably react with aldehyde moieties (MTFB) as compared with the other more sluggish side reactions which may occur. Our cross-linking results from Fig. 4 coupled with the results from mass spectroscopy (see supplemental Fig. S1) indicate that this method is effective in achieving the desired dual specificity for the H-2Kb heterodimer. Another potential limitation of the heterobifunctional cross-linking strategy described here is that both thiols and amine groups are used for coupling (cross-linker conjugation and fluorescent labeling, respectively). Thus, the peptides used in heterobifunctional cross-linking of H-2Kb complexes should not have exposed side chains from cysteine residues (to prevent peptide cross-linking) or lysine residues (to prevent direct labeling of peptide that might interfere with TCR interaction). In such cases the cysteine and/or lysine residues could be conservatively substituted to remove the reactive species. For example, substitution of the original cysteine at position 41 by methionine in the LCMV-GP34–41 used in this study does not substantially impact T cell recognition (26, 29). Alternatively, a photoexchangeable peptide strategy (30) could be employed to allow for peptide loading after fluorescent labeling.

In this study we examined a polyclonal T cell line exhibiting cross-reactivity between two different viral antigens bound to the murine class I MHC H-2Kb, one derived from LCMV and one from VV. Such T cells are known to arise after natural infection with these viruses, and are believed to play a role in heterologous immune responses observed upon infection with one virus after prior exposure to the other (20). GP34-A11R cross-reactivity has been observed for T cell lines obtained by a variety of immunization/culture protocols (5, 9). The fraction of cross-reactive cells in the overall LCMV-responsive and VV-responsive populations varies between individual mice because of the “private” nature of the T cell response (5, 9, 31), and the fraction of cross-reactive T cells present after *in vitro* culture depends on the conditions used to expand the antigen-specific cell population. The particular T cell line investigated here exhibited an unusually high degree of cross-reactivity, thus providing an opportunity to evaluate the novel MHC heterodimer staining strategy, and to characterize in detail the nature of the cross-reactive T cell population.

If MHC monomers bound to TCRs, it would be difficult to distinguish cross-reactive from singly-reactive T cell populations. In this system, we confirmed that binding of MHC monomers is not observed on the T cells bearing cross-reactive TCRs (Fig. 5B). Furthermore, the clear absence of observable binding by control MHC heterodimers carrying one cognate and one
nonspecific peptide demonstrates that both peptides need to be cognate for MHC heterodimer binding to be observed (Fig. 6B). This contrasts with an observation of binding of non-cognate MHC tetramers to human peripheral blood T cells via CD8 interactions (32), although in that study MHC oligomer staining was performed at higher concentrations and temperatures (33). We cannot exclude the possibility that exceptionally tight binding T cells, with MHC-TCR affinity significantly higher than those observed to date (28), might be able to engage MHC-peptide monomers.

Cross-reactive T populations may represent one of three situations: 1) a mixture of two T cell subpopulations, each specific for a different antigen, but with no cross-reactivity at the single cell level; 2) a population of T cells expressing two distinct TCR as a result of incomplete allelic exclusion at the TCRα locus (34) or possibly TCR sharing (35) (i.e. with cross-reactivity at the single cell but not single receptor level (36–38); or 3) a T cell population carrying T cell receptors that individually react with two (or more) different MHC-peptide complexes. Because monomeric engagement is not sufficient to observe binding of the MHC heterodimers (Figs. 5B and 6B), individual T cells staining positively with the MHC heterodimers must express receptors for both of the component MHC-peptide complexes. Thus, we can rule out point 1 as an explanation for the observed cross-reactivity of the LCMV-VV cross-reactive T cell population, i.e. the cross-reactivity is apparent at the single cell level, and not just at the population level. The MHC heterodimer staining experiment by itself cannot distinguish between cases 2 and 3, both of which have been proposed to be relevant in T cell responses to infectious agents (7, 37). However, our observation of competition between MHC tetramers in the (homo-)tetramer staining experiments indicates that, for most or all of the T cells in the LCMV-VV cross-reactive population, individual TCR able to bind H-2Kb-LCMV-GP34 also were able to bind H-2Kb-VV-A11R, i.e. case 3, the cross-reactivity is apparent at the single receptor level. We note, however, that this demonstration depends on the relative avidities of the two tetramers. For example, we could observe competition of H-2Kb-LCMV-GP34-tetramers by the tighter binding H-2Kb-VV-A11R, but not the reverse. Although it was clear from previous studies that the LCMV-VV cross-reactive T cell populations are present after subsequent infections with LCMV and VV (9, 20), and cross-reactive T cells were observed in (BL/6 × TCRα KO) F1 mice, whose T cells express only a single TCR chain and are not subject to allelic exclusion (39), the present study confirms the idea that LCMV-VV cross-reactive T cell populations utilize T cell receptors individually cross-reactive with Kb-GP34 and Kb-A11R complexes.

T cells with receptors specific for class I MHC proteins like H-2Kb typically express the CD8 co-receptor, which binds to class I MHC in a peptide-antigen independent manner through an interaction outside the peptide binding site (40, 41). The CD8-MHC interaction is substantially weaker (Kd for H-2Kb ~90 μM (42)) than that of typical MHC-TCR interactions (~10 μM) (43), but nonetheless engagement of MHC by CD8, particularly multivalent engagement, could potentiate MHC dimer binding as it does MHC tetramer binding (44–46). However, the clear absence of observable binding by control MHC heterodimers carrying one cognate and one nonspecific peptide demonstrates that both peptides need to be cognate for MHC heterodimer binding to be observed (Fig. 6B). This contrasts with an observation of binding of non-cognate MHC tetramers to human peripheral blood T cells via CD8 interactions (32), although in that study MHC oligomer staining was performed at higher concentrations and temperatures (33).

In conclusion, the heterodimerization strategy developed here for the murine class I MHC molecule H-2Kb provides specific fluorescent MHC heterodimers composed of one MHPH-linked MHC-peptide monomer and one MTFB-linked MHC monomer carrying a different peptide, with no apparent perturbation of the MHC-peptide complex or its interaction with T cell receptors. Using H-2Kb heterodimer carrying peptides VV-A11R and LCMV-GP34, we showed that a unique subset of LCMV-VV cross-reactive T cells can be characterized. Double MHC tetramer staining on these cross-reactive T cells highlighted the problems with MHC tetramer cross-competition, but also revealed that these cross-reactive T cells express a single cross-reactive TCR able to bind to both VV-A11R and LCMV-GP34 peptides. Similar strategies could be used to develop human peptide-MHC reagents for quantifying T cell cross-reactivity during human viral infections. For example, cross-reactive influenza virus-specific T cells have been observed in Epstein-Barr virus-induced infectious mononucleosis (12) and implicated in hepatitis C virus-induced fulminating hepatitis (13) and they could be quantified by this method.

Acknowledgments—We thank Dr. Jennifer Stone for initial development of the hydrazone coupling strategy and technical guidance in the early phases of this work, and Guoqi Li for kindly providing preparations of human β2-microglobulin.

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