BI-SPECIFIC MHC HETERODIMERS FOR CHARACTERIZATION OF CROSS-REACTIVE T CELLS
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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 previously have 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 crosslinkers 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 (LCMV) and vaccinia virus (VV), and used these to identify cross-reactive CD8+ T cells recognizing both LCMV 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 8-10mer 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 fragments, with additional diversity introduced by nucleotide addition at the junctions, resulting in a vast repertoire of clonally distributed TCR proteins, with hypervariable regions concentrated in the TCR loops that contact MHC and bound peptide antigen. Despite the diversity of TCR sequences, the diversity of foreign antigens is equally large or larger, and individual TCR 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 (EBV) (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 homopolymers 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, we describe here the development of novel bi-specific MHC-peptide dimers, and their use in characterization of cross-reactive T cells. 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 towards 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 crosslinking, 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) using a gradient of NaCl from 0-0.5M 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 ε_{280} = 74955 cm^{-1} M^{-1} for H-2Kb heavy chain, ε_{280} = 20003 cm^{-1} M^{-1} for β2- microglobulin light chain and varied ε_{280} 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 disulfide-linked species.

**Crosslinking 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. H-2Kb monomers were reacted with heterobifunctional cross-linkers MTFB (maleimido trioxa formyl benzaldehyde) or MHPH (3-N-maleimido-6-hydrazinumpyridine hydrochloride) in a 6-fold molar excess for 3 hours in 100 mM NaH_{2}PO_{4}, 150 mM NaCl, pH 6.0 buffer (Solulink). Cross-linker-modified H-2Kb monomers were purified away from free crosslinker 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 hours at room temperature in the presence of 5 mM aniline (Acros Organics), which was found to catalyze hydrazone formation (24). Chemically cross-linked H-2Kb dimers were purified away 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 upwards of a 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 hours at room temperature. Alexa 647 labeled H-2K\^b monomers were purified by size exclusion chromatography using a Superdex 200 column (GE healthcare). H-2K\^b homodimers and heterodimers, prepared as described above, were adjusted to a concentration of greater than 10 \(\mu\)M and labeled with a 20-fold molar excess of Alexa 647 succinimidyld ester (Invitrogen). The labeling reaction was quenched using a 50-fold molar excess of ethanolamine (Sigma), and H-2K\^b 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 \(e_{280} = 265000\) \(\text{cm}^{-1} \cdot \text{M}^{-1}\) for Alexa 647 succinimidyld ester and the above mentioned \(e_{280}\) for H-2K\^b heavy chain, \(\beta\)-microglobulin light chain and peptides. All of the H-2K\^b dimers were labeled with approximately two Alexa 647 per H-2K\^b dimer with the exception of the A11R-A11R-H-2K\^b homodimer which was labeled with approximately four Alexa 647 per dimer. For preparation of Alexa 647-labelled streptavidin-linked H-2K\^b tetramers, H-2K\^b monomers first were purified by size exclusion chromatography using NAP-5 columns (GE healthcare) to remove DTT prior to thiol modification, and then were biotinylated by reacting with a 5-fold molar excess of biotin maleimide (Pierce) for 2 hours 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-2K\^b monomers with analysis by 12% SDS-PAGE. The biotinylated H-2K\^b monomers were subsequently oligomerized by stepwise addition of Alexa 647 streptavidin (Invitrogen) to the biotinylated H-2K\^b to a final molar concentration of 1:6.

Mass spectroscopy- Alexa 647-labeled H-2K\^b tetramers/dimers/tetramers/monomers were checked for the presence of intact peptide by Matrix-Assisted Laser Desorption/Ionization (MALDI). Alexa 647-labeled H-2K\^b complexes were purified by size exclusion chromatography using a Superdex 200 column (GE healthcare) and purified fractions were mixed with UV absorbing matrix (alpha-cyano-4-hydroxy-cinnaminic acid) prior to laser desorption, ionization and detection of ionized species. The sample was analyzed on a Waters MALDI LR spectrometer.

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 (i.p.) with a non-lethal dose of 5 \(\times\) 10\(^{4}\) PFU 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 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 U/ml penicillin G, 100 \(\mu\)g/ml streptomycin sulfate, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids, 0.05 mM \(\beta\)-mercaptoethanol, and 10\% FBS for 5 days at 37\(^\circ\)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 with the panel of MHC class I H-2K\^b monomers/dimers/tetramers.

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 Becton Dickinson LSRII flow cytometer (BD Biosciences) and FlowJo software (Tree Star).

Intracellular cytokine staining (ICS)- A suspension of 10\(^6\) cells was stimulated with 1 \(\mu\)M synthetic peptide or a medium only control. Stimulations were performed for 5 h at 37\(^\circ\)C in a total volume of 200 \(\mu\)l RPMI medium supplemented with 10\% FBS, 10 U/ml of human recombinant interleukin-2 (IL-2), and 0.2 \(\mu\)M of brefeldin A (GolgiPlug; BD Pharmingen). After incubation, cell-surface antibody staining with anti-
CD8α (clone 53-6.7), and anti-CD44 (clone IM7) was performed. Thereafter, cells were washed twice with staining buffer, and then fixed and permeabilized (Cytotox/Cytoperm; BD Pharmingen). Intracellular-cytokine-producing cells were detected with PE-labeled anti-mouse interferon-gamma (IFN-γ, clone XM1G1.2) and APC-labeled anti-mouse tumor necrosis factor alpha (TNF-α, 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 crossreactive 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 (AVYNFATM) can cross-react with VV peptide A11R (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-2Kb-GP34-41 complex (26), and shows that the bound peptide places peptide side chains at the P2(V), P5(F), and P8(M) positions into pockets in the Kb 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 Kβ-restricted response to LCMV (27). The A11R peptide conforms to the Kβ-binding motif(26), and would be expected to bind similarly. No structural information is available for the Kβ-A11R complex, but the peptide is known to bind to Kb (9), forming a subdominant epitope that persists in the Kβ-restricted response to VV (5). These peptides, together with two unrelated Kβ-binding LCMV-derived peptides NP205 and GP118, and control tight-binding designed peptide SIY (4) (Table I), were used in the experiments reported below.

T cell cross-reactivity in this system was addressed first by intracellular cytokine staining (ICS) (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 Kβ-expressing RMA cells pre-pulsed with LCMV-GP34 peptide, in order 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+, CD44+ T cells secreted both TNFα (X axis) and IFNγ (Y axis) in response to the GP34 peptide. The same cells were tested for their response to other peptides. In response to VV-A11R peptide, 31.5% of T cells secreted both IFNγ and TNFα. 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 TNFα 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-2Kβ, or with control tetramers carrying an unrelated SIY peptide (SIYRYYGL). The SIY peptide is a self-antigen not expected to induce T cell responses in Kβ+ mice (4). In Figure 1b, cognate tetramer staining (blue traces) is overlaid with control-SIY tetramer staining (red traces). Staining with A11R tetramer and GP34 tetramer clearly identifies in both 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 R-phycocerythrin (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. 1) 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 to 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 positively (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 single tetramer stain, 825 and 793 in double tetramer stain with non-cognate tetramers LCMV-GP118-APC and SIY-APC, respectively, and 727 in a double tetramer stain with cognate LCMV-GP34-APC. 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 singly 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 to LCMV-GP34-APC tetramers is due to stronger binding of K\(^{b}\)-VV-A11R as compared to 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 staining experiments with LCMV-GP34 and VV-A11R, the tetramers compete with each other. This competition between MHC tetramers for binding to cross-reactive receptors on T cells interferes with the use of tetramer staining in distinguishing cross-reactive from mono-specific T cells, and seriously complicates the use of MHC tetramers in investigation of heterologous immunity.

**Construction of heterodimeric MHC-based staining reagents by keto-hydrazide coupling.** Since cross-reactive T cell populations can be difficult or impossible to identify by MHC 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), already have 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 engagement by T cell receptors (~10 \( \mu \)M, (28)) generally is too low to survive typical washing protocols (unpublished data).

Our strategy for production of a novel, bi-specific H-2K\(^{b}\) heterodimer is shown in Figure 3. This strategy takes advantage of heterobifunctional cross-linkers, MTFB and MHPH, and previously developed H-2K\(^{b}\) 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 crosslinking 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, R2) were isolated by gel filtration and subsequently cross-linked through their aldehyde (MTFB) or hydrazine (MHPH) moieties to form a hydrazone-bonded, bi-specific H-2Kb heterodimer (Fig. 3). The extent of dimerization of the MHPH-modified A11R-H-2Kb monomer with the MTFB-modified GP34-H-2Kb monomer was approximately 40% as evaluated by analytical size exclusion chromatography (“Dimer reaction mix” trace, Fig. 4A). The hydrazone-linked A11R-H-2Kb, GP34-H-2Kb heterodimer was isolated by gel filtration and labeled with the amine-reactive fluorescent dye Alexa647-succinimidyl ester, to a ratio of approximately ~2 dye per MHC molecule. Importantly, after Alexa 647 succinimidyl ester labeling, the purified, labeled MHC heterodimer exhibited the expected apparent molecular weight, as did the unlabeled dimer in the reaction mixture, with no evidence of appreciable aggregation or dissociation (Fig. 4A). Furthermore, neither MHPH-modified A11R-H-2Kb monomer nor MTFB-modified GP34-H-2Kb monomer exhibited appreciable homodimerization, which potentially would interfere with specific staining of cross-reactive cells (Fig. 4A). Because no cross-linking is observed in the absence of either MPHP or MTFB, it appears that the heterobifunctional cross-linking reaction is quite specific.

Reducing SDS-PAGE analysis also was used to characterize the maleimide coupling and hydrazone crosslinking reactions (Fig. 4B). MHPH and MTFB coupling reactions do not compromise the purity of the H-2Kb heavy chain through cleavage or degradation, as judged by SDS-PAGE, and the MHPH-modified A11R-H-2Kb and MTFB-modified GP34-H-2Kb appear to be essentially completely homogenous (the small β2m subunit and peptide components of the MHC-peptide complexes migrate with the dye front and are not visible by this analysis) (Fig. 4A). 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 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-labelled MHC monomers also exhibited no tendency to form disulfide-linked dimers (Supplemental Fig. 2).

All of the Alexa 647-labeled, H-2Kb dimers used in this study were analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy to confirm that the peptide antigens are present in unmodified form in the MHC complexes (Supplemental Fig. 3). In the A11R-H-2Kb monomer and A11R-H-2Kb homodimer, a peak at 899 daltons, corresponding to the molecular weight 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 weight of GP34 peptide, was observed. For the A11R-GP34-H-2Kb heterodimer, peaks at both 899 daltons (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, LCMV-GP34 cross-reactive T cells (Fig. 2) were re-stimulated with LCMV-GP34 peptide-pulsed targets prior to use to maintain the cells during 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 A11R tetramer (88.5%) and GP34 tetramer (80.9%), and little staining with LCMV-GP118 tetramer (2.0%) or control-SIY tetramer (3.7%), 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 after re-stimulation, MHC tetramer staining was performed on the re-stimulated line (Fig. 5A). The cells exhibited strong staining with A11R tetramer (88.5%) and GP34 tetramer (80.9%), and little staining with LCMV-GP118 tetramer (2.0%) or control-SIY tetramer (3.7%), 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.

In order 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-2K\(^b\) monomers was tested on VV-A11R, LCMV-GP34 specific T cells (Fig. 5B). No binding of any of the H-2K\(^b\) 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-2K\(^b\) dimers could engage T cells with sufficient affinity and specificity for use in conventional staining protocols. For this purpose cognate (VV-A11R, LCMV-GP34) and noncognate (control-SIY) H-2K\(^b\) 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 Figure 6a, both cognate A11R-A11R-H-2K\(^b\) homodimer and GP34-GP34-H-2K\(^b\) homodimer stain the T cells, with MFI = 1093 and 395 respectively. (The increased staining intensity for the A11R-A11R-H-2K\(^b\) homodimer reflects the greater degree of Alexa 647 labeling, approximately twice that of the other H-2K\(^b\) dimers; see Methods for details). In contrast, the non-cognate SIY-SIY-H-2K\(^b\) 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-2K\(^b\) homodimers to engage TCRs.

Finally, we wanted to evaluate whether T cells which express cross-reactive TCR could be identified using the bi-specific, cognate H-2K\(^b\) heterodimer. As shown in Figure 6b, the bi-specific, A11R-GP34-H-2K\(^b\) heterodimer is able to stain the cross-reactive T cell population (MFI = 377), with 32% positive as compared to 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-2K\(^b\) 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-2K\(^b\) heterodimers where one H-2K\(^b\) monomer is folded with a self-peptide (control-SIY) and the other with a cognate peptide (VV-A11R or LCMV-GP34), and compared staining of these reagents to the A11R-GP34-H-2K\(^b\) 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 artifactual H-2K\(^b\) “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 non-specific pMHC-TCR contact, either or both of which potentially could provide sufficient binding affinity to allow significant staining under typical experimental conditions. However, significant staining by the non-cognate A11R-SIY-H-2K\(^b\) (MFI = 162) and SIY-GP34-H-2K\(^b\) (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-2K\(^b\) 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 hetero-dimerization strategy to prepare hydrazone-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-2K\(^b\) monomers (and particularly Alexa 546-labeled MHPH-modified H-2K\(^b\) monomers) exhibited a propensity to self-react upon extended storage in higher concentrations in 4°C degrees (data not shown). For this reason, MHPH-modified H-2K\(^b\) 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 to other more sluggish side reactions which may occur. Our cross-linking results from Figure 4 coupled with the results from mass spectroscopy (see Supplementary Fig. 1), 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-crosslinking) or lysine residues (to prevent direct labeling of peptide which 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\textsubscript{41} used in this study does not substantially impact T cell recognition (26,29). Alternatively, a photo-exchangeable 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 (unpublished observations). 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, clear absence of observable binding by control MHC heterodimers carrying one cognate and one non-specific peptide demonstrates that both peptides need to be cognate in order 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 sub-populations, 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 TCR 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. Since monomeric engagement is not sufficient to observe binding of the MHC heterodimers (Fig. 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 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 but not single receptor level, 2) a mixture of two T cell sub-populations, each specific for a different antigen, but 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 x TCRα KO) F1 mice, whose T cells express only a single TCRα 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 ($K_d$ for H-2Kb ~90 $\mu$M (42) than that of typical MHC-TCR interactions (~10 $\mu$M) (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 non-specific peptide demonstrates that both peptides need to be cognate in order 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 hetero-dimerization 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 EBV-induced infectious mononucleosis (12) and implicated in hepatitis C virus-induced fulminating hepatitis (13) and they could be quantified by this method.
REFERENCES

This work was supported by NIH-U19-AI057319 (LJS) and AI081675 (RMW). University of Massachusetts Medical School FACS and Proteomics Core resources supported by the Diabetes Endocrinology Research Center grant DK32520 were used. We would like to thank Dr. Jennifer Stone for initial development of the hydrazone coupling strategy and for her technical guidance in the early phases of this work, and Guoqi Li for kindly providing preparations of human β2 – microglobulin.

The abbreviations used are: MHC, major histocompatibility complex; LCMV, lymphocytic choriomeningitis virus; VV, vaccinia virus; TCR, T cell receptor; EBV, Epstein Barr virus; MTFB, maleimido trioxa formyl benzaldehyde; MHPH, 3-N-maleimido-6-hydraziniumpyridine hydrochloride; PE, R-phycoerythrin; APC, allophycocyanin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption/ionization.
**FIGURE LEGENDS**

**Fig. 1.** CD8+ T cells specific for VV-A11R and LCMV-GP34. *A.* CD8+ T cell response against VV-A11R and LCMV-GP34 was measured by intracellular IFNγ (y axis) and TNFα (x axis) staining in response to 1 µM of the indicated peptides. T cells from an LCMV-immune mouse were isolated and expanded in vitro using LCMV-GP34 peptide, and then were analyzed for cytokine secretion in response to Kb-expressing cells pulsed with VV-A11R or LCMV-GP34 peptides, using an intracellular cytokine staining assay, with the results presented as dot plots. *B.* Visualization of CD8+ T cell response against VV-A11R and LCMV-GP34 by peptide-Kb tetramer staining using 300 nM tetramer. The T cell line from panel A was used. T cells were stained with R-phycocerythrin (PE) or allophycocyanin (APC) labeled streptavidin-based Kb tetramers folded with VV-A11R, LCMV-GP34, LCMV-GP118, or control SIY peptides. The indicated tetramer stain (blue traces) was overlaid with control-SIY tetramer stain (red traces), with results presented as histograms.

**Fig. 2.** Cross-reactive T cells engage cognate pMHC tetramers in a manner which 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 GP118 tetramer (APC); *B.* A11R tetramer (PE) and SIY tetramer (APC); *C.* GP118 tetramer (PE) and GP34 tetramer (APC); *D.* SIY tetramer (PE) and GP118 tetramer (APC); and *E.* A11R tetramer (PE) and GP34 tetramer (APC). *F.* Overlay of data shown in panels *B.* (in red) and *E.* (in blue) to facilitate comparison.

**Fig. 3.** Chemical reaction scheme of heterobifunctional crosslinkers MHPH and MTFB. Peptide-Kb monomers (R1, R2) containing a free thiol at the C-terminus of the class I heavy chain (position 282) were reacted with heterobifunctional linkers, maleimido trioxa formyl benzaldehyde (MTFB) or 3-N-maleimido-6-hydraziniumpyridine hydrochloride (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.

**Fig. 4.** Heterobifunctional crosslinking of peptide-Kb monomers. Kb monomers modified with MTFB (GP34 MTFB) or MHPH (A11R MHPH), cross-linked Kb heterodimer reaction mixture (GP34-Kb MTFB and A11R-Kb MHPH) and Alexa 647 labeled, cross-linked Kb heterodimer (A647 labeled A11R-GP34 dimer) were analyzed by size exclusion chromatography and 12% SDS-PAGE. The MTFB-MHPH chemistry is specific because the A11R MHPH Kb monomer and GP34 MTFB Kb monomers do not form cross-linked Kb dimers independently. Additionally, Alexa 647 succinimidyl ester labeling does not alter the hydrodynamic radius or purity of the cross-linked Kb dimer. *A.* In the A11R-GP34 dimer reaction mix, cross-linked Kb dimer with a retention volume ~14 mL was purified from Kb modified monomers (retention volume ~15.5 mL) by size exclusion chromatography using a Superdex 200 column. The molecular weight standards are indicated along with the approximate retention volumes. *B.* Reducing SDS-PAGE confirms the purity of the modified Kb monomers and cross-linked Kb dimers. Proteolysis is not observed after modification (GP34 MTFB, A11R MHPH) or cross-linking (A11R-GP34 dimer reaction mix). Molecular mass markers are indicated on the left.

**Fig. 5.** MHC tetramer and MHC monomer staining of cross-reactive T cells. *A.* T cells previously characterized by tetramer staining as specific for both VV-A11R and LCMV-GP34 maintained their specificity after additional restimulation with LCMV-GP34 peptide pulsed targets. After restimulation and passage in vitro, T cells were stained with the indicated streptavidin-based Kb tetramers at 300 nM (blue trace) shown overlaid with an unstained control (red trace). The MFI of the positive staining population is indicated in the upper right in each histogram. Positive staining with the VV-A11R tetramer and the LCMV-GP34 tetramer shows that the specificity of the T cells is maintained after passage. *B.* MHC monomers do not have sufficient affinity required for T cell staining. The T cell culture from panel A was stained with peptide-Kb monomers at 2 µM as indicated. The indicated monomer stain (blue trace) was overlaid with the unstained control (red trace) in each histogram. Positive staining of T cells is not observed with any of the monomers.
Fig. 6. MHC heterodimer staining of cross-reactive T cells. The T cell culture shown in Figure 5 was stained with the indicated K\textsuperscript{b}-homodimers (A.) or K\textsuperscript{b}-heterodimers (B.) at 1 \(\mu\)M. A. MHC homodimer staining demonstrates that the novel hydrazone linkage does not disturb MHC engagement of TCR and confirms that the T cells are cross-reactive for LCMV-GP34 and VV-A11R as seen in the tetramer staining experiment. B. Bispecific MHC heterodimer (A11R-GP34-K\textsuperscript{b}) staining shows that cross-reactive T cells can be readily identified. All of the indicated MHC homodimers and heterodimers (blue trace), were overlaid with an unstained control (red trace). The MFI of the positive staining population is indicated in the upper right in each histogram.
## Table 1. Relevant epitopes used in this study from VV and LCMV

<table>
<thead>
<tr>
<th>Source</th>
<th>Protein</th>
<th>Abbreviation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV</td>
<td>Nonstructural protein A11R&lt;sub&gt;198-206&lt;/sub&gt;</td>
<td>A11R</td>
<td>AIVNYANL</td>
</tr>
<tr>
<td>LCMV</td>
<td>Glycoprotein&lt;sub&gt;Gp34-41&lt;/sub&gt;</td>
<td>GP34</td>
<td>AVYNFATM&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>LCMV</td>
<td>Nucleoprotein&lt;sub&gt;NP205-212&lt;/sub&gt;</td>
<td>NP205</td>
<td>YTVKYPNL</td>
</tr>
<tr>
<td>LCMV</td>
<td>Glycoprotein&lt;sub&gt;Gp118-125&lt;/sub&gt;</td>
<td>GP118</td>
<td>ISHNFSNL</td>
</tr>
<tr>
<td>Control (designed sequence)</td>
<td>SIY</td>
<td>SIY</td>
<td>SIYRYYGL</td>
</tr>
</tbody>
</table>

*This peptide carries a C-terminal Met->Cys 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.*
Figure 3

\[
R_1\text{-SH} + \text{MTFB} \rightarrow \text{product} \quad 25^\circ\text{C}, 3\text{h}, \text{pH 6}
\]

\[
R_2\text{-SH} + \text{MHPH} \rightarrow \text{product} \quad 25^\circ\text{C}, 3\text{h}, \text{pH 6}
\]

\[
\text{product} \rightarrow \text{product} \quad 25^\circ\text{C}, 20\text{h}, \text{pH 6}, 10\text{ mM aniline}
\]

\[
\text{product} \rightarrow \text{product} \quad 25^\circ\text{C}, 20\text{h}, \text{pH 6}, 10\text{ mM aniline}
\]
Figure 6.

A. 

- A11R - A11R dimer: MFI = 1093
- GP34 - GP34 dimer: MFI = 395
- SIY - SIY dimer: MFI = 199

B. 

- SIY - A11R dimer: MFI = 162
- SIY - GP34 dimer: MFI = 215
- A11R - GP34 dimer: MFI = 377
BI-specific MHC heterodimers for characterization of cross-reactive T cells
Zu Ting Shen, Michael A. Brehm, Keith A. Daniels, Alexander B. Sigalov, Liisa K. Selin, Raymond M. Welsh and Lawrence J. Stern

J. Biol. Chem. published online August 20, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.141051

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