

Recombinant Technology

Peptide– $\beta$ 2-microglobulin–MHC fusion molecules bind  
antigen-specific T cells and can be used for  
multivalent MHC–Ig complexes

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**Abstract**

Recombinant soluble MHC molecules are widely used for visualization, activation and inhibition of antigen-specific immune responses. Using a genetic approach, we have generated two novel peptide– $\beta$ 2-microglobulin–MHC constructs. We have linked the MHC molecule with the peptide of interest, without limiting the recognition by the cognate TCR. This molecule can also be joined with the IgG heavy chain resulting in a dimeric MHC–Ig fusion protein. These molecules bind antigen-specific T cells with high specificity and sensitivity, therefore, providing a valuable tool for detection as well as enrichment of antigen-specific T cells.

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**1. Introduction**

CD8+ T lymphocytes specifically recognize antigens through the interaction of their T cell receptor (TCR) with antigenic peptides displayed on MHC class I molecules. MHC class I molecules form heterodimers of a 46-kDa heavy chain with a 12-

kDa light chain,  $\beta$ 2-microglobulin (Madden, 1995), and present antigen-derived peptides to CD8+ cytotoxic T lymphocytes (York and Rock, 1996). Peptides are transported from the cytosol into the endoplasmic reticulum in a TAP-dependent fashion, where they bind to MHC class I molecules and traffic to the cell surface. Soluble MHC class I molecules have been very useful in both structural and biological studies (McMichael and O'Callaghan, 1998; Lebowitz et al., 1999; Fahmy et al., 2001).

Recently, soluble MHC complexes have gained a lot of attention through their use as tools to identify

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*Abbreviations:* SC, single chain.

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antigen-specific T cells (Altman et al., 1996; McMichael and O'Callaghan, 1998; McMichael and Kelleher, 1999; Greten and Schneck, 2002). Multimeric MHC molecules can be generated by two different approaches. One approach is to generate tetrameric MHC class I complexes for staining antigen-specific T cells from peripheral blood (Altman et al., 1996). In that system, MHC complexes are expressed in bacteria and refolded in the presence of the peptide of interest followed by biotinylation of the MHC complexes. Multimers, presumably tetramers, are generated through the binding of avidin with four biotinylated MHCs. An alternative approach is the construction of dimeric MHC–Ig fusion proteins (Dal Porto et al., 1993) to stain antigen-specific T cells (Greten et al., 1998; Carruth et al., 1999; Selin et al., 1999; Slansky et al., 2000). Expression of MHC–Ig dimeric fusion proteins in eukaryotic cells eliminates the need for *in vitro* refolding of the MHC complex (Schneck et al., 1999). Dimeric constructs can be loaded with different peptides to efficiently stain antigen-specific T cells. Moreover, multimeric MHC complexes are not only used for the analysis of antigen-specific T cells by flow cytometric analysis, but also to enhance or block specific immune responses *in vitro* (Kambayashi et al., 2001b) and *in vivo* (O'Herrin et al., 2001). One disadvantage of these molecules, however, is that the peptide is noncovalently bound to the MHC class I portion. Therefore, efficient loading cannot always be achieved and the loaded peptide might even dissociate from the complex. There are reports that the peptide can be linked to the  $\beta$ 2-microglobulin light chain (Denkberg et al., 2000). However, this still does not prevent the peptide– $\beta$ 2-microglobulin complex from dissociating off the MHC molecule, so we investigated an alternative approach to attach the peptide to the MHC complex.

Here, we show construction of a new MHC class I molecule with covalently bound class I peptide. A single-chain unit consists of peptide– $\beta$ 2-microglobulin and an MHC complex. This recombinant peptide– $\beta$ 2-microglobulin–HLA-A2 single-chain (SC) construct is recognized by peptide-specific T cells and can be successfully fused to a murine immunoglobulin sequence. When linked to an immunoglobulin scaffold to form a dimeric SC–Ig fusion, these fusion proteins bind antigen-specific T cells specifically and can be used as sensitive tools to identify and sort antigen-specific T cells in peripheral blood.

## 2. Materials and methods

### 2.1. Isolation of PBMC, cell lines, antibodies and media

PBMC were isolated from healthy volunteers by Ficoll gradient (Biochrom, Berlin). COS-7 cells were grown in DMEM (Gibco BRL) containing 10% FCS. J558L cells were grown in RPMI-1640 containing 10% FCS (Biochrom) or serum-free media HSFM (Gibco BRL) as previously described (Greten et al., 1998). T cell lines and clones were grown in RPMI-1640 plus 10% human AB serum in the presence of 10 U/ml IL-2. PA2.1 (ATCC) and BB7.2 (ATCC) were purified from hybridoma cell supernatants. Goat anti-mouse-Fc was obtained from Cappel, the anti-human  $\beta$ 2-microglobulin mAb (B1G6) was purchased from Biodesign Int., goat anti-mouse IgG1–PE from Caltag and anti-human CD8–FITC (UCHT-4) from Sigma.

### 2.2. Construction of fusion proteins

DNA encoding the full-length human peptide– $\beta$ 2-microglobulin–HLA-A2 sequence was synthesized by a multistep PCR using a human  $\beta$ 2-microglobulin cDNA plasmid (kindly provided by M. Soloski, Johns Hopkins University) and a  $\beta$ 2-microglobulin–HLA-A2 sequence (Pascolo et al., 1997) (kindly provided by Perarnau, Paris) as templates. We first synthesized the peptide– $\beta$ 2-microglobulin fusion sequence as previously described by White et al. (1999) containing a (GGGS)<sub>3</sub> spacer sequence between the peptide and the amino-terminal end of  $\beta$ 2-microglobulin (Fig. 1A). The sequence was introduced into the pcDNA 3.1 vector and verified by sequencing. For synthesis of the peptide– $\beta$ 2-microglobulin–HLA-A2 sequence, the peptide– $\beta$ 2-microglobulin sequence and the HLA-A2 sequence were fused by PCR and the final sequence was cloned into pcDNA3.1 (Fig. 1B). Finally, we subcloned the SC– $\beta$ 2-microglobulin–HLA-A2 ( $\alpha$ 1– $\alpha$ 3 region) into the previously described pXIg vector (Fig. 1C). Correct sequence of all constructs was verified by sequencing. The dimeric SC–pXIg plasmid was electroporated into J558L cells as previously described. High expressing clones were selected and grown under 800  $\mu$ g/ml G-418 in RPMI-1640 containing 10% FCS (Seromed, Germany) and adapted to grow in serum-free media (HSFM, Gibco,

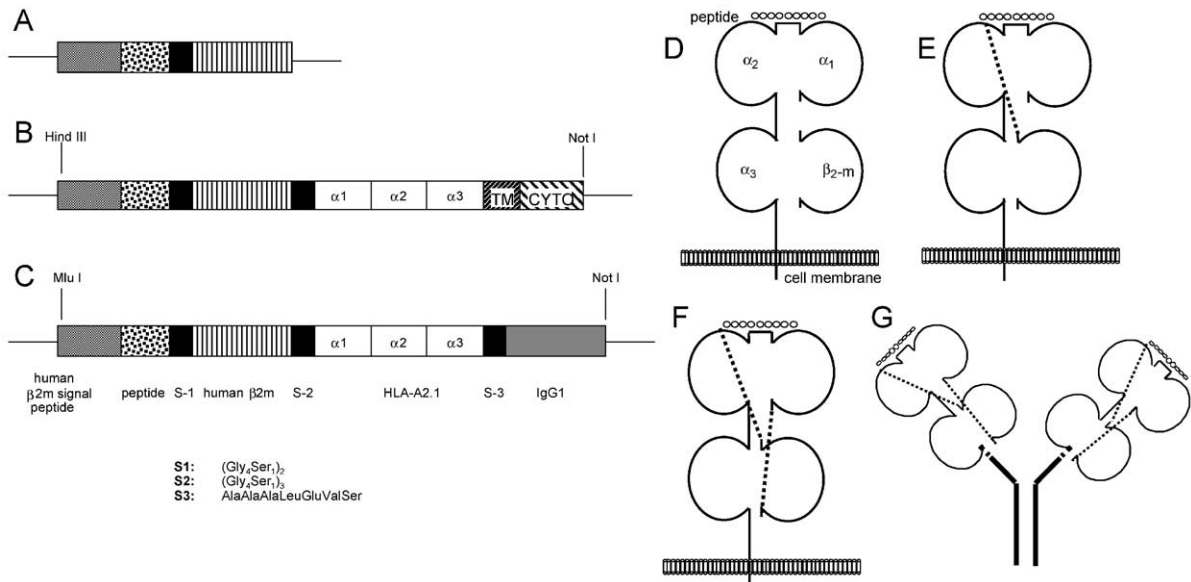


Fig. 1. Construction (A–C) and proposed conformation (D–G) of different peptide– $\beta$ 2-microglobulin–MHC constructs: (A) peptide– $\beta$ 2-microglobulin fusion construct with a (Gly<sub>4</sub>–Ser<sub>1</sub>)<sub>2</sub> spacer (S1) sequence between the peptide and the 5' end of  $\beta$ 2-microglobulin; (B) peptide– $\beta$ 2-microglobulin–MHC fusion construct including one spacer (S1) between peptide and  $\beta$ 2-microglobulin and a second spacer (S2) between  $\beta$ 2-microglobulin and the 5' end of the complete MHC heavy chain; (C) peptide– $\beta$ 2-microglobulin–MHC–Ig (SC-Ig) fusion construct including a third spacer sequence (S3) between the 3' end of the  $\alpha$ 3-chain of HLA-A2 and IgG heavy chain sequence. A scheme for the corresponding MHC constructs is shown on the right. D shows the schematic drawing of a membrane-anchored MHC class I molecule, the designed peptide– $\beta$ 2-microglobulin (as shown in A) co-expressed with MHC is shown in E. In F, a scheme of the peptide– $\beta$ 2-microglobulin–MHC (as shown in B) expressed on the cell surface is shown, and in G, the SC-Ig fusion construct (as shown in C) is drawn.

BRL) for purification. Approximately 0.5  $\mu$ g/ml of dimeric single-chain fusion protein was secreted into the cell supernatant.

### 2.3. Cytokine secretion assays

COS-7 cells were transiently transfected using DEAE dextran. After 48 h,  $1 \times 10^5$  transfected cells were added to  $1 \times 10^5$  T cells in a 96-well plate. GM-CSF release was measured from cell supernatant after 20 h of incubation at 37 °C using a commercially available ELISA (R&D). Peptide-pulsed T2 (ATCC) served as controls for specificity of the T cell lines and clones.

### 2.4. Detection of dimeric SC-IgG by ELISA

Cell supernatant from transfected J558L cells was concentrated using an Amicon concentration chamber and in some cases by affinity column purification using NIP-sepharose. For analysis of the protein by

ELISA, 96-well 1/2 area ELISA plates (Corning) were prepared as previously described and coated with a primary antibody as indicated. Antibody-bound dimeric protein was detected by an HRP-conjugated anti-mouse  $\lambda$ -light chain antibody (Southern Biotech). For some experiments, concentrated protein was further purified using an NIP-sepharose affinity column as previously described (Dal Porto et al., 1993).

### 2.5. Flow cytometric analysis and enrichment of antigen-specific T cells by MACS

For direct analysis, peptide-specific T cells were stained with dimeric SC-Ig followed by goat anti-mouse IgG1–PE and anti-human CD8–FITC. In some cases, peptide-specific T cells were further enriched using anti-PE magnetic beads (Miltenyi Biotech, Germany) according to the manufacturer's instructions. For purification of peptide-specific T cells from *in vitro* stimulated bulk cultures, PBMC

were stimulated *in vitro* once with influenza peptide (1  $\mu\text{g}/\text{ml}$ ) in the presence of 10 U/ml IL-2. After 8 days, the bulk culture was stained with M1-SC-Ig as described above. Influenza-specific T cells were enriched using anti-mouse IgG1 magnetic beads (Miltenyi Biotech) according to the manufacturer's instructions. In brief, the stained bulk culture was incubated with 20  $\mu\text{l}$  of anti-mouse IgG1 beads for 15 min at 10  $^{\circ}\text{C}$ , washed, and separated using a MiniMACS MS column (Miltenyi Biotech). Purified T cells were plated in 96-well plates and further stimulated in the presence of 0.25  $\mu\text{g}/\text{ml}$  PHA and 10 U/ml IL-2 without M1 peptide. Specificity of these clones was tested against peptide-pulsed T2 cells in a cytokine secretion assay after five stimulations.

### 3. Results

#### 3.1. Production of a peptide- $\beta$ 2-microglobulin-HLA-A2 fusion protein

Analysis of the three-dimensional structure of the human MHC class I molecule, HLA-A2 (Garboczi et al., 1992), demonstrated that the carboxy-terminal end of the peptide in the MHC class I binding groove is

close to amino-terminal end of  $\beta$ 2-microglobulin (approximately 21.8  $\text{\AA}$ ). While the ends of the MHC peptide in the binding grooves are close, a linker could extrude from the MHC complex and yet not block binding of the antigen-MHC complex with the TCR. In addition, there is also a short distance between the carboxy-terminal end of  $\beta$ 2-microglobulin and the amino-terminal end of the  $\alpha$ 1-chain of the HLA-A2 sequence, approximately 32  $\text{\AA}$ . Therefore, we developed a scheme to clone different peptide- $\beta$ 2-microglobulin-MHC complexes (Fig. 1). As a linker, a  $(\text{G}_3\text{-S}_1)_2$  sequence was placed between the peptide and  $\beta$ 2 microglobulin (Fig. 1A). A second linker  $(\text{G}_4\text{-S}_1)_3$  was introduced between the carboxy-terminal end of  $\beta$ 2-microglobulin and the  $\alpha$ 1 domain of HLA-A2 to generate the peptide- $\beta$ 2-microglobulin-HLA-A2 single-chain (SC) construct (Fig. 1C and F). Recognition of the different constructs by peptide-specific T cells was tested after transfection of the various constructs into COS cells alone or together with the HLA-A2 sequence (Fig. 1D-F). Two cell lines were used as target cells to test the specificity, one specific for Tax 11–19 (2G4) and one specific for influenza M1 peptide.

When COS cells were transfected with the peptide- $\beta$ 2-microglobulin constructs together with HLA-

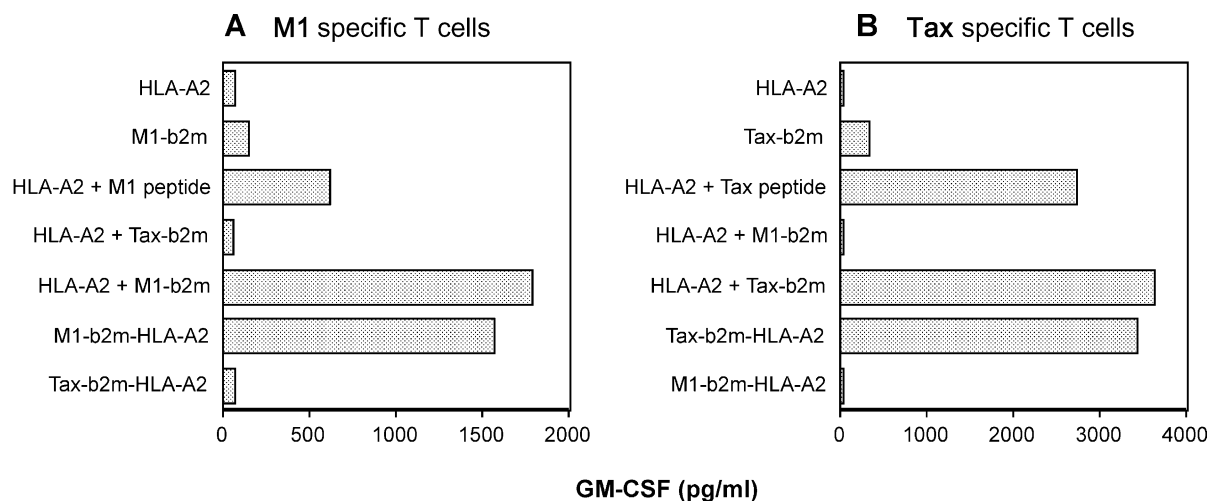


Fig. 2. Recognition of cognate peptide-MHC complexes on cell surface of COS cells. COS cells were transfected with equal amounts of plasmid as indicated on the y-axis and incubated with equal numbers of peptide-specific CD8<sup>+</sup> T lymphocytes (A: influenza M1-specific CD8 T cells; B: Tax<sub>11–19</sub>-specific T cells). GM-CSF was determined in the cell supernatant by ELISA. Both peptide-specific T cell lines recognized COS cells expressing HLA-A2 and the cognate peptide, HLA-A2 and the cognate peptide- $\beta$ 2-microglobulin sequence, as well as the cognate peptide- $\beta$ 2-microglobulin-MHC. T cells also responded to COS cells transfected with the peptide- $\beta$ 2-microglobulin in the absence of HLA-A2.

A2 (Fig. 1E), cognate T cells recognized the transfectants. Influenza-specific T cells secreted 1784 pg/ml GM-CSF in response to the corresponding peptide  $\beta$ 2-microglobulin construct and Tax-specific T cells released 3628 pg/ml of GM-CSF (Fig. 2). Therefore, T cells recognized peptide- $\beta$ 2-microglobulin constructs on the surface of HLA-A2 cotransfected antigen-presenting COS cells. Much less GM-CSF, 138 and 327 pg/ml, respectively, was detected when COS cells expressed the cognate peptide- $\beta$ 2-microglobulin sequence even in the absence of HLA-A2. This response is presumably due to cross-presentation by T cells, since they were the only source of HLA-A2 for presentation. Finally, the peptide- $\beta$ 2-microglobulin-HLA-A2 fusion (SC, Fig. 1F) DNA was transfected into COS cells. Expression of the SC constructs was confirmed by FACS analysis (data not shown). COS cells transfected with this single-chain construct were also used to stimulate peptide-specific T cells. Both M1- and Tax-specific T cells recognized the SC fusion constructs on the surface of COS cells and secreted 1536 and 3428 pg/ml GM-CSF, respectively (Fig. 2), indicating the correct conformation of the peptide- $\beta$ 2-microglobulin-HLA-A2 fusion on the cell surface of transfected cells. No construct containing an irrelevant peptide sequence induced significant GM-CSF release, while peptide-pulsed T2 cells stimulated the M1-specific T cells to release 612 pg/ml GM-CSF, or Tax-specific T cells to secrete 2729 pg/ml GM-CSF, respectively.

### 3.2. Production and characterization of the dimeric SC-Ig fusion protein

We next joined the peptide- $\beta$ 2-microglobulin-HLA-A2 ( $\alpha$ 1- $\alpha$ 3, extracellular domain) with an IgG1 sequence to generate a dimeric SC-Ig construct (Fig. 1C and G). This complex was expressed in J558L cells, and the cell supernatants were analyzed by ELISA. Antibodies specific for the Fc portion (Fig. 3A), HLA-A2 (Fig. 3B) and human  $\beta$ 2-microglobulin (Fig. 3B) bound the dimeric SC-IgG fusion protein indicating correct folding of the MHC and the presence of all subunits of the recombinant protein. SDS analysis of the purified protein revealed a distinct band at the expected molecular weight of about 98 kDa. The dimeric SC-Ig was clearly different from an HLA-A2-Ig fusion protein without the covalently

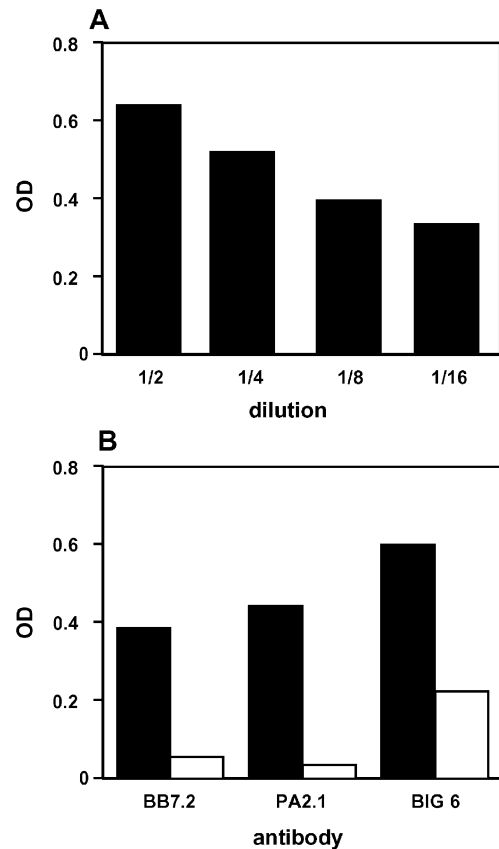


Fig. 3. Detection of chimeric SC-Ig fusion protein in supernatants of transfected cells. Ten microliters of supernatants from J558L cells transfected with M1-SC-IgG fusion proteins (black bars) and H-2K<sup>b</sup>-Ig was tested for reactivity with a goat anti-mouse immunoglobulin Fc-specific antibody (A), two HLA-A2-specific mAbs (BB7.2 and PA2.1) and a  $\beta$ 2-microglobulin (BIG6)-specific antibody (B). A murine dimeric MHC-Ig construct with a peptide and  $\beta$ 2-microglobulin was used as a negative control (white bars).

linked peptide- $\beta$ 2-microglobulin, which is approximately 15 kDa smaller (Fig. 4). Western blot analysis of the dimeric SC-Ig protein and the HLA-A2-Ig protein also demonstrated the difference in size of the two dimeric proteins with and without the peptide- $\beta$ 2-microglobulin sequence (Fig. 4).

### 3.3. Dimeric SC-Ig fusion proteins detect low frequencies of antigen-specific T cells

Multivalent MHC proteins have become valuable tools for direct ex vivo analysis of antigen-specific T

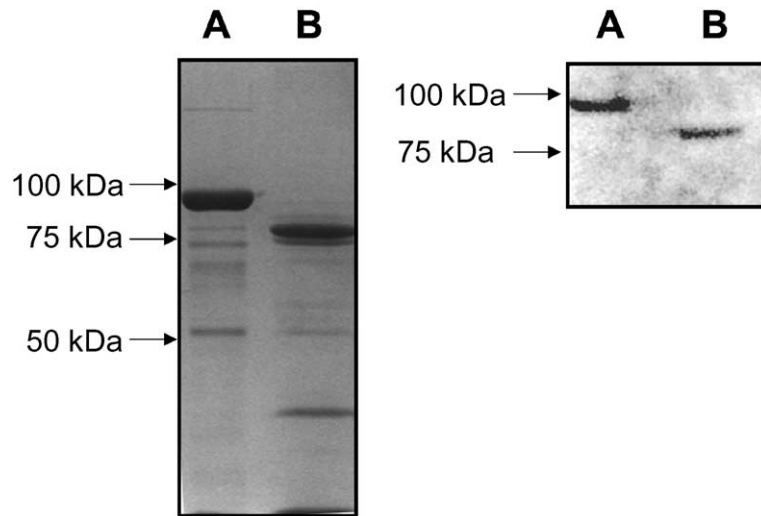


Fig. 4. Molecular mass analysis of purified SC-Ig fusion proteins (A) and HLA-A2-Ig (B) by SDS gel electrophoresis (left) and Western blot (right) using anti-mouse IgG to detect the dimeric fusion constructs. SC-Ig has the expected molecular mass of approximately 98 kDa and is approximately 15 kDa heavier than HLA-A2-Ig (B).

cell responses. Therefore, different SC-Ig proteins were generated and tested for their ability to stain peptide-specific T cells. The M1-SC-Ig protein was first tested on a V- $\beta$ 17-positive M1-specific T cell clone. M1-SC-Ig protein bound the M1-specific T cell clone specifically and as efficiently as an antibody specific for the V- $\beta$  chain of the T cell receptor. When compared to the peptide-loaded HLA-A2-Ig, the M1-SC-Ig was far more efficient in staining antigen-specific T cells than passively loaded HLA-A2-

Ig (Fig. 5). Other dimeric SC proteins specific for HTLV-1 and hepatitis showed similar results when tested on T cell bulk cultures (data not shown). CMV- or influenza-specific CD8<sup>+</sup> T cells were identified using dimeric SC-Ig constructs directly from blood without further in vitro stimulation. Fig. 6 shows a representative result of our staining results from two different HLA-A2-positive individuals (one CMV seropositive, one CMV seronegative) and an HLA-A2-negative individual by FACS analysis. Of the

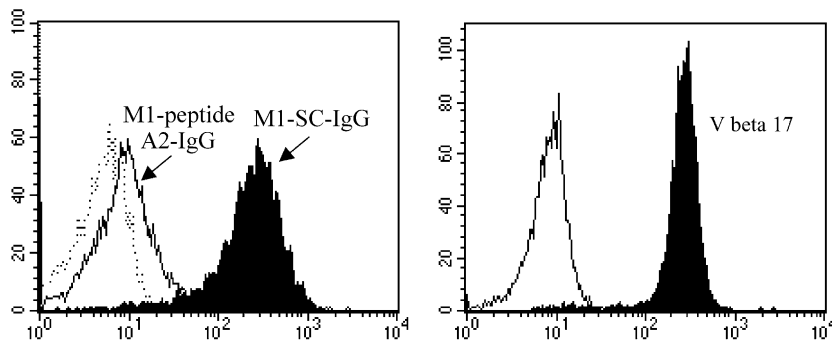


Fig. 5. Dimeric M1-SC-Ig binds stably influenza M1-specific T cell clone. The M1 peptide-specific T cell clone was stained for V $\beta$  17 (right). Three micrograms peptide-loaded HLA-A2-Ig binds M1-specific T cells; however, staining the same T cells with 300 ng M1-SC-Ig results in a more efficient separation. HLA-A2-Ig was passively loaded with a 330-fold excess of peptide which still did not lead to a complete loading of all MHC molecules with the peptide of interest resulting in a less bright staining result.

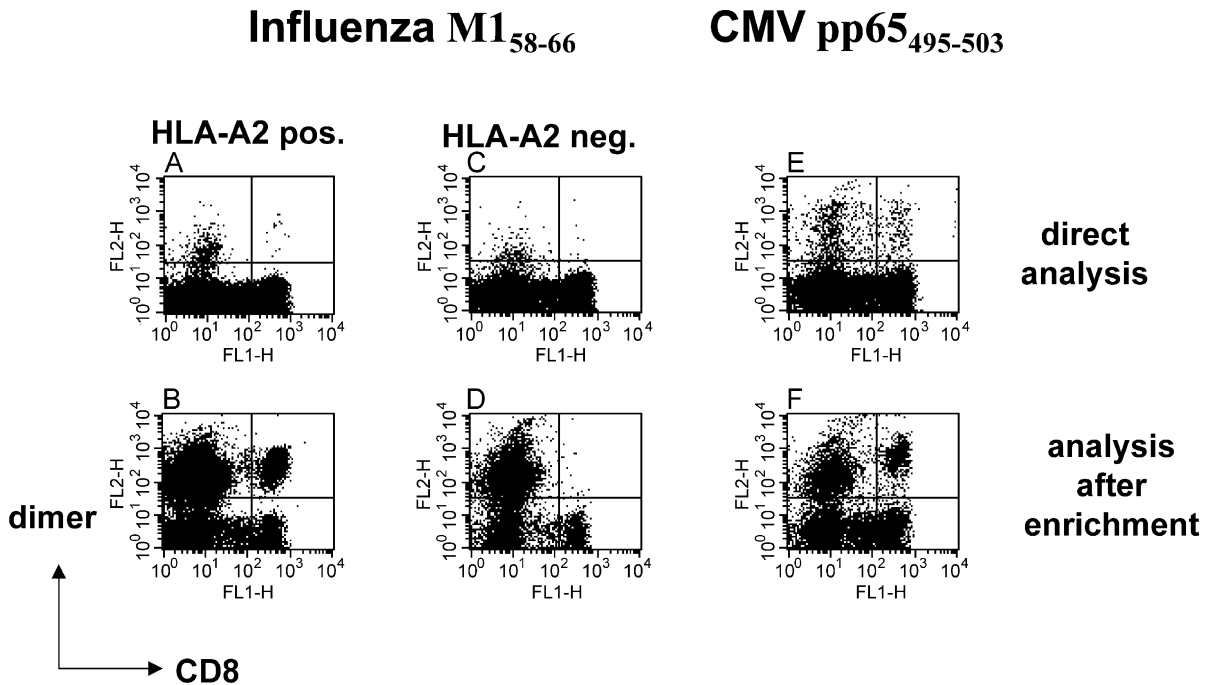


Fig. 6. Peptide–SC-Ig can be used to detect antigen-specific CD8<sup>+</sup> T cells from peripheral blood. Antigen-specific T cells were detected using M1–SC-Ig and CMV–SC-Ig and anti-IgG1–PE in parallel with anti-CD8–FITC (top). PBMC from an HLA-A2-positive donor and an HLA-A2-negative donor were stained using M1–SC-Ig (A and C), and CMV–SC-Ig was used to stain CMV–specific T cells (E). Using magnetic anti-PE beads, peptide-specific T cells were enriched and visualized after enrichment (bottom). Before enrichment, background staining was below 0.05%. One percent of the PBMC from an HLA-A2-positive, CMV antibody-positive donor were stained positive with the CMV–SC-Ig and CD8 (E). After enrichment, peptide-specific populations were found with both dimeric constructs in HLA-A2-positive donors only (B and F).

CD8<sup>+</sup> T cells, 3.8% stained positive directly out of blood using the CMV–SC-Ig fusion protein for FACS analysis. Although we were not able to identify M1-specific T cells directly from blood, we were able to enhance our staining results using a magnetic column enrichment procedure (Miltenyi Biotec). This system allows for enrichment of antibody-bound cells to levels that are detectable by FACS analysis. In individuals with low precursor frequency, the frequency of cells can be calculated back from the enriched fraction, as long as the yield from the enrichment procedure is known. Multiple determinations were done to assess the sample-to-sample variability in yield, which are taken into account when calculating an enrichment factor (data not shown). We calculated the yield from the actual number of double-positive cells after the column (in individuals where precursor frequency of antigen-specific cells was detectable by FACS, before and after the enrichment) and compared it to the expected number of cells if the yield was

100%. Thus, by knowing the yield, starting number of cells before and after enrichment and the number of antigen-specific T cells after enrichment, one can calculate back from the enriched fraction to the original frequency of antigen-specific T cells. A positive control with detectable frequency before and after enrichment is used in all experiments to test yield and enrichment. As shown in Fig. 6, after enrichment, we found 7.8% M1-positive CD8<sup>+</sup> T cells, which corresponds to approximately 0.18% of all PBMC before enrichment. This population was not found in HLA-A2-negative donors demonstrating the specificity of our assay. We also compared our staining results with an alternate method for the detection of peptide-specific T cells, a cytokine secretion-based assay for IFN- $\gamma$  (Brosterhus et al., 1999). As shown by this assay, about 0.1% of the PBMC were specific for M1 peptide with a background staining of less than 0.02% using an irrelevant peptide (data not shown). Therefore, this assay correlated very well with the

FACS results obtained by using the dimeric MHC molecule.

### 3.4. Use of SC-Ig fusion proteins to generate peptide-specific T cell lines

Currently different protocols are being used to isolate and amplify antigen-specific T cells for adoptive T cell therapy purposes (Yee et al., 1999; Becker et al., 2001). However, until today, the generation of high enough numbers of antigen-specific T cells in vitro is still a major obstacle in many therapeutic settings. We therefore isolated peptide-specific CD8<sup>+</sup> T cells from a bulk culture using the M1–SC-Ig fusion protein after one in vitro stimulation of PBMC with the influenza M1 peptide. After one in vitro stimulation, 15% of the analyzed CD8<sup>+</sup> T cells were specific for the influenza M1 peptide (Fig. 7A). Using a magnetically labeled anti-IgG1 antibody together with the M1–SC-Ig molecule, we were able to enrich influenza M1-specific T cells from the bulk culture (Fig. 7B). As analyzed by FACS, 79.33% of the cells were specific after enrichment. These cells were

cloned into 96 wells and restimulated using PHA in the absence of the specific antigenic peptide. A number of clones were randomly chosen and tested for their specificity in a cytokine secretion assay using peptide-pulsed T2 cells as targets. Eight of these clones showed specificity for the M1 peptide (Fig. 7C). Therefore, the SC-Ig molecule is a valuable reagent for isolation of antigen-specific CD8<sup>+</sup> T cells from mixed populations.

## 4. Discussion

MHC–TCR interactions take a central role in cell-mediated immune recognition. The understanding of the three-dimensional molecular structure of MHC molecules extends not only our current knowledge of structural biology of MHC, peptide and TCR, but it also helps to develop approaches to understand normal and pathologic immune responses. Here, we show the generation of a novel MHC molecule that has both the peptide and  $\beta$ 2-microglobulin covalently linked and is recognized by antigen-specific T cells when

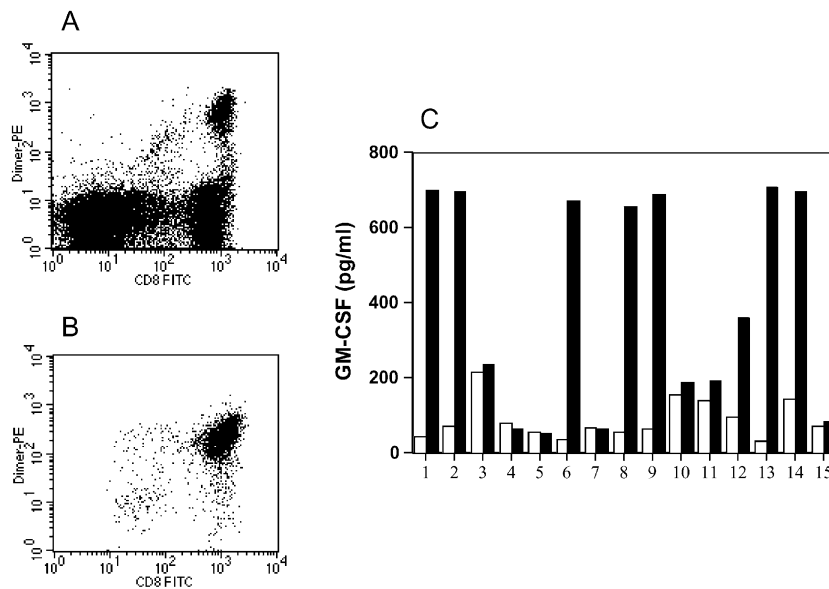


Fig. 7. Peptide-specific T cell clones can be generated from M1–SC-Ig purified T cells by nonspecific stimulation with PHA. PBMC were stimulated with M1 peptide. Peptide-specific T cells were isolated using magnetic anti-IgG1 beads and T cell bulk cultures were analyzed before (A) and after (B) purification by FACS. Purified T cells were seeded in 96-well plates and stimulated with PHA. After five stimulations, T cell clones were tested for specificity in a cytokine secretion assay using peptide-pulsed T2 cells as targets. Eight of the analyzed clones responded specifically to the M1 peptide (C).

expressed on the cell surface. Moreover, dimeric MHC complexes with covalently linked peptide and  $\beta$ 2-microglobulin were generated by joining these MHC complexes with a murine immunoglobulin sequence. These SC-IgG constructs not only can be used for identification of antigen-specific T cells but are also potentially useful tools to direct specific T cell responses *in vivo*.

A number of approaches have been described to engineer MHC molecules and link the peptide, the  $\beta$ 2-microglobulin light chain and the MHC heavy chain together. Based on the crystal structure of peptide-loaded HLA-A2, we decided to fuse the peptide to the amino-terminal end of  $\beta$ 2-microglobulin. This approach has been successfully tried with the murine MHC class I molecules D<sup>d</sup> (White et al., 1999), D<sup>b</sup>, K<sup>d</sup> (Uger and Barber, 1997, 1998; Uger et al., 1999) and the human HLA-A2 molecule with influenza matrix epitope (Tafuro et al., 2001), which can be recognized by T cells, when expressed by COS cells together with HLA-A2. We were able to confirm these results in our experiments. In addition, we also found that peptide- $\beta$ 2-microglobulin complexes were recognized by T cells in the absence of HLA-A2 on the surface of the APC. It is likely that the peptide- $\beta$ 2-microglobulin exchanged onto the HLA-A2 on T cells, since these were the only source of HLA-A2 in the culture. Therefore, we thought of an alternative method to link the peptide to the MHC molecule in order to improve the stability and loading efficiency of the MHC with a peptide of interest.

We decided to link the peptide- $\beta$ 2-microglobulin complex to the HLA-A2 heavy chain resulting in a single-chain (SC) MHC complex. A number of groups have been able to link the  $\beta$ 2-microglobulin light chain to the  $\alpha$ 1 chain of the MHC class I heavy chain (Mage et al., 1992; Lee et al., 1994, 1996; Toshitani et al., 1996; Pascolo et al., 1997) or the  $\alpha$ 3 domain (Mottez et al., 1991). However, to our knowledge, this is the first successful attempt to join the entire peptide- $\beta$ 2-microglobulin sequence to a human MHC heavy chain. Recently, expression and characterization of a similar murine SC trimer has been reported (Yu et al., 2002). Interestingly a slightly different approach failed to produce functional MHC complexes, when the peptide was linked to the amino terminus of the MHC and the  $\beta$ 2-microglobulin sequence was fused to the carboxy-terminal end of

the MHC (Sylvester-Hvid et al., 1999). It has also been reported that T cells recognize MHC molecules with a peptide linked to the amino terminus of the MHC heavy chain (Mottez et al., 1995; Kang et al., 1997). We also cloned analog constructs with the peptide directly bound to the alpha chain of the MHC. However, in our experiments, these MHC complexes could not bind stably to antigen-specific T cells (data not shown). Analysis of the three-dimensional structure of HLA-A2 demonstrates moreover that a linker between the carboxy-terminal end of the peptide and the amino-terminal end would possibly block the binding surface of the peptide with the TCR.

Soluble MHC molecules have gained a lot of attention in the past few years. Peptide-loaded multimeric MHC complexes can be used for visualization of antigen-specific T cells (McMichael and O'Callaghan, 1998; Greten and Schneck, 2002), to study  $\alpha/\beta$  TCR affinities (Kambayashi et al., 2001a) and to attenuate or induce immune responses *in vitro* and *in vivo* (Howard et al., 1999; O'Herrin et al., 2001). Different protocols have been described to generate multimeric MHC complexes. While tetrameric MHC complexes have been extensively studied for the analysis of TCR repertoires, their production is still very cumbersome. We have utilized a different approach to stain antigen-specific T cells using MHC-Ig fusion proteins (Greten et al., 1998). These proteins are expressed in eukaryotic cells and the peptide of interest is loaded by passive exchange. However, due to the dissociation of the peptide, the dimeric HLA-A2-Ig protein could not be efficiently loaded. This has impaired the quality of the reagent, which depends on a high occupancy of the MHC peptide binding groove with the peptide of interest. Therefore, we decided to join the complete peptide- $\beta$ 2-microglobulin-MHC single-chain sequence with the Ig sequence creating dimeric SC-Ig constructs. Biochemical and immunological analysis of the new construct demonstrated correct folding and the expected size of the molecule. Most importantly, it can bind specifically to peptide-specific CD8<sup>+</sup> T cells. Although the aim of this study was not to determine precursor frequencies of peptide-specific T cells from HLA-A2-positive donors, we could demonstrate that SC-Ig molecules identify rare peptide-specific T cells directly in peripheral blood. Similar results were obtained in a cytokine secretion-based assay (Bros-

terhus et al., 1999) when compared to FACS analysis using SC-Ig.

Until today, amplifying rare T cells from peripheral blood to high enough numbers so that they can be used for adoptive T cell therapies has been a very difficult task. Using the SC-Ig molecule together with anti-IgG beads, we were able to enrich for peptide-specific T cells from an in vitro generated T cell bulk culture. Isolated antigen-specific T cells were then successfully amplified using a potent stimulator such as PHA. Finally, the here described SC-Ig molecules might be very valuable tools to direct immune responses in vivo. Previous in vitro studies using peptide loaded dimeric MHC–Ig constructs suggest that they efficiently block antigen-specific T cells (O'Herrin et al., 2001). Because the peptide is covalently linked to the MHC complex, it cannot dissociate from the MHC, which will allow to use these molecules now in vivo.

In conclusion, we have engineered a single-chain HLA-A2 molecule, which is covalently bound to  $\beta$ 2-microglobulin and the peptide, each separated by a small glycine serine spacer. These peptide– $\beta$ 2-microglobulin MHC sequences are recognized by antigen-specific T cells and can be used to generate soluble MHC molecules. We have also fused single-chain MHC molecules to the IgG sequence creating dimeric single-chain MHC molecules. These molecules bind stably to antigen-specific CD8+ T cells and can be used to identify T cells directly from peripheral blood. Finally, preliminary data suggest that even more importantly, dimeric SC fusion molecules can be used to direct immune responses in vitro. This opens the possibility of future experiments using these molecules also in vivo.

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### References

- Altman, J.D., Moss, P., Goulder, P., Barouch, D.H., McHeyzer, W.M., Bell, J.I., McMichael, A.J., Davis, M.M., 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274, 94–96.
- Becker, C., Pohla, H., Frankenberger, B., Schuler, T., Assenmacher, M., Schendel, D.J., Blankenstein, T., 2001. Adoptive tumor therapy with T lymphocytes enriched through an IFN-gamma capture assay. *Nat. Med.* 7, 1159–1162.
- Brosterhus, H., Brings, S., Leyendeckers, H., Manz, R.A., Miltenyi, S., Radbruch, A., Assenmacher, M., Schmitz, J., 1999. Enrichment and detection of live antigen-specific CD4(+) and CD8(+) T cells based on cytokine secretion. *Eur. J. Immunol.* 29, 4053–4059.
- Carruth, L.M., Greten, T.F., Murray, C.E., Castro, M.G., Crone, S.N., Pavlat, M., Schneck, J.P., Silicano, R.F., 1999. An algorithm for evaluating human cytolytic T lymphocyte responses to candidate AIDS vaccines. *AIDS Res. Hum. Retrovir.* 15, 1021–1034.
- Dal Porto, J., Johansen, T.E., Catipovic, B., Parfiit, D.J., Tuveson, D., Gether, U., Kozlowski, S., Fearon, D.T., Schneck, J.P., 1993. A soluble divalent class I major histocompatibility complex molecule inhibits alloreactive T cells at nanomolar concentrations. *Proc. Natl. Acad. Sci. U. S. A.* 90, 6671–6675.
- Denkberg, G., Cohen, C.J., Segal, D., Kirkin, A.F., Reiter, Y., 2000. Recombinant human single-chain MHC–peptide complexes made from *E. coli* by in vitro refolding: functional single-chain MHC–peptide complexes and tetramers with tumor associated antigens. *Eur. J. Immunol.* 30, 3522–3532.
- Fahmy, T.M., Bieler, J.G., Edidin, M., Schneck, J.P., 2001. Increased TCR avidity after T cell activation: a mechanism for sensing low-density antigen. *Immunity* 14, 135–143.
- Garboczi, D.N., Hung, D.T., Wiley, D.C., 1992. HLA-A2–peptide complexes: refolding and crystallization of molecules expressed in *Escherichia coli* and complexed with single antigenic peptides. *Proc. Natl. Acad. Sci. U. S. A.* 89, 3429–3433.
- Greten, T.F., Schneck, J.P., 2002. The development and use of multimeric MHC molecules. *Clin. Diagn. Lab. Immunol.* 9, 216–220.
- Greten, T.F., Slansky, J.E., Kubota, R., Soldan, S.S., Jaffee, E.M., Leist, T.P., Pardoll, D.M., Jacobson, S., Schneck, J.P., 1998. Direct visualization of antigen-specific T cells: HTLV-1 Tax11–19-specific CD8(+) T cells are activated in peripheral

- blood and accumulate in cerebrospinal fluid from HAM/TSP patients. *Proc. Natl. Acad. Sci. U. S. A.* 95, 7568–7573.
- Howard, M.C., Spack, E.G., Choudhury, K., Greten, T.F., Schneck, J.P., 1999. MHC-based diagnostics and therapeutics—clinical applications for disease-linked genes. *Immunol. Today* 20, 161–165.
- Kambayashi, T., Assarsson, E., Chambers, B.J., Ljunggren, H.G., 2001a. IL-2 down-regulates the expression of TCR and TCR-associated surface molecules on CD8(+) T cells. *Eur. J. Immunol.* 31, 3248–3254.
- Kambayashi, T., Ichihara, F., Jensen, P.E., Ljunggren, H.G., 2001b. Soluble MHC class I molecules induce cellular death in a CD8+ T-cell hybridoma tumor model. *Int. J. Cancer* 91, 373–378.
- Kang, X., Robbins, P.F., Fitzgerald, E.B., Wang, R., Rosenberg, S.A., Kawakami, Y., 1997. Induction of melanoma reactive T cells by stimulator cells expressing melanoma epitope–major histocompatibility complex class I fusion proteins. *Cancer Res.* 57, 202–205.
- Lebowitz, M.S., O’Herrin, S.M., Hamad, A.R., Fahmy, T., Marguet, D., Barnes, N.C., Pardoll, D., Bieler, J.G., Schneck, J.P., 1999. Soluble, high-affinity dimers of T-cell receptors and class II major histocompatibility complexes: biochemical probes for analysis and modulation of immune responses. *Cell. Immunol.* 192, 175–184.
- Lee, L., McHugh, L., Ribaldo, R.K., Kozlowski, S., Margulies, D.H., Mage, M.G., 1994. Functional cell surface expression by a recombinant single-chain class I major histocompatibility complex molecule with a *cis*-active beta 2-microglobulin domain. *Eur. J. Immunol.* 24, 2633–2639.
- Lee, L., Loftus, D., Appella, E., Margulies, D.H., Mage, M., 1996. A recombinant single-chain HLA-A2.1 molecule, with a *cis* active beta-2-microglobulin domain, is biologically active in peptide binding and antigen presentation. *Hum. Immunol.* 49, 28–37.
- Madden, D.R., 1995. The three-dimensional structure of peptide–MHC complexes. *Annu. Rev. Immunol.* 13, 587–622.
- Mage, M.G., Lee, L., Ribaldo, R.K., Corr, M., Kozlowski, S., McHugh, L., Margulies, D.H., 1992. A recombinant, soluble, single-chain class I major histocompatibility complex molecule with biological activity. *Proc. Natl. Acad. Sci. U. S. A.* 89, 10658–10662.
- McMichael, A.J., Kelleher, A., 1999. The arrival of HLA class II tetramers. *J. Clin. Invest.* 104, 1669–1670.
- McMichael, A.J., O’Callaghan, C.A., 1998. A new look at T cells. *J. Exp. Med.* 187, 1367–1371.
- Mottez, E., Jaulin, C., Godeau, F., Choppin, J., Levy, J.P., Kourilsky, P., 1991. A single-chain murine class I major transplantation antigen. *Eur. J. Immunol.* 21, 467–471.
- Mottez, E., Langlade-Demoyen, P., Gournier, H., Martinon, F., Maranski, J., Kourilsky, P., Abastado, J.P., 1995. Cells expressing a major histocompatibility complex class I molecule with a single covalently bound peptide are highly immunogenic. *J. Exp. Med.* 181, 493–502.
- O’Herrin, S.M., Slansky, J.E., Tang, Q., Markiewicz, M.A., Gajewski, T.F., Pardoll, D.M., Schneck, J.P., Bluestone, J.A., 2001. Antigen-specific blockade of T cells *in vivo* using dimeric MHC peptide. *J. Immunol.* 167, 2555–2560.
- Pascolo, S., Bervas, N., Ure, J.M., Smith, A.G., Lemonnier, F.A., Perarnau, B., 1997. HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. *J. Exp. Med.* 185, 2043–2051.
- Schneck, J.P., Slansky, J., O’Herrin, S., Greten, T.F., 1999. Use of MHC–Ig dimers for visualizing antigen specific T cells. *Current Protocol in Immunology*. Wiley, New York, p. 17.3.
- Selin, L.K., Lin, M.Y., Kraemer, K.A., Pardoll, D.M., Schneck, J.P., Varga, S.M., Santolucito, P.A., Pinto, A.K., Welsh, R.M., 1999. Attrition of T cell memory: selective loss of LCMV epitope-specific memory CD8 T cells following infections with heterologous viruses. *Immunity* 11, 733–742.
- Slansky, J.E., Rattis, F.M., Boyd, L.F., Fahmy, T., Jaffee, E.M., Schneck, J.P., Margulies, D.H., Pardoll, D.M., 2000. Enhanced antigen-specific antitumor immunity with altered peptide ligands that stabilize the MHC–peptide–TCR complex. *Immunity* 13, 529–538.
- Sylvester-Hvid, C., Nielsen, L.L., Hansen, N.J., Pedersen, L.O., Buus, S., 1999. A single-chain fusion molecule consisting of peptide, major histocompatibility gene complex class I heavy chain and beta2-microglobulin can fold partially correctly, but binds peptide inefficiently. *Scand. J. Immunol.* 50, 355–362.
- Tafuro, S., Meier, U.C., Dunbar, P.R., Jones, E.Y., Layton, G.T., Hunter, M.G., Bell, J.I., McMichael, A.J., 2001. Reconstitution of antigen presentation in HLA class I-negative cancer cells with peptide-beta2m fusion molecules. *Eur. J. Immunol.* 31, 440–449.
- Toshitani, K., Braud, V., Browning, M.J., Murray, N., McMichael, A.J., Bodmer, W.F., 1996. Expression of a single-chain HLA class I molecule in a human cell line: presentation of exogenous peptide and processed antigen to cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* 93, 236–240.
- Uger, R.A., Barber, B.H., 1997. Presentation of an influenza nucleoprotein epitope incorporated into the H-2Db signal sequence requires the transporter-associated with antigen presentation. *J. Immunol.* 158, 685–692.
- Uger, R.A., Barber, B.H., 1998. Creating CTL targets with epitope-linked beta 2-microglobulin constructs. *J. Immunol.* 160, 1598–1605.
- Uger, R.A., Chan, S.M., Barber, B.H., 1999. Covalent linkage to beta2-microglobulin enhances the MHC stability and antigenicity of suboptimal CTL epitopes. *J. Immunol.* 162, 6024–6028.
- White, J., Crawford, F., Fremont, D., Marrack, P., Kappler, J., 1999. Soluble class I MHC with beta2-microglobulin covalently linked peptides: specific binding to a T cell hybridoma. *J. Immunol.* 162, 2671–2676.
- Yee, C., Savage, P.A., Lee, P.P., Davis, M.M., Greenberg, P.D., 1999. Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide–MHC tetramers. *J. Immunol.* 162, 2227–2234.
- York, I.A., Rock, K.L., 1996. Antigen processing and presentation by the class I major histocompatibility complex. *Annu. Rev. Immunol.* 14, 369–396.
- Yu, Y.Y., Netuschil, N., Lybarger, L., Connolly, J.M., Hansen, T.H., 2002. Cutting edge: single-chain trimers of MHC class I molecules form stable structures that potentially stimulate antigen-specific T cells and B cells. *J. Immunol.* 168, 3145–3149.