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### Impact of Antigen Presentation on TCR Modulation and Cytokine Release: Implications for Detection and Sorting of Antigen-Specific CD8<sup>+</sup> T Cells Using HLA-A2 Wild-Type or HLA-A2 Mutant Tetrameric Complexes

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# Impact of Antigen Presentation on TCR Modulation and Cytokine Release: Implications for Detection and Sorting of Antigen-Specific CD8<sup>+</sup> T Cells Using HLA-A2 Wild-Type or HLA-A2 Mutant Tetrameric Complexes<sup>1</sup>

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Soluble MHC class I molecules loaded with antigenic peptides are available either to detect and to enumerate or, alternatively, to sort and expand MHC class I-restricted and peptide-reactive T cells. A defined number of MHC class I/peptide complexes can now be implemented to measure T cell responses induced upon Ag-specific stimulation, including CD3/CD8/ $\zeta$ -chain down-regulation, pattern, and quantity of cytokine secretion. As a paradigm, we analyzed the reactivity of a Melan-A/MART-1-specific and HLA-A2-restricted CD8<sup>+</sup> T cell clone to either soluble or solid-phase presented peptides, including the naturally processed and presented Melan-A/MART-1 peptide AAGIGILTV or the peptide analog ELAGIGILTV presented either by the HLA-A2 wild-type (wt) or mutant (alanine→valine aa 245) MHC class I molecule, which reduces engagement of the CD8 molecule with the HLA-A2 heavy chain. Soluble MHC class I complexes were used as either monomeric or tetrameric complexes. Soluble monomeric MHC class I complexes, loaded with the Melan-A/MART-1 peptide, resulted in CD3/CD8 and TCR  $\zeta$ -chain down-regulation, but did not induce measurable cytokine release. In general, differences pertaining to CD3/CD8/ $\zeta$ -chain regulation and cytokine release, including IL-2, IFN- $\gamma$ , and GM-CSF, were associated with 1) the format of Ag presentation (monomeric vs tetrameric MHC class I complexes), 2) wt vs mutant HLA-A2 molecules, and 3) the target Ag (wt vs analog peptide). These differences are to be considered if T cells are exposed to recombinant MHC class I Ags loaded with peptides implemented for detection, activation, or sorting of Ag-specific T cells. *The Journal of Immunology*, 2002, 168: 2766–2772.

The advent of soluble MHC class I complexes loaded with antigenic peptides has opened new perspectives for the analysis of T cell biology (1). Tetrameric class I complexes can be used to 1) enumerate Ag-specific T cells without the need of ex vivo expansion (2), 2) combine the detection of Ag-specific T cells with T cell marker analysis and define immune effector functions by intracellular cytokine staining (3), 3) sort Ag-specific T cells (4), 4) expand immune effector cells if the appropriate MHC class I molecule is attached to a solid surface combined with a costimulatory molecule (5), or 5) detect MHC-restricted and Ag-specific T cells in situ (6).

One of the major advantages of soluble MHC class I molecules is that a defined amount of Ag can be used to stimulate or to sort Ag-specific T cells. Earlier studies showed that the amount of Ag and the exposure time of T cells to their nominal target Ag critically influence the quality and the quantity of cytokines released after the interaction of the TCR with the MHC/peptide complex

(7). In addition, the encounter of T cells with the target epitope leads to TCR down-regulation, which represents a pivotal event in T cell activation (8). In general, CD3  $\epsilon$  and the T cell coreceptor CD4 or CD8 are down-regulated in a parallel fashion (9). The same is true for the TCR  $\zeta$ -chain, which is promptly degraded after TCR triggering (10). Some authors postulated that the magnitude of TCR down-regulation is correlated with T cell effector functions (11). Others suggested that TCR engagement and down-regulation may not be associated directly with T cell responses (12). Thus, events associated with TCR engagement and ultimately TCR down-regulation are not only of interest in the context of T cell biology, but also in the experimental and clinical application of tetramer-guided sorting of Ag-specific T cells.

We have examined CD3/CD8 and TCR  $\zeta$ -chain down-regulation, implementing the Ag-specific CD8<sup>+</sup> T cell clone SK-Mel-29.1.1, which is directed against the HLA-A2-restricted and melanoma-associated differentiation Ag Melan-A/MART-1 (AAGIGILTV) (13). This T cell clone appears to be associated with immunosurveillance and protection from tumor recurrence in the patient SK29 (14). Soluble as well as solid-phase bound monomeric and tetrameric HLA-A2 complexes were loaded with the naturally processed and presented Melan-A/MART-1 Ag AAGIGILTV (15), or the peptide analog ELAGIGILTV, which has been reported to induce enhanced immune effector functions in anti-Melan-A/MART-1-directed T cells (16). HLA-A2 molecules were used as the wild-type (wt)<sup>3</sup> HLA-A2.1 molecule or, alternatively,

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<sup>3</sup> Abbreviations used in this paper: wt, wild type; ITAM, immunoreceptor tyrosine-based activation motif; m, mutant.

as an HLA-A2 mutant protein, which reduces nonspecific background binding of nonspecific T cells due to an altered interaction of the CD8 molecule with the HLA-A2  $\alpha 3$  domain (17). We tested whether these reagents affect CD3/CD8/ $\zeta$ -chain modulation and T cell immune effector functions defined by cytolysis and cytokine release.

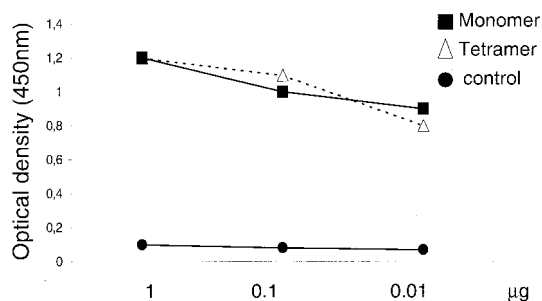
## Materials and Methods

### Tetramer complexes

HLA-A2 wt or HLA-A2 mutant tetramer complexes loaded either with AAGIGILTV or ELAGIGILTV were prepared as described in detail recently (17). Mutated (alanine $\rightarrow$ valine at position 245, termed HLA-A2 m) HLA-A2 tetrameric complexes have been shown to reduce the background staining of nonspecific CD8-binding T cells by affecting the interaction of the CD8 molecule with the MHC class I heavy chain. Substitution at position 245, located on the fifth  $\beta$ -pleated sheet, may either directly affect the interaction of the TCR/CD8 with MHC class I, or it may alter the MHC class I conformation at a distant site, i.e., the major CD8 contact site that includes position 227 in the HLA-A2 molecule (18). T cells were either exposed to monomeric or tetrameric HLA-A2 complexes in solution or, alternatively, attached to a solid surface. The anti- $\beta_2$ -microglobulin mAb clone B1G3 (IgG2a) obtained from Beckman Coulter (Krefeld, Germany) was dissolved at 2  $\mu\text{g}/\text{ml}$  in PBS, and 50  $\mu\text{l}$  of this solution was used per well of a 96-well microtiter plate (Maxisorb; Nunc, Wiesbaden, Germany) and incubated for 2 h at 37°C. Plates were washed twice with PBS before adding either monomeric or tetrameric class I complexes, as indicated in individual experiments.

To ensure that an equal number of MHC/peptide complexes, composed of either monomers or tetramers, are presented to CTL, the anti- $\beta_2$  mAb was attached to 96-well plates, as described above, followed by incubation of monomer or tetramer MHC/peptide complexes either at 1, 0.1, or 0.01  $\mu\text{g}$  MHC/peptide equivalent per well for 1 h at room temperature and washed twice with PBS, followed by incubation with the biotin-conjugated mAb w6/32 (mIgG2A; Leinco Technologies, Ballwin, MO), 50  $\mu\text{l}/\text{well}$ , a 1/400 dilution of a 0.1 mg/ml concentration for 30 min, followed by two washing steps with PBS/0.005% Tween. This mAb defines a monomorphic determinant on MHC molecules that is dependent on the correctly folded MHC heavy chain/peptide/ $\beta_2$ -microglobulin complex (19). A total of 50  $\mu\text{l}$  avidin-peroxidase was added for 1 h at room temperature, washed four times with PBS-Tween, and developed with tetramethylbenzidine. The reaction was terminated using 50  $\mu\text{l}$  1.8 N  $\text{H}_2\text{SO}_4$ , and the OD was determined at 450 nm (Fig. 1). The titration of monomer or tetramer complexes attached to plastic surfaces yielded a similar number of MHC/peptide complexes, as defined by the mAb w6/32.

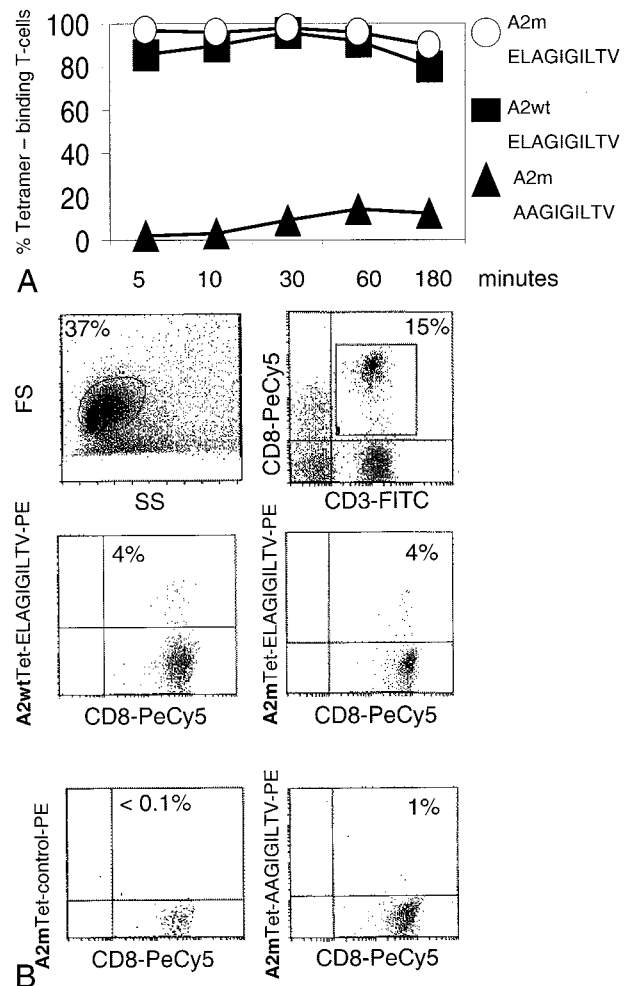
Plates were incubated for 1 h at room temperature and washed once with PBS before adding the T cell clone SK-Mel-29.1.1 in RPMI supplemented with 10% FCS, glutamine, and streptomycin/penicillin (complete medium; obtained from Life Technologies, Eggenstein, Germany). T cells were harvested at different time points and tested for CD3, CD8, or CD3  $\zeta$ -chain



**FIGURE 1.** Multimeric Ag arrays composed of monomeric or tetrameric MHC complexes are composed of similar numbers. Monomeric or tetrameric MHC/peptide complexes were titrated at 1, 0.1, or 0.01  $\mu\text{g}$  MHC/peptide equivalents, as described in *Materials and Methods*, in 96-well microtiter plates. Correctly folded MHC/peptide complexes (HLA-A2 m loaded with ELAGIGILTV) were defined using the biotin-labeled mAb w6/32. The number of correctly folded MHC/peptide complexes can be compared using arbitrary units (OD at 450 nm) defined by the mAb w6/32 in an ELISA system. The primary mAb (anti- $\beta_2$ -microglobulin) without MHC/peptide complexes was used as a control.

expression determined by flow cytometry. Alternatively, T cells were exposed to MHC class I complexes, to the HLA-A2<sup>+</sup> and Melan-A/MART-1-positive target cell line Mel624, or to plate-bound anti-CD3 mAb (Okt3, 2  $\mu\text{g}/\text{ml}$ , 50  $\mu\text{l}/\text{well}$ ), and supernatants were harvested for detection of IFN- $\gamma$ , GM-CSF, or IL-2 by ELISA, according to the manufacturer's instructions.

The staining pattern of HLA-A2 molecules loaded either with the naturally processed and presented Melan-A/MART-1 peptide AAGIGILTV or, alternatively, with the T cell superagonist ELAGIGILTV loaded either onto HLA-A2m or HLA-A2wt molecules, was defined in a time course experiment using the SK-Mel-29.1.1 T cell clone as effector cells. No differences between A2wt or A2m molecules presenting the superagonist peptide, but reduced staining of the T cell clone implementing the AAGIGILTV peptide (Fig. 2A), could be observed. This staining pattern could also be reproduced using tetramer complexes to stain Ag-specific T cells in



**FIGURE 2.** A and B, Binding characteristics of HLA-A2 wt or m MHC molecules loaded with Melan-A/MART-1 peptides. A, The T cell clone SK-Mel-29.1.1 was incubated with mutant (A2m) or wild-type (A2wt) PE-labeled tetrameric MHC complexes loaded with either the peptide analog ELAGIGILTV or the naturally processed and presented epitope AAGIGILTV. Aliquots were harvested and the percentage of tetramer-staining T cells was evaluated. No major difference between A2wt or A2m tetramer complexes loaded with ELAGIGILTV was observed. In contrast, only 20% of the T cell clone stained after 60 min with the AAGIGILTV peptide presented by HLA-A2 m. CD3/CD8 or CD3  $\zeta$ -chain modulation was evaluated in the tetramer-positive T cell population. B, Representative staining of tetramer-positive CD3<sup>+</sup>CD8<sup>+</sup> T cells in PBL from an HL-A2<sup>+</sup> patient with melanoma. No difference pertaining to A2 wt vs A2 m molecules loaded with ELAGIGILTV. A lower number of CD8<sup>+</sup> PBL stains positive for the AAGIGILTV peptide. Tetramer-positive cells are reported as the percentage of positive-staining T cells in the CD3<sup>+</sup>CD8<sup>+</sup> T cell pool (15% of PBL). FS, forward scatter; SS, side scatter.

PBL (Fig. 2B). A2wt or A2m molecules loaded with ELAGIGILTV stain 4% of the CD3<sup>+</sup> CD8<sup>+</sup> T cell population from a patient with melanoma. In contrast, the A2m tetramer loaded with AAGIGILTV stained only 1% of T cells. We have been able to show earlier that the TCR repertoire binding to either HLA-A2wt or HLA-A2m MHC/peptide complexes loaded with ELAGIGILTV is almost identical in patients with melanoma (20).

### Flow cytometry

T cells were stained with an anti-CD3  $\epsilon$  (clone UCHT1) mAb labeled with energy-coupled dye, with anti-CD8  $\alpha$ -chain mAb (clone B9.11, murine IgG1) labeled with PE-Cyanin (PC5) for 15 min at 4°C, and flow cytometric analysis was performed using a Beckman Coulter Epics XL flow cytometer. Intracellular TCR  $\zeta$ -chain detection was carried out after staining cells with cell surface markers, followed by permeabilization with Intraprep (Beckman Coulter) using the anti- $\zeta$ -chain mAb directly labeled with FITC (clone 6B10.2, mouse IgG1) obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The human T cell clone SK-Mel-29.1.1 expresses the TCR V region  $\alpha$ -chain 2S1 and V region  $\beta$ -chain 13S3, and has already been described in detail (13).

### Functional assays

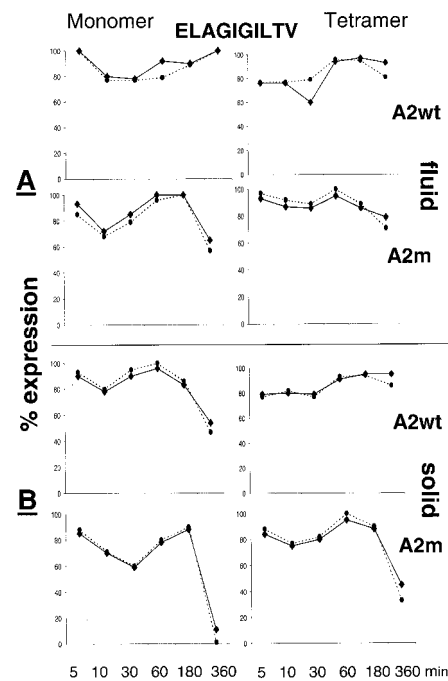
Cytotoxicity was analyzed in a standard 4-h <sup>51</sup>Cr release assay, and supernatants were tested for IL-2, IFN- $\gamma$ , or GM-CSF by ELISA. Target cells were HLA-A/B<sup>+</sup> B cells (C1R) transfected with either the A2wt or A2m molecule (18). The E:T ratio was 10:1, unless otherwise indicated.

## Results

### TCR down-regulation upon Ag contact in fluid phase

The anti-Melan-A/MART-1-specific T cell clone SK-Mel-29.1.1 was incubated with monomeric or tetrameric HLA-A2 wt or HLA-A2 m MHC class I complexes loaded with the Melan-A/MART-1 peptide analog ELAGIGILTV. In each experiment, tetramer complexes as well as monomers were at 1  $\mu$ g in a reaction volume of 0.5 ml with  $5 \times 10^5$  T cells. Aliquots of T cells were harvested at different time points ranging from 5 to 360 min, and CD3/CD8 expression as well as intracellular TCR  $\zeta$ -chain expression was evaluated and compared with CD3/CD8/ $\zeta$ -chain expression before Ag exposure (i.e., 100%). Differences were observed in T cells exposed to tetramer complexes compared with monomeric MHC class I molecules (Fig. 3A). CD3/CD8 expression was down-regulated on T cells exposed to monomeric HLA-A2 wt molecules 10 min after Ag exposure (80% of the original expression, or 20% reduction, respectively). After 360 min, T cells regained similar expression levels as compared with TCR expression before Ag exposure. HLA-A2wt tetramer complexes resulted already after 5 min in 80% of the original CD3/CD8 and  $\zeta$ -chain expression and ~90% of the original CD3, CD8, or  $\zeta$  TCR expression was observed 360 min after Ag exposure.

In contrast to the HLA-A2wt-presenting molecule, the HLA-A2m induced a different behavior in the T cell clone SK-Mel-29.1.1 pertaining to TCR expression levels. After exposure to monomeric (mutant) soluble HLA-A2 complexes, CD3/CD8 and  $\zeta$  down-regulation was observed after 10 min, similarly to the HLA-A2 wt molecule. In contrast, CD3/CD8 and TCR  $\zeta$ -chain expression was reduced to 60% of the original expression levels after 360 min of Ag exposure to monomeric HLA-A2 m complexes. Similarly, HLA-A2 m tetramer complexes resulted in ~70% of the original TCR expression level after 360 min of Ag contact. Thus, exposure of the anti-Melan-A/MART-1 T cell clone to HLA-A2wt class I complexes resulted in restoration of the TCR expression level after 360 min of Ag exposure, and the mutant HLA-A2 molecule yielded a more pronounced down-regulation of the TCR complex, as defined by CD3/CD8 and  $\zeta$ -chain expression. Notably, the stimulating Ag (i.e., monomer or tetramer) was present throughout the entire experiment (Fig. 3A). However, exposure of T cells for 10 min to tetramer complexes, followed by



**FIGURE 3.** A and B, Parallel down-regulation of CD3 and CD8 ( $\blacklozenge$ ) together with the intracellular  $\zeta$ -chain ( $\bullet$ ) stimulated with either monomeric or tetrameric MHC class I complexes loaded with ELAGIGILTV in fluid phase (A) or attached to a solid phase (B). The stimulating MHC class I molecule was present throughout the assay. T cells were harvested at different time points and stained for CD3, CD8, and intracellular TCR  $\zeta$ -chain expression by flow cytometry. Results are depicted as the percentage of cell surface expression, CD3/CD8, and  $\zeta$ -chain expression is 100% before Ag exposure. Representative data from three independent experiments.

two washing steps, showed similar results regarding CD3/CD8 and  $\zeta$ -chain down-regulation, respectively (data not shown).

### Pronounced TCR down-regulation in T cells exposed to solid-phase monomeric or tetrameric class I complexes

In a control experiment, monomeric or tetrameric HLA-A2 complexes were attached to a solid phase using an anti- $\beta_2$ -microglobulin mAb, as described in *Materials and Methods*. Thus, Ag presentation occurred as multimeric HLA-A2 complexes composed of either monomeric complexes or tetrameric complexes (Fig. 3B). We tested whether Melan-A/MART-1-specific T cells reacted 1) similarly or differently as defined by CD3/CD8 and  $\zeta$ -chain expression by comparing HLA-A2 wt or HLA-A2 m molecules, and 2) differently to monomer or tetramer complexes in fluid phase as compared with MHC class I Ag-presenting molecules attached to a solid phase. Two patterns of reactivity emerged: CD3/CD8 and  $\zeta$ -chain expression was greatly reduced at 360 min after Ag contact, yielding ~50% of TCR expression in T cells exposed to solid-phase HLA-A2wt monomeric complexes or HLA-A2m tetramer complexes. HLA-A2m monomeric complexes ablated CD3/CD8 and TCR expression after exposure of 360 min. A different pattern was observed in HLA-A2wt tetramer complexes: CD3/CD8/ $\zeta$ -chain expression was reduced to 80% after 5 min and completely regained TCR expression after 60 min.

### Cytokine expression in T cells reacting to fluid- or solid-phase monomer or tetramer complexes

Aliquots from supernatants of each experiment (Fig. 3, A and B) were harvested at 3, 6, and 24 h after Ag exposure and tested for



Table I. TCR down-regulation and cytokine release induced by antigenic stimulation is dependent on the format of Ag presentation<sup>a</sup>

		ELAGIGILTV				AAGIGILTV			
		HLA-A2wt		HLA-A2m		HLA-A2m		A2wt	OKT3
		Monomer	Tetramer	Monomer	Tetramer	Monomer	Tetramer	Mel624	
Ag presentation in "fluid phase" assays									
TCR-expression	30	79%	60%	82%	92%	100%	100%	n.d. <sup>b</sup>	100%
	80	100%	96%	68%	81%	100%	20%	n.d.	100%
Cytokine-release (pg/ml)	IL-2	0	>400	0	>400	0	<200	n.d.	0
	IFN- $\gamma$ or GM-CSF	0	>250	0	>250	0	<100	n.d.	0
Ag presentation in "solid phase" assays									
TCR-expression	30	83%	80%	59%	78%	n.d.	n.d.	90%	80%
	180	58%	97%	16%	44%	n.d.	n.d.	42%	63%
Cytokine-release (pg/ml)	IL-2	>400	>400	>400	<200	>400	<200	>400	>400
	IFN- $\gamma$ or GM-CSF	<100	<100	<100	<100	<100	<100	>250	>250

<sup>a</sup> The T cell clone SK-Mel-29.1.1 was exposed to antigenic stimulation as described in detail in Figs. 1 and 3 and tested a various time points (5–360 min) for cellular CD3/CD8 cell surface and intracellular  $\zeta$ -chain expression. Supernatants were harvested at 3, 6, and 24 h after initiation of Ag contact and tested for IL-2, IFN- $\gamma$  and GM-CSF by ELISA. The table represents a description of TCR modulation and cytokine release. CD3/CD8 levels are given at either 30 (30') or 180 min (180') after Ag exposure as compared to CD3/CD8 cell surface expression on nonstimulated T cells. Cytokine release data (IL-2 and IFN- $\gamma$  or GM-CSF) represent values at 24 h after Ag exposure and are given in picograms per milliliter.

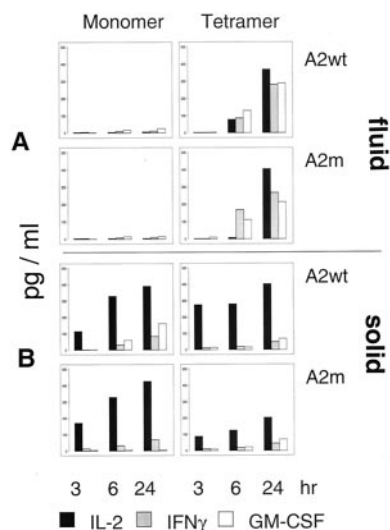
<sup>b</sup> n.d., Not done.

IL-2, IFN- $\gamma$ , or GM-CSF by ELISA (Fig. 4, A and B). No significant cytokine release could be detected in T cells exposed to monomeric HLA-A2 complexes in fluid phase independent of the HLA-A2wt or HLA-A2m Ag-presenting molecule (Fig. 4A). In contrast, tetrameric complexes in fluid phase resulted in IL-2, IFN- $\gamma$ , and GM-CSF secretion independent of the HLA-Awt- or HLA-A2m-presenting molecules (Fig. 4A). HLA-A2m monomeric complexes induced higher IL-2 levels as compared with the HLA-A2m tetrameric molecules (up to 420 pg/ml in 24 h using monomeric complexes) as compared with 200 pg IL-2 in 24 h elicited by HLA-A2 tetramer complexes (Fig. 4B). Differences regarding cytokine secretion were observed for IL-2, but not for IFN- $\gamma$  or GM-CSF. Note that alterations in CD3/CD8 or  $\zeta$ -chain down-regulation (e.g., T cells exposed to solid-phase tetrameric HLA-A2m mole-

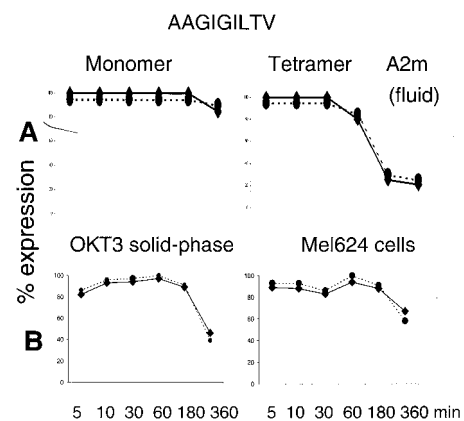
cules) were not associated with elevated levels of cytokine secretion.

#### TCR down-regulation and cytokine secretion in response to naturally processed and presented MHC class I epitopes

The previous set of experiments was performed using a Melan-A/MART-1 peptide analog ELAGIGILTV, which has been described to represent a superagonistic peptide leading to enhanced cytolysis and more efficient cytokine release as compared with the wt epitope (E)AAGIGILTV (Fig. 5A). As a control, we used the naturally processed and presented Melan-A/MART-1 peptide AAGIGILTV, the plate-bound anti-CD3 mAb OKT3, as well as the Melan-A/MART-1<sup>+</sup> and HLA-A2<sup>+</sup> melanoma cell line Mel624 (Fig. 5B) to activate T cells. Solid-phase monomeric HLA-A2 m molecules loaded with AAGIGILTV yielded only 10% reduction



**FIGURE 4.** A and B, Cytokine release in response to Ag stimulation. Supernatants from T cells stimulated with either monomeric or tetrameric MHC class I complexes loaded with ELAGIGILTV from fluid phase (A) or solid phase (B) were harvested at 3, 6, and 24 h and tested for IL-2, IFN- $\gamma$ , and GM-CSF by ELISA.

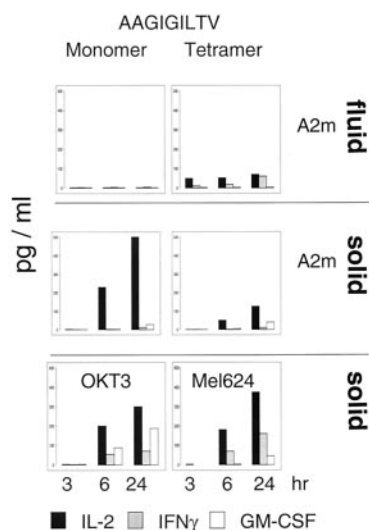


**FIGURE 5.** A and B, Parallel down-regulation of CD3 and CD8 ( $\blacklozenge$ ) together with the intracellular  $\zeta$ -chain ( $\bullet$ ) upon Ag exposure to the naturally processed and presented Melan-A/MART-1 Ag AAGIGILTV. This epitope is presented by the A2m molecule in a fluid phase (A). As a control, the anti-CD3 mAb OKT3 or HLA-A2<sup>+</sup> Melan-A/MART-1<sup>+</sup> melanoma cells ( $10^6$ /tumor cells/well and  $10^6$  T cells) were tested for the capacity to induce down-regulation of TCR components (B). Representative data from two independent experiments.

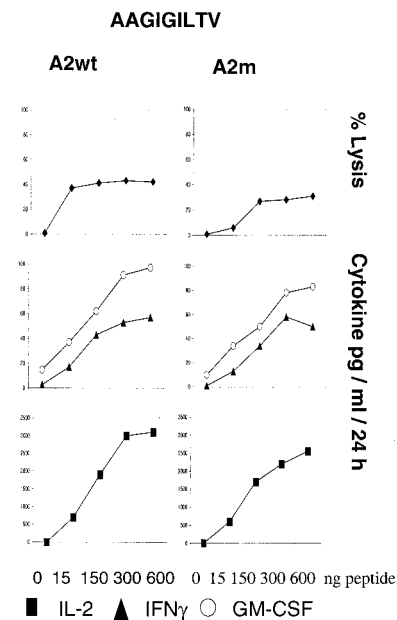
of CD3/CD8/ $\zeta$ -chain expression after 360 min of Ag exposure. In contrast, tetrameric HLA-A2 m molecules, plate-bound anti-CD3 Ab, or melanoma cells Mel624 showed a similar pattern of TCR reactivity: the TCR expression level remained stable up to 60 min after Ag exposure and was followed by a significant TCR down-regulation: 20% of the original expression level as a reaction to HLA-A2m molecules loaded with AAGIGILTV, 40% induced by anti-CD3, or 60% as a response to melanoma cells. Evaluation of cytokine secretion (Fig. 6) in supernatants harvested at 3, 6, and 24 h of Ag exposure elicited by TCR engagement resulted in significant IL-2 secretion: 300 pg/ml/24 h upon OKT3 stimulation, 370 pg/ml/24 h upon exposure to Mel624 cells, and 500 pg/ml/24 h upon exposure to solid-phase monomeric HLA-A2m molecules loaded with the AAGIGILTV peptide. Similar to solid-phase HLA-A2m tetramer complexes loaded with ELAGIGILTV (Fig. 3B), tetramer HLA-A2m molecules loaded with AAGIGILTV yielded less IL-2 secretion (120 pg/ml/24 h) as compared with the multimeric class I complexes composed of monomeric HLA-A2 molecules. As a control, fluid-phase HLA-A2m monomeric molecules loaded with AAGIGILTV did not yield cytokine secretion. Similar to the (fluid-phase) HLA-A2m tetramer complexes loaded with ELAGIGILTV (Fig. 4A, top panel), the HLA-A2m tetramer molecule loaded with AAGIGILTV (Fig. 6) resulted in IL-2, IFN- $\gamma$ , and GM-CSF secretion (e.g., 80 pg IL-2/ml/24 h), albeit to a lesser extent as compared with the ELAGIGILTV peptide analog (i.e., 420 pg IL-2/ml/24 h).

#### HLA-A2wt or HLA-A2m molecules do not impact on cytolytic capacity of T cells

HLA-A2wt and HLA-A2m tetrameric molecules showed substantial differences as compared with CD3/CD8/ $\zeta$ -chain expression in T cells after Ag exposure (Fig. 3, A and B; Fig. 5, A and B) as well as differences in cytokine release (Fig. 4, A and B; Fig. 6). However, these experiments do not address potential differences in the cytolytic T cell effector function. To test for cytolytic capacity, HLA-A, B-negative C1R cells transgenic for HLA-A2wt or the HLA-A2m molecules were loaded with the ELAGIGILTV or the AAGIGILTV peptide and tested for cytolysis and cytokine release



**FIGURE 6.** Cytokine release in response to antigenic stimulation with the peptide AAGIGILTV. Supernatants from T cells stimulated with monomeric or tetrameric MHC class I complexes either in fluid phase or solid phase were harvested at different time points and tested for IL-2, IFN- $\gamma$ , and GM-CSF. As a control, supernatants from T cells stimulated with the anti-CD3 mAb OKT3 or Mel624 cells served as a control.



**FIGURE 7.** No differences in the wt or m HLA-A2 molecule expressed as a transgene in HLA-A and HLA-B<sup>-</sup> C1R cells as Ag-presenting molecules for the peptide AAGIGILTV. C1R-A2wt or C1R-A2m cells were pulsed with the peptide AAGIGILTV from 15 to 600 ng in the presence of  $\beta_2$ -microglobulin and tested as targets in a standard <sup>51</sup>Cr release assay, as well as in a 24-h cytokine release assay. Similar data were obtained with the peptide analog ELAGIGILTV as the target epitope (data not shown).

(Fig. 7). No significant differences in IL-2, IFN- $\gamma$ , or GM-CSF secretion as well as for cytolysis could be observed in HLA-A2wt or HLA-A2m molecules presenting the peptide AAGIGILTV. Similar data were obtained for the Melan-A/MART-1 peptide analog ELAGIGILTV (data not shown).

## Discussion

### Monomeric complexes result in TCR down-regulation

One of the salient findings of this study is that monomeric HLA-A2 complexes loaded with the nominal target epitope are able to induce TCR down-regulation, but not T cell effector functions, e.g., cytokine release (Fig. 3, A and B; Fig. 4, A and B). A recent study in a murine system showed that T cell clones are indeed able to respond to monomeric MHC class I complexes, as defined by calcium influx or proliferation if the coreceptor CD8 chain is available at the cell surface (21).

An early step in T cell activation is the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs), either in the TCR  $\epsilon$ - or  $\zeta$ -chain mediated by Lck. Once phosphorylated, the ITAMs recruit the tyrosine kinase ZAP-70, which facilitates further phosphorylation and leads to more downstream signaling events and ultimately to cytokine secretion (for review, see Ref. 22). The observation that anti-CD3 cross-linking (using the anti-CD3  $\epsilon$  mAb OKT3) is able to induce T cell activation supports this model (Figs. 5B and 6): the signaling cascade can be triggered by tyrosine kinases that are already associated with the  $\zeta$ -chains in the responsive T cell. This model is supported by experiments in which dimers of peptide/MHC complexes are able to elicit IL-2 secretion in CD8-negative T cells; thus, in a minimalistic model, TCR homodimerization is able to trigger T cells (23).

Nonmutually exclusive, the initial triggering event is mediated by the apposition of ITAM-bound ZAP-70 with the coreceptor Lck. This model is supported by the observation that bispecific Abs (directed against CD3 and the coreceptor) are able to induce

T cell activation (24). Thus, homodimerization of the TCR chain and/or heterodimerization of the TCR with the coreceptor are also able to induce T cell activation.

Because TCR and CD8 coreceptor down-regulation occurs exclusively upon Ag exposure, the T cell clone SK-Mel-29.1.1 was apparently activated by monomeric MHC class I/peptide complexes (Fig. 3A). However, this did not lead to significant downstream events, i.e., cytokine secretion (Fig. 4B). It is interesting to note that the CD3/CD8 or TCR  $\zeta$ -chain down-regulation was more pronounced in T cells exposed to the monomeric class I complexes with the mutation in position 245. This mutation reduces interaction of the CD8 molecule with the MHC class I complex (18, 21): at first glance surprising, since we observed a stronger TCR/CD3 down-regulation as a response to a HLA-A2 (mutant) molecule with reduced capacity to engage with the TCR as compared with the HLA-A2 wt molecule. The existence of CD8-independent T cell clones (25) suggests that this observation is not unique and that CD8 may, at least in some cases, not be able to play its role as a coreceptor due to an orientation of the TCR to its ligand that is not compatible with CD8 binding to HLA-A2 (discussed in Ref. 21). The mutation at position 245 in HLA-A2 affecting the CD8 binding site (18) may thus allow for a better engagement of the TCR/CD8 complex to HLA-A2 peptide targets, resulting in TCR down-regulation.

However, coordinate TCR and CD8 down-regulation occurs also if the coreceptor CD8 cannot engage with the MHC class I molecule: the interaction of the coreceptor CD8 in initiating coordinate TCR/CD8 down-regulation may be dispensable if T cells are stimulated by optimal ligands (26, 27). We prove that this is operational in the Melan-A/MART-1-reactive T cell clone: the coordinate TCR/CD8 down-regulation is only observed as a response to the peptide analog (superagonist) ELAGIGILTV presented by a monomeric MHC class I complex (see Fig. 3A), but not to the monomeric class I peptide complex loaded with the naturally processed and presented peptide AAGIGILTV (see Fig. 5A). The proof that the monomeric MHC class I/peptide (AAGIGILTV) complexes are properly folded is provided by the control experiment, in which the association of monomeric complexes loaded with the peptide AAGIGILTV to multimers by attachment to plastic leads to pronounced TCR down-regulation and biologically meaningful secretion of cytokines (Figs. 5 and 6).

In addition, the number of MHC/peptide complexes is identical independent of monomeric or tetrameric MHC complexes (see Fig. 1). However, even if the number of MHC/peptide complexes is identical, the spatial arrangement of these Ag-presenting molecules impacts on different spatial arrangements of TCR-associated molecules, e.g., CD8 or the adapter molecule linker for activation of T cells (28), and ultimately on T cell effector functions (29, 30). Thus, the spatial arrangement of MHC/peptide molecules may represent an important aspect of the *in vivo* interactions between T cells and APCs.

CD3/CD8 and TCR  $\zeta$ -chain down-regulation occurs only if the T cell clone is exposed to the tetrameric complex, but not to monomers loaded with the Melan-A/MART-1 peptide AAGIGILTV (Fig. 5). Thus, subtle differences in response to either HLA-A2wt or HLA-A2m peptides are observed if the dynamics of TCR down-regulation are measured. These differences did not impact on downstream T cell effector functions: the T cell clone secretes IL-2, IFN- $\gamma$ , as well as GM-CSF in response to multimeric MHC class I/peptide complexes and shows no differences regarding cytolytic T cell responses if C1R-A2wt or C1R-A2m molecules are expressed as a transgene in C1R APCs. Thus, at least in the Melan-A/MART-1-specific T cell clone SK-Mel-29.1.1, TCR down-regulation cannot be used as a measure to predict the biological out-

come of T cell activation. This notion is supported by additional experimental data provided in this study: exposure of the T cell clone to Melan-A/MART-1<sup>+</sup> and HLA-A2<sup>+</sup> melanoma cells did not show a pronounced CD3/CD8 reduction, but resulted in a similar cytokine secretion pattern as compared with multimeric HLA-A2 complexes loaded with the ELAGIGILTV peptide.

In addition, cell surface CD3/CD8 down-modulation in the Melan-A/MART-1-specific effector cell clone exhibited a biphasic pattern, i.e., CD3/CD8 down-modulation was observed after 10–30 min of Ag exposure, followed by recovery of CD3/CD8 cell surface expression and a subsequent CD3/CD8 down-modulation after 180 min (Fig. 3). Several mechanisms may account for this phenomenon: the first CD3/CD8 down-modulation may result from directly engaged receptors mediated by endocytosis. In contrast, the CD3/CD8 down-modulation may represent nondirectly engaged bystander TCRs that are internalized by activation of intracellular signaling mediators, i.e., protein tyrosine kinase, phosphatidylinositol 3-kinase, or protein kinase C (31, 32). Down-modulated CD3/CD8 complexes may impact on the effector function(s) of freshly isolated Ag-specific T cells using tetramer complexes. A strong antigenic stimulus may render these T cells anergic or, alternatively, may induce apoptosis if tetramer-reactive T cells are re-exposed to the nominal target Ag.

Differences in TCR down-regulation in the Melan-A/MART-1-specific T cell clone in response to the naturally processed and presented peptide AAGIGILTV as compared with the peptide analog ELAGIGILTV may also be associated with the nature of this individual T cell clone: this clone has been repeatedly isolated from the melanoma patient SK29, who is still in complete remission (13, 14). Recent evidence suggests that the time course of TCR down-regulation is different in naive and primed T cells (discussed in Ref. 33) and shows different requirements for costimulation.

In conclusion, we have been able to show that 1) monomeric class I complexes are able to induce T cell activation, as defined by coordinate TCR/CD8 down-regulation if an optimal T cell ligand is used as the stimulating epitope, 2) multimeric class I complexes, either in solution or attached to a solid phase, are able to induce cytokine secretion in responding T cells, 3) CD3/CD8 and TCR  $\zeta$ -chain down-regulation does not correlate with immune effector functions, and 4) differences pertaining to HLA-A2wt or HLA-A2m molecules exist if CD3/CD8 down-regulation is analyzed, but both HLA-A2 molecules are able to effectively induce cytokine secretion and cytotoxicity in Melan-A/MART-1-reactive T cells. These observations impact on the implementation of soluble class I molecules in either detection or sorting of MHC class I/peptide-specific T cells. In addition, monomeric ligands may be used to gauge the affinity of the clonotypic TCR to the respective peptide epitope: high affinity TCRs may respond with TCR down-regulation even to monomeric MHC class I/peptide complexes.

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