

Optimizing the exogenous antigen loading of monocyte-derived dendritic cells

Detlef Dieckmann^{1*}, Erwin. S. Schultz^{1*}, Bernhard Ring¹, Patrick Chames², Gerhard Held³, Hennie. R. Hoogenboom⁴ and Gerold. Schuler¹

¹Department of Dermatology, University Hospital Erlangen, Erlangen, Germany

²Cellectis SA, Paris, France

³Department of Medicine, University of the Saarland, Homburg, Germany

⁴Merus BV, Utrecht, The Netherlands

Keywords: antigen presentation, human, peptide, T cells, vaccination

Abstract

Dendritic cell (DC) vaccination, i.e. the adoptive transfer of antigen-loaded DC, is still at an early stage and requires standardization. In this study, we investigated the exogenous loading of monocyte-derived DCs with HLA class I- and II-restricted peptides, as despite widespread use, little effort has been put into its pre-clinical validation. We found that only mature DCs (m-DC) but not immature DCs (im-DC) could be sufficiently loaded with exogenous class I-restricted peptides and were by far superior in expanding CD8⁺ primary (Melan-A.A2 peptide-specific) and recall [Influenza matrix peptide (IMP) A2-specific] T cell responses. Primary stimulation with peptide-loaded im-DCs even down-regulated antigen-specific T cell responses. Our results indicate that stimulation with m-DCs is superior in terms of quantity and quality compared with im-DCs, supporting their preferred use in clinical DC trials. Loading of m-DCs with high (10 µM) concentrations generated clearly more Melan-A effectors than loading with 1 or 0.1 µM without any negative effect on the quality (affinity) of the resulting T cells. In contrast to the findings with the Melan-A peptide loading with 10 µM IMP was counter-productive, induced apoptosis and yielded fewer specific T cells of inferior affinity as compared with loading with 1 or 0.1 µM. In sharp contrast to the situation for HLA class I, much higher levels and longer half-lives of peptide–HLA class II complexes were obtainable upon loading of im-DCs with exogenous peptide, but m-DCs were functionally preferable to induce T_H1 responses *in vitro*. Another surprising finding was that, while presentation to T cells upon simultaneous loading of several peptides with highly varying affinities and competing for the same class I or II molecule was possible, in priming experiments peptide competition clearly inhibited T cell induction. Although peptides will obviously vary in their individual properties, our study clearly points to some important principles that should be taken into account.

Introduction

The rational development of dendritic cell (DC)-based vaccination against cancer was made possible by the identification of human tumor antigens (1, 2). The adoptive transfer of antigen-loaded DCs represents a promising approach to induce anti-tumor immunity in cancer patients, but the optimal vaccination strategy still has to be defined.

To date >1000 patients have been vaccinated with DCs, largely monocyte-derived DCs loaded with either tumor cell lysates or defined peptides (3). Encouraging clinical responses have often been observed, yet require confirmation

in larger randomized trials. Recent reviews (4, 5) have emphasized that the information that can be extracted from all the trials performed is, unfortunately, rather limited, as only few studies used standardized DC vaccines and established immunomonitoring. Nevertheless, the proof of principle, that tumor-specific CTL can be induced by DC vaccination, was clearly demonstrated in several trials (4). However, the induced immune responses are rather weak compared with those occurring in natural viral infections and the overall clinical responses still remain limited. Many efforts have been

*These authors contributed equally to this study.

Correspondence to: D. Dieckmann; E-mail: detlef.dieckmann@derma.imed.uni-erlangen.de

Transmitting editor: R. Steinman

Received 6 December 2004, accepted 20 February 2005

2 Peptide loading of dendritic cells

undertaken to optimize DC vaccination (6, 7), but a major hurdle has been the absence of reagents to quantify MHC-peptide complexes on antigen-loaded DCs. As a consequence, to date there have been no systematic studies addressing the peptide loading of DCs in the human system. The present study was performed to systematically establish the optimal conditions to induce both, antigen-specific CD8⁺ and CD4⁺ T cells by peptide-loaded DCs. Antigen-specific T cell clones were used as the conventional strategy to monitor HLA-peptide complexes on the surface of DCs. In addition, we took advantage of only recently developed antibodies specific for a given HLA class I-peptide complex (8–10) to quantitatively detect HLA-peptide complexes on the cell surface and thus to study the influence of peptide concentration and time of antigen loading on the presentation of HLA class I peptides on DCs. These analyses were accompanied by *in vitro* priming and recall assays to determine the effects on priming or expansion of T cells specific for certain model antigens.

To further simplify DC vaccines it would be desirable to load as many antigenic peptides onto one DC batch as possible. DCs loaded simultaneously with several peptides with different affinity (even including high-affinity binding analogues) have indeed been used in clinical trials without pre-clinical verification of simultaneous efficient presentation of all the desired epitopes (11, 12). Several concerns have kept other investigators from loading several peptides onto one DC batch. Exogenous peptide has to compete with an endogenously processed antigen for access to HLA molecules. Using more than one peptide binding to the same HLA molecule for loading on DCs would further increase this competition and probably lead to preferred binding of the peptide with the highest affinity and lowest off-rate for the HLA molecule. Another possible drawback of loading with several peptides arises from the proven competition of T cells for access to the antigen-presenting cell (APC) (13, 14). A recent study using HLA-A2 transgenic mice has demonstrated that the simultaneous presence of several immunodominant epitopes on an APC skews the immune response towards the single epitope for which CTL precursor frequencies are highest (13, 14). We, therefore, also carefully analyzed the effects of simultaneously loading DCs with several peptides competing for a given HLA molecule.

Methods

Culture medium

RPML 1640 (Bio Whittaker) supplemented with 1% heat-inactivated autologous plasma, 20 μ M gentamicin (Sigma) and 2 mM L-glutamine (Bio Whittaker) was used for the generation of DCs. CD8⁺ T cells were cultured in X-VIVO-20 (Bio Whittaker) supplemented with 1% heat-inactivated single-donor human serum and CD4⁺ T cells were cultured in RPML 1640 supplemented with 10% heat-inactivated human pool serum. In both cases 20 μ g ml⁻¹ gentamicin and 2 mM L-glutamine was supplemented.

Cytokines

All cytokines used in this study were recombinant human proteins. Final concentrations were 1000 U ml⁻¹ granulocyte

macrophage colony-stimulating factor (GM-CSF) (LeukomaxTM, Novartis) and 800 U ml⁻¹ IL-4 (Sandoz); IL-2 (ProleukinTM, Chiron Corp.) was used at the concentrations indicated. For DC maturation, we used a cocktail consisting of 2 ng ml⁻¹ IL-1 β (Sigma), 1000 U ml⁻¹ IL-6 (Sandoz), 10 ng ml⁻¹ tumor necrosis factor- α (TNF- α) (Bender, Vienna, Austria) and 1 μ g ml⁻¹ PGE₂ (Sigma) (15).

Antibodies

For immunostaining PE- and FITC-conjugated antibodies (all from BD Pharmingen) against CD3 (UCHT 1), CD4 (RPA-T4), CD8 (RPA-T8), CD14 (M5E2), CD80 (L307.4), CD83 (HB15e) and CD86 (FUN-1), unconjugated antibodies (all from BD Pharmingen) against HLA class I (W6/32 HL), HLA-DR (L243), HLA-DQ (SPVL3), HLA-DP (B7/21), HLA-DM (MaP.DM1) and the respective mouse isotype controls were employed. As a second antibody a FITC-conjugated polyclonal goat anti-mouse antibody (BD Pharmingen) was used. Phage fd-Fab-Hyb3, G8 or H2 were produced as described before (8) and used as described in Chames *et al.* (16).

Peptides

All peptides used were >95% pure, of good manufacturing practice quality, purchased from Clinalfa (Läufelfingen, Switzerland) and used at the indicated concentrations.

HLA-A*0201-restricted peptides: Influenza matrix peptide (IMP) A2: GILGFVFTL (17); Melan-A.A2 native: EAAGIGILTV (18); Melan-A.A2 analogue (ana): ELAGIGILTV (19); gp-100.A2 ana: IMDQVPFSV (20).

HLA-A1-restricted peptides: MAGE-1.A1: EADPTGHSY (21), MAGE-3.A1: EVDPIGHLY (22); Tyrosinase.A1 ana: KSDICTDEY (23).

HLA-DP4-restricted peptides: MAGE-3.DP4: KKLLTQH-FVQENY (24), Tetanus toxin (TT).DP4: FNNFTVSFWLRVPK (25), NY-ESO-1.DP4: SLLMWITQCFLPVF (26).

To quantitate antigen-specific, IFN- γ -releasing, peptide-specific effector T cells, an enzyme-linked immunospot (ELISPOT) assay was used as described (27, 28).

Cell isolation and DC generation

DCs were generated from whole-blood or leukapheresis products of HLA-A*0201-, HLA-A1- or HLA-DP4-positive donors. Leukapheresis was obtained from the Department of Transfusion Medicine from healthy donors after informed consent was given as described (29, 30). In brief, PBMC were isolated by Ficoll density gradient centrifugation. Monocytes were isolated by plastic adherence and cultured in RPML medium, supplemented with IL-4 and GM-CSF. At day 6, a maturation cocktail (IL-1 β , IL-6, PGE₂ and TNF- α) was added (15). At day 7 for leukapheresis products or day 8 for whole-blood non-adherent cells were harvested and constituted mature dendritic cells (m-DC) that were >90% double-positive for co-stimulatory molecules (CD80, CD86) and CD83. Immature dendritic cells (im-DC) were cultured with GM-CSF and IL-4 for 7 days without the addition of the maturation cocktail.

CD8⁺ and CD4⁺ T cells were isolated from PBMC with anti-CD8 or anti-CD4 magnetic beads (Miltenyi Biotec). Purity was assessed by FACS.

Flow cytometric analysis

For immunofluorescence staining cells were washed and stained for 20 min at 4°C with optimal dilution of each antibody. Cells were washed again and analyzed by flow cytometry (FACSScan™ and CELLQuest™ software; Becton Dickinson).

For analysis with HLA-peptide-specific Fab/Phage constructs HLA-A1-positive DCs were pulsed with 10 µM of HLA-A1-binding peptide MAGE-1.A1 or MAGE-3.A1 as an irrelevant control for 1 h. DCs were washed two times in PBS (Bio Whittaker) and re-suspended at 10^6 cells ml⁻¹. All staining procedures were performed at 4°C. DCs were incubated for 30 min with fd-Fab-Hyb3, G8 or H2, washed again and incubated with an anti-M13 coat protein antibody (Zytomed) for an additional 30 min. After two rounds of washing in PBS, DCs were incubated with goat anti-mouse PE Fab fragments (Caltag) for 15 min. Cells were washed again and analyzed by flow cytometry (FACSScan™ and CELLQuest™ software; Becton Dickinson). Cultured EBV-transformed B cells (EBV B cells) were pulsed with the MAGE-1.A1 peptide or irrelevant MAGE-3.A1 peptide (10 µg ml⁻¹) for 1 h at 37°C and washed two times with PBS before staining.

Recognition assay with peptide-loaded DCs

Im-DCs and m-DCs from healthy HLA-DP4⁺ or HLA-A1⁺ donors were loaded for 1 h with different amounts of peptide MAGE-3.DP4 or MAGE-1.A1 and washed. HLA-DP4⁺ DCs were used to stimulate a MAGE-3.DP4-specific CD4⁺ T_H clone which had been isolated from the blood of a vaccinated melanoma patient, hereafter referred to as clone R12-57 (31). HLA-A1⁺ DCs were used to stimulate a MAGE-1.A1-specific CD8⁺ T cell line, generated from the blood of a vaccinated melanoma patient. CD4⁺ and CD8⁺ T cells (4×10^3) were co-cultured with 1.5×10^4 peptide-loaded DCs in 96 round-bottomed microwells for indicated time points. Eighteen hours after the co-culture, IFN-γ was measured in the supernatants by ELISA using reagents from Medgenix Diagnostics-Biosource (Fleurus, Belgium). For the peptide competition assay m-DCs were loaded for 1 h with the MAGE-3.DP4 or MAGE-1.A1 peptide alone or in the presence of other peptides competing for the same HLA molecule at indicated concentrations.

Induction/expansion of antigen-specific T cells

HLA class I. Forty eight-well plates were set up with HLA-A2⁺ CD8⁺ T cells (2×10^6 per well) with autologous m-DCs and im-DCs (10^5 per well) loaded with Melan-A.A2 or IMP.A2 (10 µM if not indicated differently for 1 h). In some experiments m-DCs were loaded with 10 µM peptide for 1 h, washed thoroughly and used either directly or after an additional incubation period without peptide for 24–48 h. IL-2 (20 U ml⁻¹) was added every other day and two re-stimulations at weekly intervals were performed with im-DCs or m-DCs as indicated. Seven days after the last stimulation cells were harvested for tetramer and ELISPOT analysis.

For peptide competition assays HLA-A2⁺ CD8⁺ T cells (2×10^6 per well) were co-cultured with autologous, m-DCs (10^5 per well) loaded either alone with a Melan-A.A2 peptide (native peptide or ana at 10 µM) or in the presence of the gp-100.A2 ana peptide at the indicated concentrations. IL-2 (20 U ml⁻¹)

was added every other day. After two weekly re-stimulations cells were used for tetramer staining.

In some experiments antigen-specific CD8⁺ T cells were sorted. Therefore, cells were stained with specific tetramers as described above, washed and incubated with anti-PE microbeads (Miltenyi Biotech) according to the manufacturer's instructions. Purity was assessed by FACS and routinely >95%. These cells were then used for an ELISPOT assay.

HLA class II. CD4⁺ T cells (5×10^4 cells per 96 microwell) of HLA-DP4⁺ healthy donors stimulated once either with autologous im-DCs or m-DCs (1×10^4 per well) were loaded with the TT.DP4 peptide (5 µM). On days 7 and 14 fresh medium supplemented with IL-2 (10 U ml⁻¹) and IL-7 (5 ng ml⁻¹) was added and on day 21 aliquots of each well were assayed in duplicates for their capacity to produce IFN-γ, IL-4 and IL-10 when stimulated with 1×10^4 autologous EBV B cells loaded with TT.DP4 peptide or an irrelevant peptide as a negative control. Microwells were considered positive when the cytokine production was more than two times the mean value of the negative control plus standard deviation.

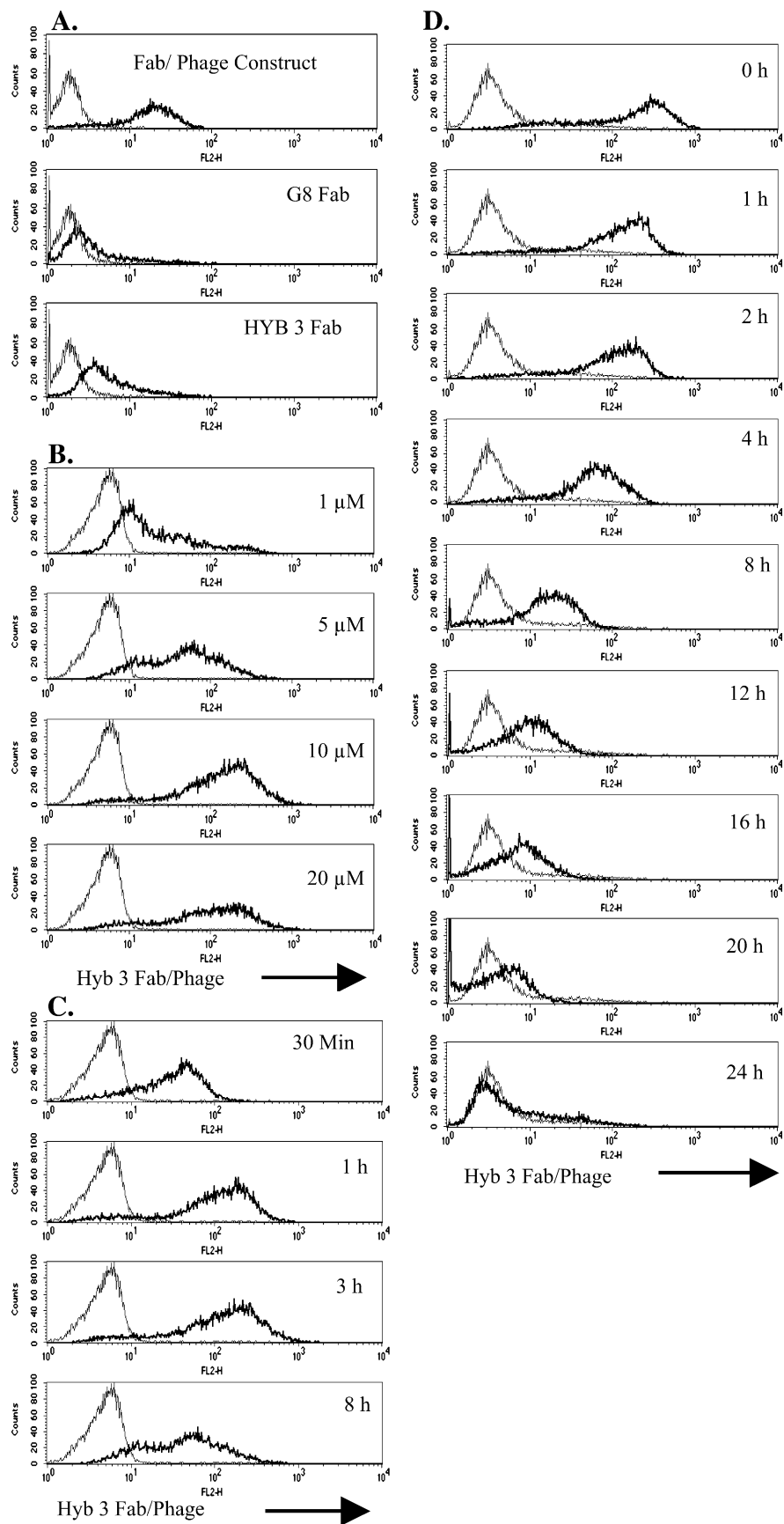
To study the influence of peptide competition CD4⁺ T cells (2×10^6 per 24 well) were co-cultured with autologous m-DCs (2×10^4 per well) loaded with the TT.DP4 peptide in the presence or absence of the MAGE-3.DP4 peptide as a competing peptide (both peptides at 5 µM). On day 7, T cells were re-stimulated and on day 14 (1.5×10^5 per microwell) these cells were analyzed for their IFN-γ production upon stimulation with m-DCs (1.5×10^4 per microwell) loaded either with the tetanus peptide or the NY-ESO-1.DP4 peptide as a negative control by ELISPOT.

Results

HLA class I presentation

Staining of DCs with the anti-MAGE-1.A1-specific Fab/Phage to define conditions for plateau HLA class I peptide loading. The use of antigen-specific T cell clones as the traditional approach to detect defined HLA class I-peptide complexes on the surface of APC does not allow for an exact quantification as <100 MHC-peptide complexes are sufficient to activate antigen-specific T cells (32). To address critical variables that might define the peptide-loading efficiency of DCs, we took advantage of the fact that it has recently become possible to select TCR-like antibodies from a large Fab/Phage library (8, 9). The TCR-like antibody used in our study recognizes a MAGE-1 peptide (EADPTGHSY) bound to HLA-A1. To this end we have performed extensive pilot experiments which revealed that neither the natural (G8) nor the affinity-matured (Hyb3) Fab fragment (8) was able to stain peptide-loaded DCs sufficiently. Only when Fab displayed on the filamentous phage surface was used (Fab/Phage), unequivocal and reproducible staining became possible, in particular for the affinity-matured version of the antibody, Hyb3 (Fig. 1A). This is likely related to the fact that a fraction of the phage displays multiple Fab fragments that can avidly bind to the DCs, and that the antibody used in the secondary staining detects the phage capsid protein (M13) present at several thousand copies, thereby increasing the sensitivity of the staining (Fig. 1A).

4 Peptide loading of dendritic cells



Phage particles displaying Fab's were then used to characterize optimal peptide-loading conditions, showing that 10 μ M of peptide (Fig. 1B) with a loading time of 1 h (Fig. 1C) is sufficient to reach plateau loading. Under these conditions, HLA class I-peptide complexes can be readily detected for 12–16 h with the Fab/Phage construct (Fig. 1D). A loading temperature of 37°C when compared with 4°C and room temperature was found optimal (data not shown), while the effect of using different media (PBS, HBSS, RPMI, X-VIVO all $\pm$ human serum albumin) was minimal (data not shown). Other loading schemes like loading overnight in combination with loading for a few hours next day as used in some studies (12) did not improve loading of DCs (data not shown).

When m-DCs and im-DCs were compared by Fab/Phage staining in pulse-chase experiments, m-DCs showed higher mean fluorescence intensity (MFI) and a longer time of peptide presentation. Staining vanished almost completely after only 4 h on im-DCs (Fig. 2A), suggesting that m-DCs can be loaded more efficiently with exogenously added class I peptides than im-DCs. Similar data were obtained, when a MAGE-1.A1-specific T cell line was used. With im-DCs T cell stimulation was completely lost after 12 h even at the highest peptide dose used, whereas on using m-DCs T cell recognition was still detectable after 48 h (Fig. 2B).

Induction of HLA class I-specific naive and memory CD8⁺ T cells is more efficient with m-DCs compared with im-DCs. To test the relative *in vitro* immunostimulatory potency of m-DCs versus im-DCs, CD8⁺ T cells of human volunteers were co-cultured with autologous DCs loaded with the HLA-A2-restricted Melan-A ana peptide or IMP as model peptides for naive (33) and recall responses (34), respectively. After each round of stimulation, cells were analyzed by tetramer staining and IFN- γ ELISPOT (Fig. 3). Tetramer staining revealed that T cells primed with m-DCs yielded $\sim$ 3.5 times higher numbers of antigen-specific T cells compared with those primed with im-DCs, regardless of whether DCs were loaded with Melan-A (3.2 versus 0.8% tetramer-positive cells) or IMP (12.2 versus 3.4%). After one re-stimulation this quantitative difference was still clearly visible for IMP (37.7 versus 10.8%), but less obvious for Melan.A (14.5 versus 10.2%). Re-stimulation with m-DCs after priming with im-DCs only partially reverted the quantitative outcome compared with cells stimulated with im-DCs alone. In our hands these effects were more pronounced, when IMP.A2 peptide was used as (recall) antigen. The above-mentioned effects were also seen if absolute cell numbers were analyzed. Analysis of IFN- γ production by ELISPOT revealed more pronounced effects. IFN- γ production was markedly reduced in all CD8⁺ T cells primed with im-DCs, regardless of whether im-DCs or m-DCs were used for re-stimulation. Priming with m-DCs on the other hand resulted in CD8⁺ T cells producing higher amounts of IFN- γ

not influenced by the DCs used for re-stimulation. These results were seen with the Melan-A.A2 and the IMP.A2 peptides alike (Fig. 3). Measurement of IL-10 production did not reveal marked differences between the differently stimulated T cells and was generally low (data not shown). Because Dhodapkar *et al.* have shown antigen-specific inhibition of effector function in humans after injection of im-DCs (35), we also analyzed our antigen-specific CD8⁺ T cells for inhibitory function on CD4⁺ and CD8⁺ T cells, but could not detect any inhibitory effect (data not shown).

Peptide concentration used for loading affects primary and recall CD8⁺ T cell responses differently. While we demonstrated that higher peptide concentrations (up to 10 μ M) increase the density of HLA-peptide complexes on the surface of DCs, it remained unclear how this HLA-peptide density would influence the DCs for priming and expansion of CD8⁺ T cells. Therefore m-DCs were loaded with different concentrations of Melan-A.A2 ana and IMP.A2 which are well-characterized model antigens/epitopes (33, 36). In the Melan-A model, the number of tetramer-positive T cells declined with decreasing peptide concentrations, while the number of Annexin V-positive, apoptotic cells increased (Fig. 4). Tetramer-positive cells were sorted to 95% purity and used at equal amounts in an ELISPOT assay with titrated amounts of peptide. Analysis revealed that tetramer-positive cells secreted equal amounts of IFN- γ regardless of the peptide concentration used for priming (Fig. 4).

Expansion of memory IMP.A2-specific CD8⁺ T cells showed a completely different behavior. Here, the lowest amount of peptide (0.1 μ M) yielded the highest amount of tetramer-positive T cells, while with high concentrations lower numbers of tetramer-positive T cells accompanied by an increase in Annexin V staining were observed. Tetramer staining already revealed a clear increase in MFI with decreasing concentration of peptide, suggesting the generation of higher affinity T cells. When tetramer-sorted T cells were used in an IFN- γ ELISPOT assay, the highest amounts of IFN- γ were indeed produced by the T cell induced with the lowest peptide concentration (Fig. 4).

Influence of the longevity of HLA-peptide complexes on the induction/expansion of antigen-specific CD8⁺ T cells. Recent studies indicate that monocyte-derived DCs upon intracutaneous injection need between 1 and 2 days after vaccination to reach in substantial numbers the draining lymph node, where they get in contact with T cells (37; our own unpublished observations). In addition, our model studies employing both a TCR-like antibody and a T cell line specific for MAGE-1.A1 suggested a short half-life of HLA-peptide complexes, similar to data observed for loading with other unmodified class I-restricted peptides (Fig. 2) (38). Therefore, we performed

Fig. 1. Staining of DCs with the MAGE-1.A1-/HLA-A1-specific Fab/Phage construct Hyb3 and defining optimal peptide-loading conditions for DCs. (A) DCs were loaded with MAGE-1.A1 (thick line) or MAGE-3.A1 (10 μ M) (thin line) as negative control for 1 h, washed and stained with the MAGE-1.A1-/HLA-A1-specific Fab fragment G8, the affinity-matured Fab fragment Hyb3 or the Fab/Phage construct Hyb3 as described in Methods and analyzed by FACS. (B) DCs were loaded with indicated concentrations of MAGE-1.A1 peptide or irrelevant control peptide for 1 h and stained with the Fab/Phage construct Hyb3. (C) DCs were loaded with 10 μ M of MAGE-1.A1 or irrelevant controls for indicated time points and stained with the Fab/Phage construct Hyb3. (D) DCs were pulsed with 10 μ M MAGE-1.A1 or irrelevant controls for 1 h and washed two times. Staining with the Fab/Phage construct Hyb3 was performed at indicated time points after washing. Solid lines always represent loading with the MAGE-1.A1 peptide. Thin lines represent DCs loaded with the irrelevant control MAGE-3.A1. One representative result of five independent experiments is shown.

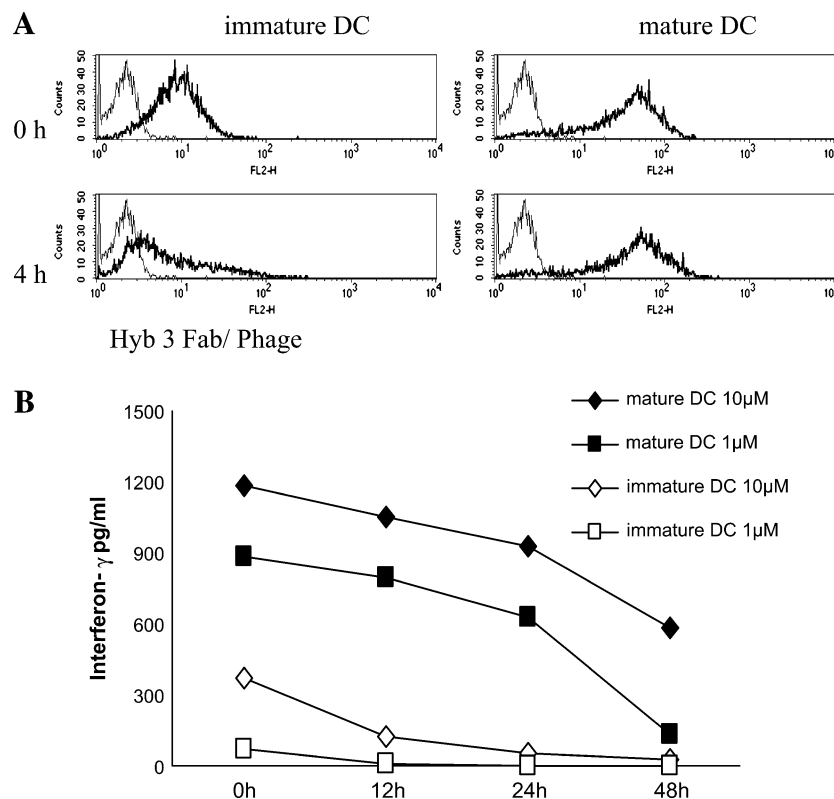


Fig. 2. Analyzing the peptide loading of m-DCs versus im-DCs with the Hyb3 Fab/Phage construct and a MAGE-1.A1-specific CTL line. M-DCs and im-DCs were generated as described in Methods. (A) M-DCs and im-DCs were pulsed with 10 μ M MAGE-1.A1 or irrelevant control for 1 h, washed two times and stained with Fab/Phage Hyb3 at indicated time points. Solid lines represent loading of DCs with MAGE-1.A1. In (B) m-DCs and im-DCs were loaded with indicated concentrations of MAGE-1.A1 for 1 h. Cells were washed two times and co-cultured at indicated time points after washing with a MAGE-1.A1-specific CTL line (4000 CTL, 15 000 DCs). After 18 h of culture IFN- γ production was analyzed by ELISA. Values shown are the mean of triplicate determinations. Standard deviation was <5% of mean values. One representative result of four independent experiments is shown.

pulse-chase experiments to analyze the ability of DCs after chase periods of 24 and 48 h to prime/expand Melan-A- and IMP-specific T cells. M-DCs were loaded with 10 μ M of peptide, washed and used either directly or after 24 and 48 h of chase (e.g. after washing, without the addition of further peptides). The number of Melan-A-specific CD8 $^{+}$ T cells decreased with longer chase periods from 7.6 to 4.6% (decrease of 34%) (Fig. 5). These values are still well above background levels, indicating that after 48 h enough peptide is retained on the surface of m-DCs for sufficient priming (Fig. 5). Expansion of IMP-specific T cells also was increased with longer chase periods from 2.4 to 3.8% (increase of 37%) (Fig. 5). If lower concentrations of IMP were used for pulsing (0.1 μ M) results similar to the Melan-A-priming experiments were seen in that at the 48-h time point a slight reduction occurred (results not shown).

Antigenic competition for a given HLA class I molecule influences the induction of antigen-specific CD8 $^{+}$ T cells by peptide-loaded DCs. For the clinical application of DC vaccines it would be desirable to load several different peptides onto the same DC batch. To address these issues we used as a model for HLA class I the simultaneous loading of DCs with the MAGE-1.A1 and the MAGE-3.A1 or the Tyrosinase.A1 ana peptide. DCs were then analyzed with the Hyb3 Fab/Phage construct and a MAGE-1.A1-specific CTL

line. As shown in Fig. 6(A) concurrent loading leads to a decreased staining by Fab/Phage, accompanied to a lesser extend by a reduction in T cell recognition (Fig. 6B). Nevertheless, loading was still detectable even if 10 times higher concentrations of a competing peptide were used. As expected, the effects were more pronounced when the Tyrosinase.A1 ana peptide with a higher affinity for the HLA class I molecule was used. Altogether, however, we were surprised by the relatively marginal effects observed.

In a further set of experiments we wished to determine the effects of concurrent loading of DCs with competing peptides on CD8 $^{+}$ T cell priming in bulk T cell cultures as this should be more comparable with the physiological situation *in vivo*. For this purpose we have chosen the well-characterized model antigens Melan-A and gp-100, as both native and ana peptides have been described and the induction of T cell responses is feasible using blood samples of healthy volunteers (34). In addition, Melan-A precursors have been proven to be naive in the blood of healthy volunteers and are yet often detectable at high frequencies (33). DCs were either loaded with Melan-A.A2 native peptide or the Melan-A.A2 ana peptide and concurrently with the gp-100.A2 ana peptide. These differently loaded DCs were then used to stimulate CD8 $^{+}$ T cells. Tetramer stainings were performed before each cycle of re-stimulation. When Melan-A.A2 ana and gp-100.A2

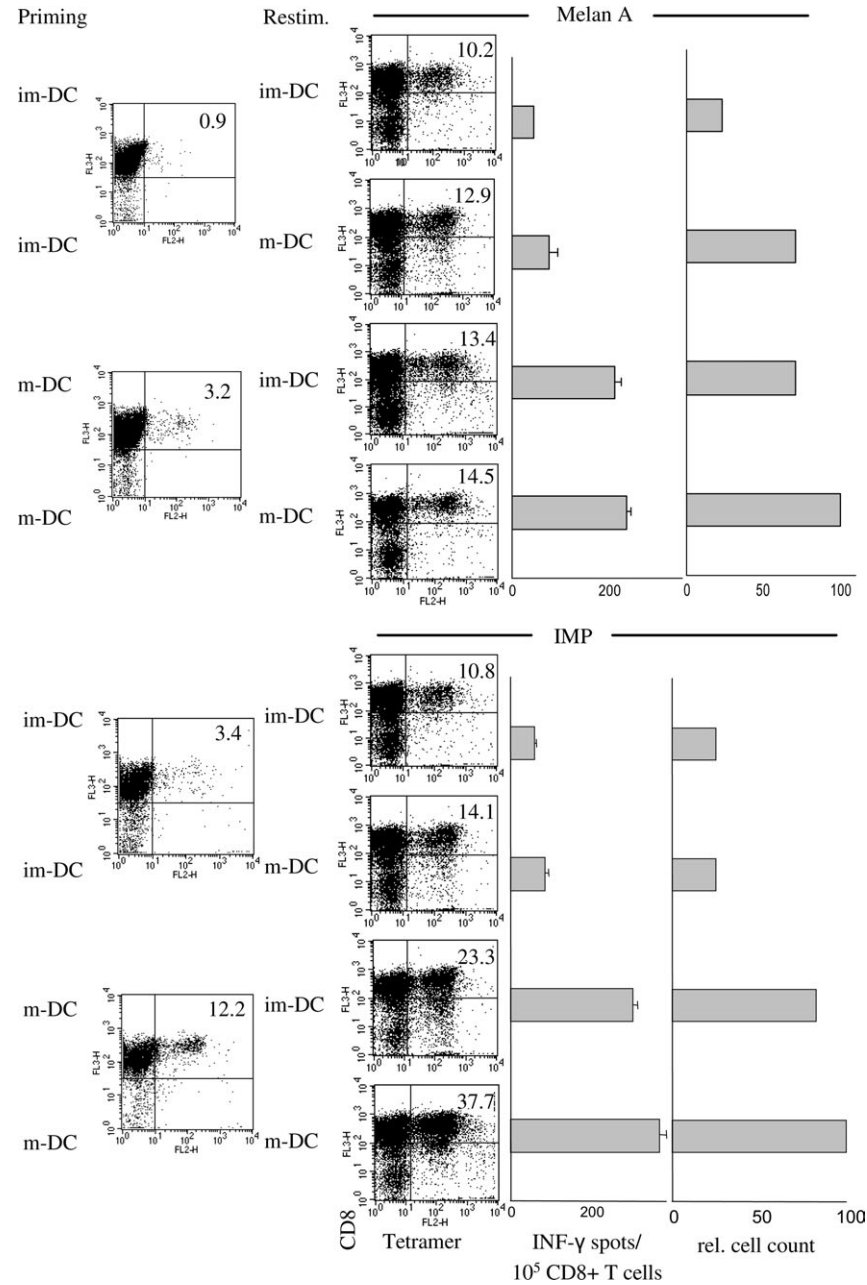


Fig. 3. Induction/Expansion of Melan-A.A2 and IMP.A2-specific CTL by peptide-loaded m-DCs versus im-DCs. HLA-A*0201⁺ m-DCs and im-DCs were pulsed with Melan-A.A2 and IMP.A2 peptides (10 μ M for 1 h) and co-cultured with autologous CD8⁺ T cells at a 1 : 20 ratio. IL-2 was added at 20 U ml⁻¹ every other day. Two weekly re-stimulations were performed with m-DCs or im-DCs as indicated. One week after the last stimulation T cells were harvested for tetramer staining. IFN- γ production was analyzed in ELISPOT (depicted as spots above background) and IL-10 production was analyzed in the supernatant by ELISA (not shown). One representative result of six independent experiments is shown.

ana peptides were pulsed onto the same batch of DCs, a sizeable reduction in Melan-A tetramer staining was observed only with higher concentrations (50 and 100 μ M) of gp-100.A2 ana, while only very faint staining with the gp-100.A2 tetramer was observed even at highest concentrations of the gp-100.A2 ana peptide (Fig. 7). The combination of gp-100.A2 ana with Melan-A native peptide leads to a more pronounced decrease in Melan-A tetramer staining, readily detectable at the lowest amount of gp-100.A2 ana added and resulting in a 4-fold decrease of staining with the highest

amount of gp-100.A2 ana (100 μ M). On the other hand, gp-100.A2 tetramer-positive cells were already clearly generated at the lowest concentration of gp-100.A2 ana if combined with the wild-type Melan-A.A2 peptide and increased with higher concentrations of gp-100.A2 ana peptide used (Fig. 7). Still gp-100 tetramer staining was markedly suppressed when gp-100.A2 ana and Melan-A.A2 native were simultaneously loaded, compared with gp-100.A2 ana loaded only. This indicates that both peptide affinity and precursor frequency play a role in the induction of tumor-specific CTL.

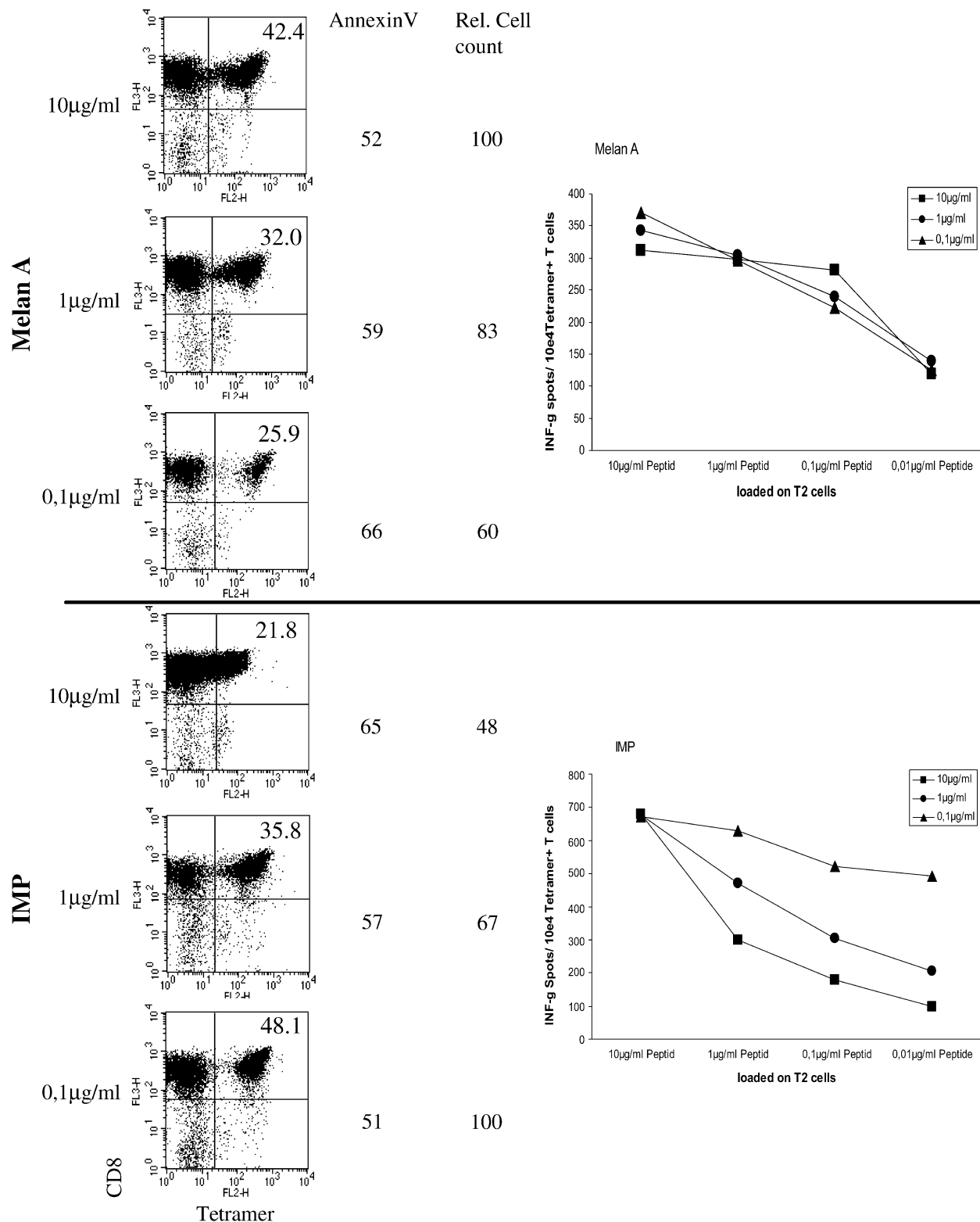


Fig. 4. Induction/Expansion of antigen-specific CD8⁺ T cells with m-DCs loaded with different concentrations of antigenic peptide. CD8⁺ T cells were stimulated with syngeneic m-DCs loaded with different concentrations of Melan-A.A2 and/or IMP.A2 peptide at a 20 : 1 ratio. IL-2 (20 U ml⁻¹) was added every other day. Seven days after the second re-stimulation cells were harvested, counted and stained with specific tetramers and Annexin V. Part of the tetramer-labeled T cells were sorted with anti-PE microbeads and used in a standard IFN-γ ELISPOT assay against T2 cells loaded with graded peptide concentrations. One representative result of five independent experiments is shown.

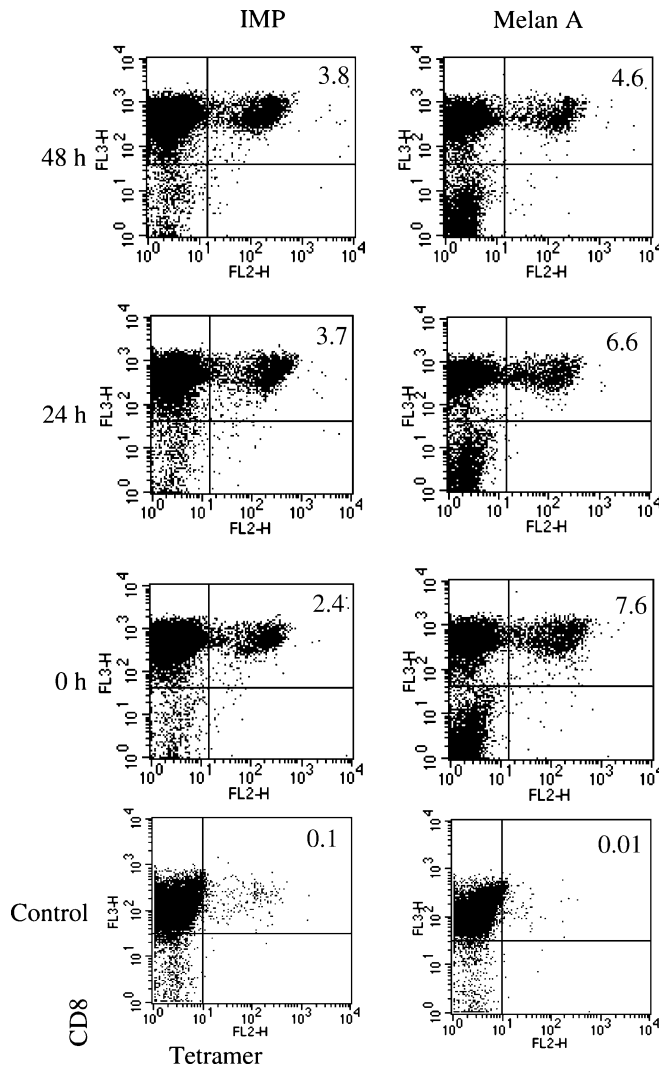
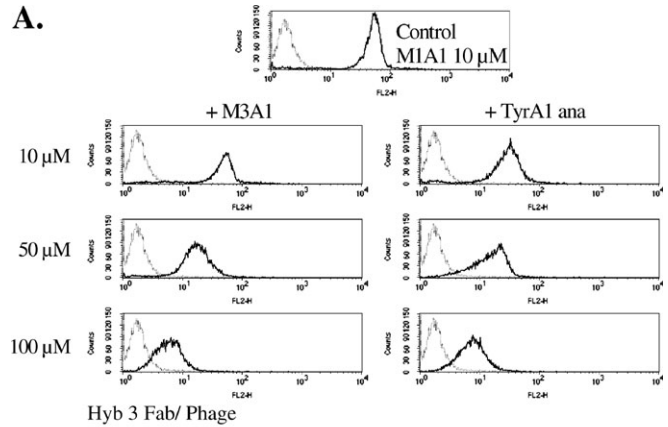


Fig. 5. Effects of different chase durations on the induction/expansion of antigen-specific CD8⁺ T cells. DCs were loaded with 10 μ M of Melan-A.A2 ana and IMP.A2 peptides for 1 h. Subsequently, cells were washed extensively and used either directly or after another incubation period of 24 to 48 h, without further addition of peptide. Co-culture with syngeneic CD8⁺ T cells was set up at a 1 : 20 ratio. After two rounds of re-stimulation T cells were harvested, stained with the respective tetramer and analyzed by FACS. One representative result of five independent experiments is shown.

HLA class II presentation

Im-DCs present exogenous peptide more efficiently to CD4⁺ T_H than m-DCs. HLA-DPB1*0401 expressing im-DCs and m-DCs were loaded for 1 h with either 1 or 10 μ M of peptide MAGE-3.DP4 and then monitored right away or after chase periods up to 96 h using the MAGE-3.DP4-specific CD4⁺ T cell clone R12-57 (31). As shown in Fig. 8(A) production of IFN- γ by the T cells was much stronger when stimulated with im-DCs as compared with m-DCs. Furthermore, im-DCs were able to stimulate the T cells significantly longer (up to 96 h) after peptide loading than m-DCs. This difference in antigen presentation could be partially overcome using higher peptide concentrations (10 μ M) (Fig. 8B). As it has been shown that im-DCs rapidly and massively up-regulate their HLA class II–

A.



B.

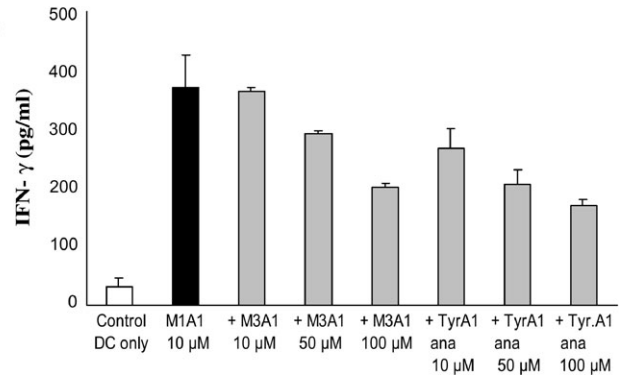
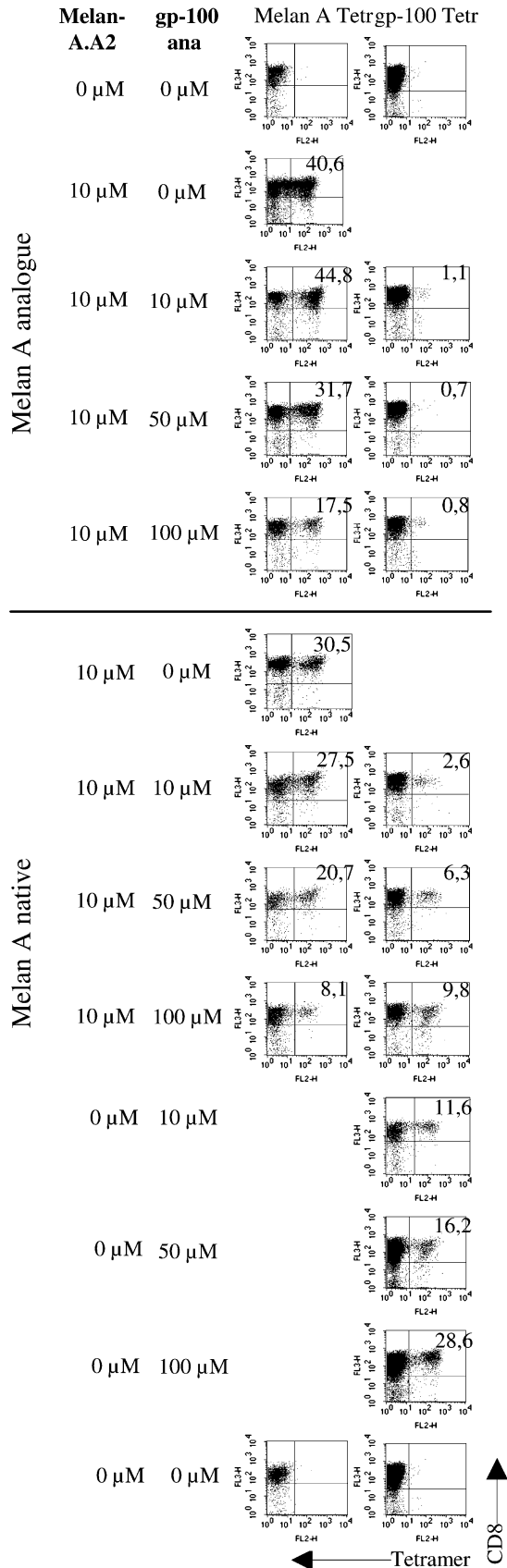


Fig. 6. Antigenic competition on DCs simultaneously loaded with different peptides competing for the same MHC class I molecule. HLA-A1⁺ m-DCs were loaded with either 10 μ M of MAGE-1.A1 alone or 10 μ M of MAGE-1.A1 and MAGE-3.A1 or Tyrosinase.A1 ana as competing peptides at indicated concentrations for 1 h. Thereafter, DCs were co-incubated with a MAGE-1.A1-specific CTL line and the supernatant was analyzed by IFN- γ ELISA after 18 h of co-culture (A) or stained with Fab/Phage Hyb3 and analyzed by FACS (B). One representative result of three independent experiments is shown.

peptide complexes on the cell surface upon maturation (39–41) we wondered whether it would be optimal to load peptide onto im-DCs and subsequently mature them overnight in the continued presence of peptide. Surprisingly, this procedure did not enhance the stimulation of the T cell clone as compared with peptide-loaded m-DCs (data not shown).

HLA-DM is expressed on the surface of im-DCs but not m-DCs. It had already been demonstrated that im-DCs in contrast to m-DCs express functional surface HLA-DM, a molecular chaperone which serves as an editor for loading empty HLA class II molecules with high-stability peptides (42). Because this might be one explanation for our observations we wondered whether this was also true for the monocyte-derived DCs used in our system. Indeed, as shown in Fig. 9, im-DCs expressed HLA-DM on their cell surface while m-DCs did not.

Expansion of tetanus toxoid-specific T_H by peptide-loaded im-DCs versus m-DCs. While im-DCs and m-DCs were similarly effective in the induction of peptide-specific IFN- γ -secreting-CD4⁺ T cells (24 versus 21 positive wells out of 96), significantly more IL-4-producing (10 versus 4 positive wells out of 96) and IL-10-producing (15 versus 8 positive wells out of 96) CD4⁺



T cells were induced by the im-DCs (Fig. 10). Despite the superior loading of im-DCs with exogenous peptide these results support the use of m-DCs for vaccination, as tumor-specific T_H1 responses are thought to be more effective in anti-tumor immunity. Therefore, further experiments were only performed with m-DCs.

Antigenic competition for a given HLA class II molecule influences the expansion of antigen-specific $CD4^+$ T cells by peptide-loaded DCs. Loading DCs with the prototype class II peptide MAGE-3.DP4 in the presence of other competing peptides also restricted by HLA-DP4 reduced the activation of the MAGE-3.DP4-specific $CD4^+$ T cell clone R12-57 in a dose-dependent manner (Fig. 11). Nevertheless, there was still a remarkably significant stimulation even when the DCs were loaded with two additional peptides competing for the same HLA molecule. Next, we used a bulk T cell stimulation assay rather than a T cell clone to explore the potential impact of peptide competition in a more demanding setting of a recall assay. Bulk $CD4^+$ T cells of healthy donors were stimulated with autologous m-DCs loaded with the TT.DP4 peptide in the presence or absence of an HLA-DP4-binding irrelevant peptide. Although we observed a clear recall response in both settings (with or without antigen competition), activation of TT-specific $CD4^+$ T_H appeared less effective in the presence of a competing peptide (Fig. 12) and occasionally (data not shown) became essentially negative.

Discussion

The detection of HLA class I-peptide complexes was carried out with a TCR-like antibody that was selective for a MAGE-1 peptide presented by HLA-A1. While rare, suitable TCR-like antibodies generated by conventional strategies have been available for some time in mice (43, 44). Antibodies specifically detecting human HLA class I-peptide complexes have only recently become available by the Fab/Phage library approach (9). This is the first study evaluating such an antibody as a new tool for direct monitoring of HLA class I-peptide complexes on the cell surface. A TCR-like antibody specifically recognizing HLA-A1 molecules loaded with a MAGE-1 peptide was the first to be described (8). Fab/Phage fragments directly rather than the purified affinity-matured Fab fragments proved feasible for FACS staining of DCs.

The phage antibody Hyb3 was used to determine relative differences in the quantity of HLA-peptide complexes on DCs. Differences in levels of HLA-peptide complexes were apparent in the concentration range of 1–10 μ M, and a plateau of HLA-peptide complex formation was reached with 10 μ M of peptide loaded for 1 h at 37°C in RPMI supplemented with 1%

Fig. 7. Influence of simultaneous loading of peptides competing for the same MHC class I molecule on the induction of antigen-specific $CD8^+$ T cells by peptide-loaded DCs. HLA-A2 $^+$ DCs were either loaded simultaneously with 10 μ M Melan-A.A2 native/ana and gp-100.A2 ana at indicated concentrations or with the respective peptides alone at 10 μ M. Co-cultures were set up with autologous $CD8^+$ T cell at a 1 : 20 ratio. IL-2 was added at 20 U ml $^{-1}$ every other day and two restimulations were performed at weekly intervals. Seven days after the last stimulation cells were harvested for tetramer analysis. One representative result of six independent experiments is shown.

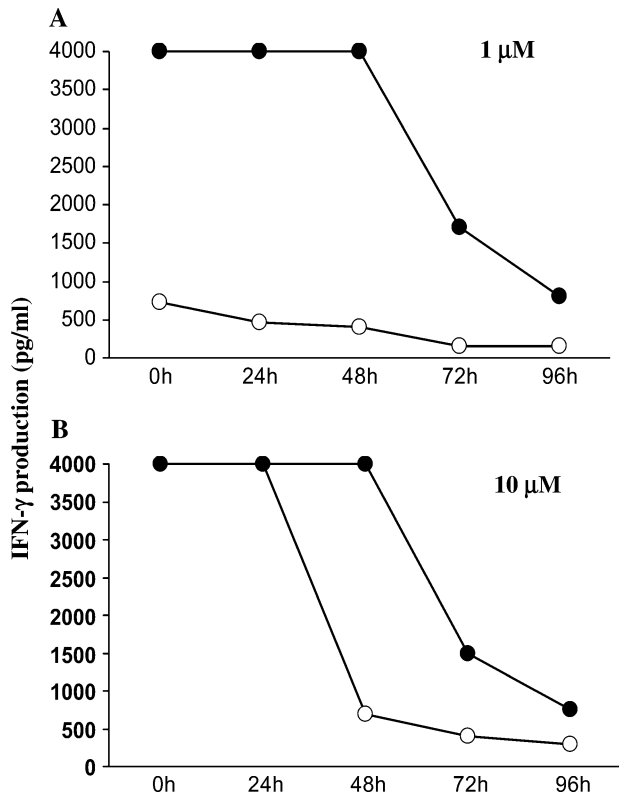


Fig. 8. Stimulation of MAGE-3-specific CD4⁺ T_h by peptide-loaded im-DCs versus m-DCs. HLA-DP4 expressing im-DCs (filled circle) and m-DCs (open circle) were loaded with different concentrations of peptide for 1 h, washed and used as stimulator cells at the indicated time points. The MAGE-3.DP4-specific T cell clone R12-57 (4000 cells) was co-cultured with 15 000 DCs and 18 h later IFN- γ secreted in the supernatant was measured by ELISA. Values shown are the mean of triplicate determinations. One representative result of five independent experiments is shown.

heat-inactivated autologous plasma (Fig. 1). Loading at 20°C or 4°C was inferior while loading in serum/plasma-free salt solutions made no difference, and longer loading time or higher peptide concentrations did not augment the maximum amount of HLA-peptide complexes on the surface of DCs. This finding was reassuring in that such loading conditions are widely used for exogenous peptide pulsing. Of note is that loading overnight during maturation did not bear an advantage in our model setting and may actually be inferior in case of unstable peptides (45). The stability of HLA-peptide complexes is a critical determinant of immunogenicity *in vitro* and *in vivo* (19, 46), and analogue peptides with increased HLA-binding properties have been developed to prolong the short half-life of HLA-peptide complexes (19). Using this semi-quantitative TCR-like antibody approach, we found that unequivocal antibody staining was detectable only for up to 24 h on m-DCs pulsed with 10 μ M peptide, suggesting that the longevity of the HLA-peptide complex was indeed rather short. As T cell clones detect 100 or less MHC-peptide complexes (32, 47, 48) we also used a MAGE-1.A1-specific CTL line and found that complexes were still clearly detectable at 48 h on DCs pulsed with 10 μ M but surprisingly no longer on DCs pulsed with just 1 μ M at onset. These data underscore that besides the properties of the particular peptide, other

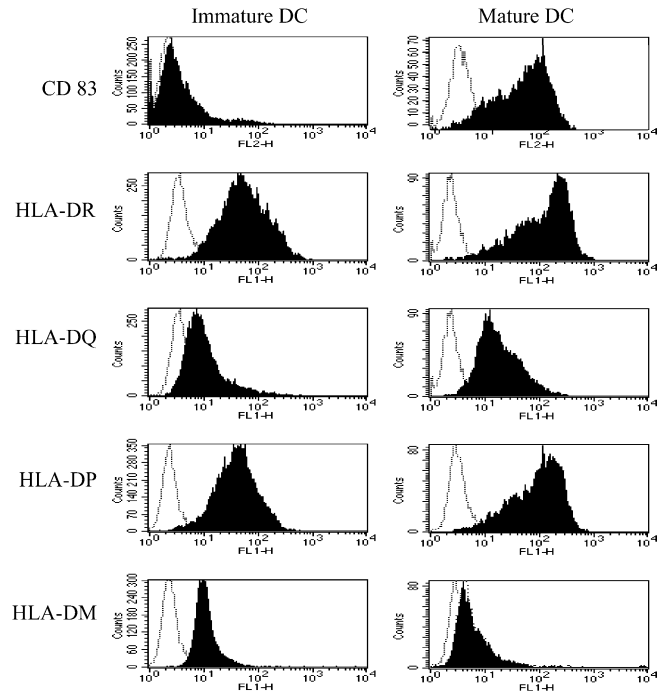


Fig. 9. Differential surface expression of HLA-DM by im-DCs and m-DCs. Im-DCs and m-DCs were labeled with antibodies against CD83, HLA-DM, HLA-DR, HLA-DQ and HLA-DP and expression was analyzed by flow cytometry.

factors including, peptide concentration, duration of pulse and time until encounter with T cells, will determine the actual amount of HLA-peptide complexes available for T cell stimulation and will directly influence the immunogenicity (49). These facts must, therefore, now be considered in designing and interpreting vaccination protocols. This holds true, for example for comparison of various routes such as intracutaneous, intranodal/intralympathic or intravenous. Indeed the reported higher immunogenicity of the intracutaneous over the intravenous administration of peptide-loaded DCs might in fact have simply been caused by significant differences in the amount of HLA-peptide complexes due to a much longer time interval until encounter of intravenously administered DCs with T cells (50). Our experiments also indicate that TCR-like antibodies will be preferred tools to quantitate HLA-peptide complexes for validating DC vaccines and for evaluating various potential strategies to prolong the half-life of immunogenic complexes (e.g. exogenous loading with peptide analogues, loading from the inside of the cell upon delivery of long peptides or coupling to translocating peptides, protein as immune complexes, transfection, etc.).

Many DC trials including even more recent ones have employed immature monocyte-derived DCs and some researchers continue to favor them even though there is a well-founded concern that such DCs might be either only weakly immunogenic or even tolerogenic (35, 51, 52). We directly compared the peptide-loading and immunostimulatory properties of im-DCs with those of m-DCs *in vitro*. Im-DCs proved less efficient in presenting exogenous HLA class I-restricted peptide in line with observations in the mouse and human system (39, 44, 53). Im-DCs showed fewer HLA-peptide

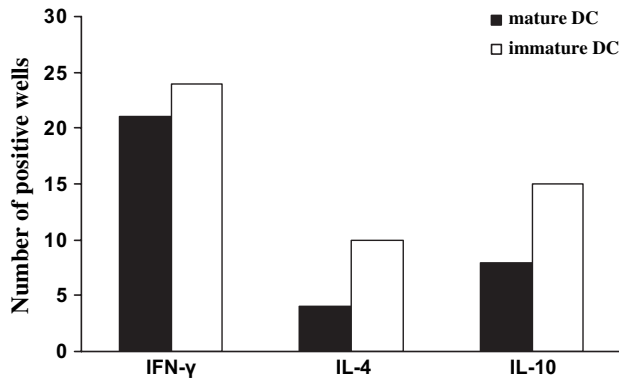


Fig. 10. Induction of TT-specific T_h by peptide-loaded m-DCs versus im-DCs. $CD4^+$ T cells were stimulated twice with im-DCs or m-DCs loaded with the TT peptide presented by HLA-DP4. On day 14 microcultures were analyzed for their ability to produce cytokines after stimulation with peptide by ELISA. Microwells were considered positive when the cytokine production was more than two times the mean value of the negative control plus standard deviation. The columns represent the number of microwells containing TT-specific T cells out of 96 microwells. One representative result of three independent experiments is shown.

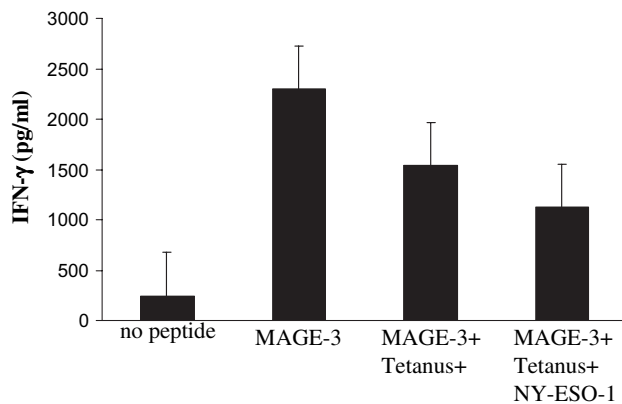


Fig. 11. Antigenic competition on DCs simultaneously loaded with different peptides competing for the same HLA class II molecule. M-DCs were loaded for 1 h with the MAGE-3.DP4 peptide alone or in the presence of other peptides competing for HLA-DP4 molecules at a concentration of 5 μ M for each peptide. After washing, DCs (15 000) were co-cultured with the MAGE-3.DP4-specific $CD4^+$ T cell clone R12-57 and 16 h later IFN- γ secreted in the supernatant was measured by ELISA. Values shown are the means of triplicate determinations. One representative result of four independent experiments is shown.

complexes on their surface which in addition had a very short half-life and were no longer detectable after a few hours (Fig. 2). The reduced level of HLA-peptide complexes on im-DCs can be partially explained by a constitutively lower HLA class I expression on im-DCs compared with m-DCs as detected by flow cytometric analysis using a class I-specific antibody (data not shown). In addition, it has been shown that im-DCs have a higher membrane turnover, resulting in a shorter half-life of HLA-peptide complexes (53). As expected, im-DCs were also inferior in priming naive (Melan-A-specific) as well as memory (IMP-specific) $CD8^+$ T cell responses (Fig. 3). Of note was that the type of DC used for initial stimulation had the biggest impact on $CD8^+$ T cell responses. The use of im-DCs for initial stimulation resulted in reduced numbers of tetramer-

positive T cells, lower levels of IFN- γ production and lower absolute cell numbers. After re-stimulation the quantitative difference in tetramer-positive cells was still clearly visible for IMP but less obvious for Melan-A. However, ELISPOT analysis clearly demonstrated a difference in the IFN- γ production in both models. These findings suggest that priming of naive T cells (Melan-A) with im-DCs can result in the induction of non-functional T cells.

Dhodapkar *et al.* have observed that im-DCs loaded with IMP peptide down-regulated IFN- γ -producing IMP-specific $CD8^+$ T cells in the blood and induced IL-10-producing IMP-specific $CD8^+$ regulatory T cells which were also suppressive *in vitro* (35, 54). We could not detect IL-10-producing and -suppressive T cells in our *in vitro* system so that the mechanisms (*in vivo* versus *in vitro*) responsible for the decreased responsiveness of the T cells exposed to im-DCs remain to be established. Our data provide additional arguments that im-DCs if used for vaccination will be insufficient to induce immunity. In fact in a recent two-armed clinical study immature peptide-loaded DCs proved essentially non-immunogenic (55). In contrast to the authors of this study we would, however, not conclude that DC vaccination is less effective than simple peptide vaccination. Rather, im-DCs appear not suitable and should not be used for inducing immunity, yet it should be systematically explored whether they are effective for inducing antigen-specific T cell tolerance.

In a further set of experiments we sought to evaluate the influence of the HLA-peptide complex density on priming/expansion of antigen-specific T cells by m-DCs. IMP served as a model for expansion of memory $CD8^+$ T cells. Melan-A was chosen to study the priming of naive T cell responses as it has been shown that in healthy donors sizeable amounts of truly naive Melan-A.A2-specific $CD8^+$ T cells exist (33). Interestingly, we found marked differences regarding the effect of HLA-peptide density between the two antigens which have not been emphasized so far. Priming of Melan-A-specific $CD8^+$ T cells was quantitatively (relative and absolute numbers of tetramer-positive T cells) increased with higher peptide concentrations, while expansion of IMP-specific $CD8^+$ T cells was more efficient at 10- (1 μ M) to 100-fold (0.1 μ M) lower peptide concentrations used for DC loading (Fig. 4). The markedly lower percentage and absolute numbers of IMP tetramer-positive $CD8^+$ T cells generated upon DC loading with the higher peptide concentration were paralleled by an increase in apoptosis rate as measured by Annexin V staining (Fig. 4), probably reflecting activation-induced cell death. In an ELISPOT assay with titrated amounts of peptides we could show that IMP-specific T cells primed with lower amounts of peptide seem to develop a higher affinity, as markedly lower peptide concentrations were sufficient to induce high amounts of IFN- γ production. This was also supported by an increase of the MFI of tetramer staining indicating higher affinity TCR (Fig. 4). Instead of loading DCs with different peptide concentrations we also chased DCs pulsed with 10 μ M for up to 48 h (Fig. 5) to mimic the time required for migration to lymph nodes upon intracutaneous injection which would be accompanied by a decrease in MHC-peptide complexes. Interestingly, the results were as implied by our preceding experimental data in that we saw a 40% reduction in Melan-A priming, yet an increase in expansion of IMP-specific T cells.

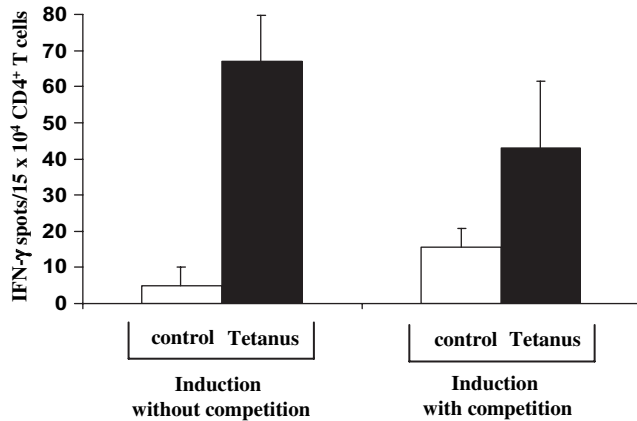


Fig. 12. Influence of antigen competition on the induction of TT-specific CD4⁺ T_H by peptide-loaded m-DCs. CD4⁺ T cells were stimulated weekly with autologous m-DCs loaded with the TT.DP4 peptide in the presence or absence of the MAGE-3.DP4 peptide as a peptide competing for HLA binding. On day 14 IFN- γ secretion by T cells after stimulation with the tetanus peptide or the NY-ESO-1.DP4 peptide as a negative control was measured by ELISPOT. Values shown are the means of duplicate determinations. One representative result of three independent experiments is shown.

Differences between the priming of a naive T cell response and the expansion of memory T cells are probably the explanation for our findings. It may be important in the future to measure the HLA-peptide density and to take it into account in the design and interpretation of human DC vaccines. Indeed, Ludewig *et al.* had reported previously that higher antigen concentrations are advantageous for priming of T cells against autoantigens by murine DCs which may need a certain threshold for the induction of specific T cells (56). If DC vaccines are used to boost primed responses, high amounts of antigens might, however, be counter-productive either by selection of low-affinity instead of high-affinity T cells (57, 58) or in case of supra-optimal peptide loading by even causing activation-induced cell death as previously described for T cell clones *in vitro* (59).

For HLA class II presentation im-DCs were—in sharp contrast to the findings for class I peptides—much more efficient in presenting exogenously loaded peptide to CD4⁺ T_H than m-DCs as also reported by other groups (42, 60). Plateau stimulation of a MAGE-3.DP4-specific T cell clone occurred upon pulsing with just 1 μ M of peptide and was still significant after a 96-h chase. In case of m-DCs higher peptide concentrations (10 μ M) were necessary to obtain efficient T cell stimulation (Fig. 8). At first glance these findings are in contrast to the well-established fact that DCs up-regulate the synthesis and cell surface expression of long-lived MHC class II-peptide complexes during their maturation (39, 41). Of note, we studied the loading of DCs with exogenous peptide which is a scenario different from the classical MHC class II presentation pathway of DCs. Indeed, it has been shown that im-DCs express abundant empty class II molecules and HLA-DM on their cell surface (42, 60), providing the prerequisites for an extracellular mode of antigen presentation. Furthermore, secretion of proteases by DCs, able to cleave a variety of protein antigens and to generate antigenic peptide fragments for presentation on class II molecules, has been demonstrated (60). Im-DCs may thus be equipped to sample the extracellular milieu by

producing and binding peptides. In this setting HLA-DM acts as a peptide editor, catalyzing the loading of empty class II peptides with suitable peptides. Once a peptide is bound with a sufficient affinity to the class II molecules it would no longer be degraded and its half-life increased, explaining the data from our pulse-chase experiments. Thus, the suggested alternative pathway might be an explanation for the efficient loading of im-DCs with exogenous peptides observed in this study. It is actually tempting to speculate that this property of im-DCs plays a role in their induction of regulatory T cells *in vivo* (51, 61, 62). M-DCs on the contrary have no empty class II molecules and no surface HLA-DM as their HLA class II molecules are all filled with peptides selected for binding in the endosomal loading compartments. Are im-DCs therefore the better candidates for peptide-based vaccination therapy with regard to the induction of tumor-specific CD4⁺ T_H? The answer is no, because repetitive stimulation with im-DCs might lead to the induction/expansion of non-proliferating IL-10-producing CD4⁺ T cells which could result in anergy or tolerance against tumor antigens (63). Furthermore, despite the fact that im-DCs were quite effective in the expansion of T_H1 memory responses, significantly more IL-4- and IL-10-producing TT-specific CD4⁺ T cells were induced *in vitro* as compared with m-DCs (Fig. 10). Therefore, despite the excellent presentation of exogenous peptide by im-DCs, we strongly recommend the use of m-DCs for vaccination. Very importantly, the peptide dose should be at least 10 μ M to assure sufficient loading of m-DCs with class II peptides. Indeed, m-DCs loaded with such doses of class II peptides have proven effective in rapidly inducing T_H1 responses in melanoma patients (31, 64).

We finally addressed the implications of concurrent loading of DCs with several peptides competing for the same HLA class I or II molecule. When we evaluated the simultaneous binding of several HLA class I peptides we were quite surprised to find rather marginal effects even if high-affinity binding analogue peptides were included and a TCR-like antibody was used which allows a more direct quantification of MHC complexes as compared with T cell clones (Fig. 6B). A similar situation was observed upon simultaneous loading of several HLA class II peptides restricted by the same HLA class II molecule (Fig. 11). Thus, loading of HLA molecules on DC surfaces cannot be simply predicted from the binding properties of peptides as calculated by computer algorithms. It was possible to expand tetanus toxoid-specific CD4⁺ T cells even when DCs were simultaneously loaded with the tetanus-toxoid peptide and a second HLA-DP4-binding peptide, but the expansion was reduced, indicating that competition of the two peptides for the HLA-DP4 molecules does even play a role in the expansion of strong memory T cell responses where the T cell frequency should be high (Fig. 12).

We used Melan-A.A2 native and the high-affinity Melan-A.A2 ana concurrently with the high-affinity binder gp-100.A2 ana and used only volunteer blood exhibiting sizeable CTL precursor frequencies that would allow unambiguous priming after a single stimulation *in vitro*. Induction of Melan-A-specific CTL was more abundant when Melan-A.A2 ana was used and though a decrease in CTL quantity was observed with increasing concentrations of gp-100.A2 ana, this effect was much more pronounced when Melan-A.A2 native competed with gp-100 ana (Fig. 7), indicating that concurrent loading

with different peptides favors the induction of CTL specific for the peptide with the highest affinity. The induction of gp-100.A2-specific CTL was virtually abolished by the concurrent loading of with Melan-A.A2 ana. Even if the lower affinity Melan-A.A2 native peptide was used, a reduction in gp-100.A2 CTL quantity by a factor of 12 could be observed. These results demonstrate that at least for mechanistic studies it is not advisable to load multiple peptides on one batch of DCs, as it is not possible to overcome lower binding affinity of a given peptide by increasing the concentration and the individual precursor frequency also plays a very important role. The precursor frequency is less predictable than peptide affinities, as it varies considerable from patient to patient and antigen to antigen.

In summary, the formation and half-life of HLA-peptide complexes upon loading of DCs depends on various parameters and has marked, discriminate and in part negative influences on ensuing T cell responses. DC vaccines have so far not been validated with respect to the amount and half-life of the HLA-peptide complexes. Our study not only shows that such validation will be critical for the further systematic development of this vaccination strategy but it also demonstrates that the use of TCR-like antibodies specific for a particular HLA-peptide complex might be the tool of choice.

Acknowledgements

We thank Mrs. H. Ploettner, S. Dotterweich, S. Emmerling and E. Müller for their excellent technical assistance. This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (SCHU 1264/2-1/2) (to E.S.S.).

Abbreviations

ana	analogue
APC	antigen-presenting cell
DC	dendritic cell
EBV B cells	EBV-transformed B cells
ELISPOT	enzyme-linked immunospot
GM-CSF	granulocyte macrophage colony-stimulating factor
im-DC	immature dendritic cell
IMP	Influenza matrix peptide
m-DC	mature dendritic cell
MFI	mean fluorescence intensity
TNF	tumor necrosis factor
TT	tetanus toxin

References

- Wang, R. F. 2001. The role of MHC class II-restricted tumor antigens and CD4⁺ T cells in antitumor immunity. *Trends Immunol.* 22:269.
- van der, B. P., Zhang, Y., Chaux, P. *et al.* 2002. Tumor-specific shared antigenic peptides recognized by human T cells. *Immunol. Rev.* 188:51.
- Ridgway, D. 2003. The first 1000 dendritic cell vaccinees. *Cancer Invest.* 21:873.
- Cerundolo, V., Hermans, I. F. and Salio, M. 2004. Dendritic cells: a journey from laboratory to clinic. *Nat. Immunol.* 5:7.
- Figdor, C. G., De Vries, I. J., Lesterhuis, W. J. and Melief, C. J. 2004. Dendritic cell immunotherapy: mapping the way. *Nat. Med.* 10:475.
- Steinman, R. M. and Dhodapkar, M. 2001. Active immunization against cancer with dendritic cells: the near future. *Int. J. Cancer* 94:459.
- Schuler, G., Schuler-Thurner, B. and Steinman, R. M. 2003. The use of dendritic cells in cancer immunotherapy. *Curr. Opin. Immunol.* 15:138.
- Chames, P., Hufton, S. E., Coulie, P. G., Uchanska-Ziegler, B. and Hoogenboom, H. R. 2000. Direct selection of a human antibody fragment directed against the tumor T-cell epitope HLA-A1-MAGE-A1 from a nonimmunized phage-Fab library. *Proc. Natl Acad. Sci. USA* 97:7969.
- Hoogenboom, H. R. 2002. Overview of antibody phage-display technology and its applications. *Methods Mol. Biol.* 178:1.
- Cohen, C. J., Denkberg, G., Lev, A., Epel, M. and Reiter, Y. 2003. Recombinant antibodies with MHC-restricted, peptide-specific, T-cell receptor-like specificity: new tools to study antigen presentation and TCR-peptide-MHC interactions. *J. Mol. Recognit.* 16:324.
- Nestle, F. O., Alijagic, S., Gilliet, M. *et al.* 1998. Vaccination of melanoma patients with pep. *Nat. Med.* 4:328.
- Banchereau, J., Palucka, A. K., Dhodapkar, M. *et al.* 2001. Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. *Cancer Res.* 61:6451.
- Palmowski, M. J., Choi, E. M., Hermans, I. F. *et al.* 2002. Competition between CTL narrows the immune response induced by prime-boost vaccination protocols. *J. Immunol.* 168:4391.
- Kedl, R. M., Kappler, J. W. and Marrack, P. 2003. Epitope dominance, competition and T cell affinity maturation. *Curr. Opin. Immunol.* 15:120.
- Jonuleit, H., Kuhn, U., Muller, G. *et al.* 1997. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol.* 27:3135.
- Chames, P., Willemsen, R. A., Rojas, G. *et al.* 2002. TCR-like human antibodies expressed on human CTLs mediate antibody affinity-dependent cytolytic activity. *J. Immunol.* 169:1110.
- Bhardwaj, N., Bender, A., Gonzalez, N., Bui, L. K., Garrett, M. C. and Steinman, R. M. 1994. Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human CD8⁺ T cells. *J. Clin. Invest.* 94:797.
- Romero, P., Gervois, N., Schneider, J. *et al.* 1997. Cytolytic T lymphocyte recognition of the immunodominant HLA-A*0201-restricted Melan-A/MART-1 antigenic peptide in melanoma. *J. Immunol.* 159:2366.
- Valmori, D., Fonteneau, J. F., Lizana, C. M. *et al.* 1998. Enhanced generation of specific tumor-reactive CTL *in vitro* by selected Melan-A/MART-1 immunodominant peptide analogues. *J. Immunol.* 160:1750.
- Parkhurst, M. R., Salgaller, M. L., Southwood, S. *et al.* 1996. Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. *J. Immunol.* 157:2539.
- Traversari, C., van der, B. P., Luescher, I. F. 1992. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J. Exp. Med.* 176:1453.
- Gaugler, B., Van den, E. B., van der, B. P. *et al.* 1994. Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J. Exp. Med.* 179:921.
- Kittlesen, D. J., Thompson, L. W., Gulden, P. H. *et al.* 1998. Human melanoma patients recognize an HLA-A1-restricted CTL epitope from tyrosinase containing two cysteine residues: implications for tumor vaccine development. *J. Immunol.* 160:2099.
- Schultz, E. S., Lethe, B., Cambiaso, C. L. *et al.* 2000. A MAGE-A3 peptide presented by HLA-DP4 is recognized on tumor cells by CD4⁺ cytolytic T lymphocytes. *Cancer Res.* 60:6272.
- Panina-Bordignon, P., Tan, A., Termijtelen, A., Demotz, S., Corradin, G. and Lanzavecchia, A. 1989. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur. J. Immunol.* 19:2237.
- Zeng, G., Wang, X., Robbins, P. F., Rosenberg, S. A. and Wang, R. F. 2001. CD4(+) T cell recognition of MHC class II-restricted epitopes from NY-ESO-1 presented by a prevalent HLA DP4 allele: association with NY-ESO-1 antibody production. *Proc. Natl Acad. Sci. USA* 98:3964.

- 27 Herr, W., Linn, B., Leister, N., Wandel, E., Meyer zum Buschenfelde, K. H. and Wolfel, T. 1997. The use of computer-assisted video image analysis for the quantification of CD8⁺ T lymphocytes producing tumor necrosis factor alpha spots in response to peptide antigens. *J. Immunol. Methods* 203:141.
- 28 Thurner, B., Haendle, I., Roder, C. *et al.* 1999. Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J. Exp. Med.* 190:1669.
- 29 Feuerstein, B., Berger, T. G., Maczek, C. *et al.* 2000. A method for the production of cryopreserved aliquots of antigen-preloaded, mature dendritic cells ready for clinical use. *J. Immunol. Methods* 245:15.
- 30 Thurner, B., Roder, C., Dieckmann, D. *et al.* 1999. Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. *J. Immunol. Methods* 223:1.
- 31 Schultz, E. S., Schuler-Thurner, B., Stroobant, V. *et al.* 2004. Functional analysis of tumor-specific Th cell responses detected in melanoma patients after dendritic cell-based immunotherapy. *J. Immunol.* 172:1304.
- 32 Reay, P. A., Matsui, K., Haase, K., Wulfig, C., Chien, Y. H. and Davis, M. M. 2000. Determination of the relationship between T cell responsiveness and the number of MHC-peptide complexes using specific monoclonal antibodies. *J. Immunol.* 164:5626.
- 33 Pittet, M. J., Valmori, D., Dunbar, P. R. *et al.* 1999. High frequencies of naive Melan-A/MART-1-specific CD8(+) T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2 individuals. *J. Exp. Med.* 190:705.
- 34 Fonteneau, J. F., Larsson, M., Somersan, S. *et al.* 2001. Generation of high quantities of viral and tumor-specific human CD4⁺ and CD8⁺ T-cell clones using peptide pulsed mature dendritic cells. *J. Immunol. Methods* 258:111.
- 35 Dhodapkar, M. V., Steinman, R. M., Krasovsky, J., Munz, C. and Bhardwaj, N. 2001. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J. Exp. Med.* 193:233.
- 36 Dhodapkar, M. V., Steinman, R. M., Sapp, M. *et al.* 1999. Rapid generation of broad T-cell immunity in humans after a single injection of mature dendritic cells. *J. Clin. Invest.* 104:173.
- 37 De Vries, I. J., Krooshoop, D. J., Scharenborg, N. M. *et al.* 2003. Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. *Cancer Res.* 63:12.
- 38 Kawakami, Y. and Rosenberg, S. A. 1996. T-cell recognition of self peptides as tumor rejection antigens. *Immunol. Res.* 15:179.
- 39 Cella, M., Engering, A., Pinet, V., Pieters, J. and Lanzavecchia, A. 1997. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* 388:782.
- 40 Pierre, P., Turley, S. J., Gatti, E. *et al.* 1997. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature* 388:787.
- 41 Inaba, K., Turley, S., Iyoda, T. *et al.* 2000. The formation of immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. *J. Exp. Med.* 191:927.
- 42 Arndt, S. O., Vogt, A. B., Markovic-Plese, S. *et al.* 2000. Functional HLA-DM on the surface of B cells and immature dendritic cells. *EMBO J.* 19:1241.
- 43 Porgador, A., Yewdell, J. W., Deng, Y., Bennink, J. R. and Germain, R. N. 1997. Localization, quantitation, and *in situ* detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity* 6:715.
- 44 Kukutsch, N. A., Rossner, S., Austyn, J. M., Schuler, G. and Lutz, M. B. 2000. Formation and kinetics of MHC class I-ovalbumin peptide complexes on immature and mature murine dendritic cells. *J. Invest. Dermatol.* 115:449.
- 45 Amoscato, A. A., Prenovitz, D. A. and Lotze, M. T. 1998. Rapid extracellular degradation of synthetic class I peptides by human dendritic cells. *J. Immunol.* 161:4023.
- 46 Romero, P., Valmori, D., Pittet, M. J. *et al.* 2002. Antigenicity and immunogenicity of Melan-A/MART-1 derived peptides as targets for tumor reactive CTL in human melanoma. *Immunol. Rev.* 188:81.
- 47 Harding, C. V. and Unanue, E. R. 1990. Quantitation of antigen-presenting cell MHC class II-peptide complexes necessary for T-cell stimulation. *Nature* 346:574.
- 48 Kimachi, K., Croft, M. and Grey, H. M. 1997. The minimal number of antigen-major histocompatibility complex class II complexes required for activation of naive and primed T cells. *Eur. J. Immunol.* 27:3310.
- 49 Walter, S., Herrgen, L., Schoor, O. *et al.* 2003. Cutting edge: predetermined avidity of human CD8 T cells expanded on calibrated MHC/anti-CD28-coated microspheres. *J. Immunol.* 171:4974.
- 50 Eggert, A. A., Schreurs, M. W., Boerman, O. C. *et al.* 1999. Biodistribution and vaccine efficiency of murine dendritic cells are dependent on the route of administration. *Cancer Res.* 59:3340.
- 51 Steinman, R. M., Hawiger, D. and Nussenzweig, M. C. 2003. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* 21:685.
- 52 Jonuleit, H., Giesecke-Tuettenberg, A., Tuting, T. *et al.* 2001. A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. *Int. J. Cancer* 93:243.
- 53 Zehn, D., Cohen, C. J., Reiter, Y. and Walden, P. 2004. Extended presentation of specific MHC-peptide complexes by mature dendritic cells compared to other types of antigen-presenting cells. *Eur. J. Immunol.* 34:1551.
- 54 Dhodapkar, M. V. and Steinman, R. M. 2002. Antigen-bearing immature dendritic cells induce peptide-specific CD8(+) regulatory T cells *in vivo* in humans. *Blood* 100:174.
- 55 Slingluff, C. L., Jr, Petroni, G. R., Yamshchikov, G. V. *et al.* 2003. Clinical and immunologic results of a randomized phase II trial of vaccination using four melanoma peptides either administered in granulocyte-macrophage colony-stimulating factor in adjuvant or pulsed on dendritic cells. *J. Clin. Oncol.* 21:4016.
- 56 Ludewig, B., McCoy, K., Pericin, M. *et al.* 2001. Rapid peptide turnover and inefficient presentation of exogenous antigen critically limit the activation of self-reactive CTL by dendritic cells. *J. Immunol.* 166:3678.
- 57 Bullock, T. N., Colella, T. A. and Engelhard, V. H. 2000. The density of peptides displayed by dendritic cells affects immune responses to human tyrosinase and gp100 in HLA-A2 transgenic mice. *J. Immunol.* 164:2354.
- 58 Bullock, T. N., Mullins, D. W. and Engelhard, V. H. 2003. Antigen density presented by dendritic cells *in vivo* differentially affects the number and avidity of primary, memory, and recall CD8⁺ T cells. *J. Immunol.* 170:1822.
- 59 Alexander-Miller, M. A., Derby, M. A., Sarin, A., Henkart, P. A. and Berzofsky, J. A. 1998. Supraoptimal peptide major histocompatibility complex causes a decrease in Bcl-2 levels and allows tumor necrosis factor alpha receptor II-mediated apoptosis of cytotoxic T lymphocytes. *J. Exp. Med.* 188:1391.
- 60 Santambrogio, L., Sato, A. K., Carven, G. J., Belyanskaya, S. L., Strominger, J. L. and Stern, L. J. 1999. Extracellular antigen processing and presentation by immature dendritic cells. *Proc. Natl Acad. Sci. USA* 96:15056.
- 61 Lutz, M. B. and Schuler, G. 2002. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol.* 23:445.
- 62 Apostolou, I. and von Boehmer, H. 2004. *In vivo* instruction of suppressor commitment in naive T cells. *J. Exp. Med.* 199:1401.
- 63 Jonuleit, H., Schmitt, E., Schuler, G., Knop, J. and Enk, A. H. 2000. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J. Exp. Med.* 192:1213.
- 64 Schuler-Thurner, B., Schultz, E. S., Berger, T. G. *et al.* 2002. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. *J. Exp. Med.* 195:1279.