Optimizing the exogenous antigen loading of monocyte-derived dendritic cells

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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 (10⁻⁰⁰ M) concentrations generated clearly more Melan-A effectors than loading with 1 or 0.1 M 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 Th1 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
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 skew 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

RPMI 1640 (Bio Whittaker) supplemented with 1% heat-inactivated autologous plasma, 20 μM 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 RPMI 1640 supplemented with 10% heat-inactivated human pool serum. In both cases 20 μg 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) (Leukomax™, Novartis) and 800 U ml−1 IL-4 (Sandoz); IL-2 (Proleukin™, Chiron Corp.) was used at the concentrations indicated. For DC maturation, we used a cocktail consisting of 2 ng ml−1 IL-1β (Sigma), 1000 U ml−1 IL-6 (Sandoz), 10 ng ml−1 tumor necrosis factor-α (TNF-α) (Bender, Vienna, Austria) and 1 μg 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 Cinsalia (Laufelfingen, 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); gp100-A2 ana: IMDQVPSV (20).


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 RPMI medium, supplemented with IL-4 and GM-CSF. At day 6, a maturation cocktail (IL-1β, 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 Biotech). 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^+ Tp, 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 \times 10^5) were co-cultured with 1.5 \times 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 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).
Phage particles displaying Fab’s were then used to characterize optimal peptide-loading conditions, showing that 10 μM 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 ± 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 ~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 μM) 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 μM) 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 μM) (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 μM 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 μM 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.
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 μM 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 μM) 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. 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 μM 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.
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 μM) 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 μM). 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. 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 ana and IMP.A2 peptides (10 μM 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.
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 an 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.
HLA class II presentation

Im-DCs present exogenous peptide more efficiently to CD4+ Tn, than m-DCs. HLA-DPB1*0401 expressing im-DCs and m-DCs were loaded for 1 h with either 1 or 10 μM 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 peptide-loaded m-DCs and subsequently matured 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 Tn 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 peptide-loaded im-DCs as compared to m-DCs (32).
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<sub>h</sub>1 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> Th 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 μM, and a plateau of HLA–peptide complex formation was reached with 10 μM of peptide loaded for 1 h at 37°C in RMPI supplemented with 1%
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 μM 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 μM but surprisingly no longer on DCs pulsed with just 1 μM at onset. These data underscore that besides the properties of the particular peptide, other 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/intralymphatic 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 showed 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

![Image of Figure 8](image-url)

**Fig. 8.** Stimulation of MAGE-3-specific CD4+ Tn 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.

![Image of Figure 9](image-url)

**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-DR, HLA-DQ and HLA-DP and expression was analyzed by flow cytometry.
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 μM) to 100-fold (0.1 μM) 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 μM 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. 10.** Induction of TT-specific T₈ 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 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 μM for each peptide. After washing, DCs (15 000) were co-cultured with the MAGE-3.DP4-specific CD8+ 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.
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 cells 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 μM of peptide and was still significant after a 96-h chase. In case of m-DCs higher peptide concentrations (10 μM) 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 m-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 cells? 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 T1 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 μM 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 T1 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 considerably 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.

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Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ana</td>
<td>analogue</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>EBV B cells</td>
<td>EBV-transformed B cells</td>
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<tr>
<td>ELISpot</td>
<td>enzyme-linked immunospot</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony-stimulating factor</td>
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<td>im-DC</td>
<td>immature dendritic cell</td>
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<tr>
<td>m-DC</td>
<td>mature dendritic cell</td>
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<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>TT</td>
<td>tetanus toxin</td>
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References

Peptide loading of dendritic cells


