Efficiency of peptide presentation by dendritic cells compared with other cell types: implications for cross-priming

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Abstract

Dendritic cells (DCs) play a key role in the induction of cellular immune responses by harvesting antigens from peripheral tissue for cross-priming CD8⁺ T cells. It has been demonstrated that apoptotic bodies, whole- or degraded-cell-associated or soluble antigens as well as heat shock protein-bound peptides can be taken up, processed and cross-presented by DCs. Since cells are continuously releasing peptides from their surface MHC molecules, DCs in the tissues are exposed to such peptides and might process and present them to T cells as an additional pathway for cross-priming. To investigate this possibility, we compared and characterized the presentation of exogenous peptides by DCs and other cell types employing novel recombinant antibodies with TCR-like specificities for specific peptide–MHC complexes (pMHCs). These analyses reveal that loading of immature and mature DCs with peptide is far less efficient than it is for monocytes, T and B lymphocytes, B-lymphoblastoid, melanoma and TAP-deficient T2 cells. This inefficiency of peptide transfer to the MHC molecules of DCs makes it unlikely that these cells recycle peptides released from the MHC molecules of other cells and may explain why cross-presentation of such peptides has not yet been observed.

Introduction

Cross-presentation of antigens by dendritic cells (DCs) is likely the most important mechanism for inducing CD8 T cells responses (1). A number of studies have been carried out to elucidate both the mechanisms involved in cross-priming and the sources of cross-presented epitopes. It was reported that, in principle, soluble as well as cell-associated antigens could be cross-presented (1). For cell-associated antigens, attempts to identify the precursor molecules that are taken up and processed by DCs have precipitated contradicting findings pointing at chaperone-bound peptides (2), whole proteins (3), proteasome substrates (4) or other intermediates of the antigen-processing pathway (5). In addition to these sources, MHC-bound peptides could be recycled for cross-representation by DCs. Several studies have demonstrated large amounts of degenerated MHC heavy chain-only molecules at cell surfaces, indicating frequent dissociation of the MHC–peptide complexes and, thereby, release of MHC-bound peptides (6–8). It is, thus, likely that DCs in the tissues are exposed to such peptides. However, published data suggest that functional representation of MHC-bound peptides does not occur in vivo (4). To study the possibilities for the transfer of MHC-bound peptides from tissue cells to DCs, we analyzed uptake and loading of free soluble peptides onto the MHC class I molecules of immature dendritic cells (iDCs) and mature dendritic cells (mDCs) in comparison to B lymphocytes, CD4 T lymphocytes, monocytes, SK-mel-24 (SK) melanoma cells, B-lymphoblastoid JY and TAP-deficient T2 cells. Using recently developed recombinant antibodies with TCR-like specificities for defined HLA-A*0201–peptide complexes (9–11), we established the dose–response relationships between the peptide concentration used for pulsing the different cell types and the resulting surface densities of pMHCs and the responses of cognate T cells. Thereby, we observed that the efficiency of loading peptides onto...
HLA-A*0201 of both iDCs and mDCs is much lower than it is for the other cell types which might explain why functional presentation by DCs of peptides released from other cells has not been observed.

**Methods**

**Cells**

For DCs generation (12), PBMC of healthy donors were incubated for 1 h at 37°C in RPMI medium with 50 µM 2-mercaptoethanol (2-ME), 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin (all Gibco-Invitrogen, Karlsruhe, Germany) and 1% heat-inactivated human plasma in a tissue culture flask (Nalge Nunc International, Wiesbaden, Germany). Non-adherent cells were washed off with PBS. The adherent cells were cultured in the above medium plus 1000 U ml⁻¹ granulocyte macrophage colony-stimulating factor (Leukomax®, Essex Pharma, München, Germany) and 2000 U ml⁻¹ IL-4 (PromoCell, Heidelberg, Germany) and collected from the day 6 DC cultures before maturation. The DCs were harvested for the experiments between 24 and 30 h later. The iDCs were replenished after 48 h. On day 5, non-adherent cells were harvested, washed with PBS and cultured in fresh medium and cytokines as before. After 24 h, tumor necrosis factor α (10 ng ml⁻¹), IL-1β (10 ng ml⁻¹), IL-6 (25 ng ml⁻¹; all Strathmann Biotech, Hamburg, Germany) and PGE-E2 (0.2 µg ml⁻¹; Minprostin® E2, Pharmacia, Erlangen, Germany) were added to induce maturation. The mDCs were harvested and depleted with anti-CD11c microbeads (Miltenyi Biotec), respectively. T2, JY and SK were cultured in the above medium plus 1000 U ml⁻¹ granulocyte macrophage colony-stimulating factor (Leukomax®, Essex Pharma, München, Germany) and dissolved at 20 µg ml⁻¹ protein TAX were custom-synthesized by EMC microcollection (Tübingen, Germany) and dissolved at 20 µg ml⁻¹ in DMSO (Pierce, Weiterstadt, Germany). For peptide loading, the cells were washed once with DMEM/0.5% BSA and, unless stated otherwise, incubated in DMEM/0.5% BSA with the peptides at the indicated concentrations for 90 min at 26°C followed by 90 min at 37°C. After pulsing, the cells were washed to remove free peptide and kept at 4°C until analysis.

**Detection of pMHCs**

The recombinant Fab 4A9 with specificity for complexes of HLA-A*0201 with ILA (A2/ILA) was used for the analysis of pMHC with biotinylated polyclonal rabbit anti-human Fab IgG antibody (Acris/DPC Biermann, Hiddenhasen, Germany) as secondary and streptavidin–rPE (BD-Biosciences) as tertiary reagents. The biotinylated Fabs G2D12 and T3F2 with specificities for HLA-A*0201 complexes with KTW (A2/KTW) and LLF (A2/LLF), respectively, were tetramerized with streptavidin–rPE or streptavidin–APC (Molecular Probes, Leiden, Netherlands) and then used for cell staining. Where indicated, rPE staining was quantified with QuantiBRITE™ PE (BD-Biosciences). Total HLA-A*0201 expression was analyzed with FITC-labeled BB7.2 (BD-Biosciences) and quantified using Quantum Fluorescence Kit for the determination of ‘molecules of equivalent soluble fluorochrome’ units (Sigma).

**Generation and analysis of peptide-specific T cells**

T cells in freshly isolated PBMC were primed with peptide-loaded DCs from healthy donors. On day 2, 50 U ml⁻¹ IL-2 (Proleukin, Chiron, München, Germany) was added and refreshed every 2–3 days. After 14 days, CD4⁺ cells were removed by magnetic cell sorting and the CD8⁺ T cells re-stimulated with peptide-pulsed irradiated PBMC in the presence of IL-2. For the measurement of proliferation, T cells were labeled with 1 µM CSFE (Sigma) for 5 min at room temperature, washed and cultured together with peptide-loaded APC and 50 U ml⁻¹ IL-2. After 2 days, fresh IL-2 was added. After 4 days, the cells were harvested and counterstained with anti-CD8 APC (BD-Biosciences). The CSFE fluorescence signals of CD8⁺ T cells were analyzed by flow cytometry. FlowJo® analysis software was used to calculate the proliferation index which gives the average number of cell divisions in the proliferating population.

**Results**

**Specific detection of MHC–peptide complexes with recombinant Fabbs**

A major problem in studying antigen presentation is that pMHCs can be detected only by T cells so that it is neither possible to quantify the amount of peptide presented by an antigen-presenting cell nor possible to separate the antigen presentation from the co-stimulatory aspects of T cell activation. To overcome these limitations, we made use of recently developed recombinant antibodies with TCR-like specificities. The recombinant Fabbs employed in this study had been isolated from a large naive human Fab phage display library by repeated panning for clones that specifically bind complexes of soluble recombinant HLA-A*0201 and defined T cell epitopes (9–11). To confirm the specificities of the Fabbs, we analyzed their reactivity to the cell types used in this study after pulsing with the cognate epitope for the respective Fabbs or control peptides. Staining of T2 cells that, because of their TAP-deficiency, largely lack endogenously processed peptides served as additional background control. With T2, the
A2/KTW-specific Fab G2D12 only stains the KTW- but not ILA-pulsed cells (Fig. 1A) and inversely, the A2/ILA-specific Fab 4A9 only ILA-pulsed cells (Fig. 1H). In both cases, the staining for the negative control peptides was the same as for the unpulsed T2 cells proving that the increased cell surface expression of HLA-A*0201 by stabilization of the HLA molecules with the synthetic peptides does not increase nonspecific staining of the cells. Also, the staining of cells with normal TAP and, thereby, HLA expression was dependent on the cognate peptide for the respective Fab. G2D12 (Fig. 1B and G), 4A9 (Fig. 1I and J) and T3F2 (Fig. 1K and L) stained HLA-A*0201-expressing cells only after pulsing with their cognates epitope KTW, ILA and LLF, respectively, but not cells pulsed with other peptides. The cells tested were lymphoblastoid JY cells (Fig. 1B, I and K), SK melanoma cells (Fig. 1C), mDCs (Fig. 1D, J and L), monocytes (Fig. 1E), B lymphocytes (Fig. 1F) and T lymphocytes (Fig. 1G). A representative example of the phenotype of the mDCs used in this study is shown in the plots M and N.

Kinetics of peptide uptake and loading onto the MHC molecules of different cell types

The histograms in Fig. 1 already suggest that different cell types take up and present T cell epitopes with different efficiency. For a more accurate quantification of the efficiency of peptide handling, we pulsed the cells with serial dilutions of the KTW and determined the amount of specific A2/KTW complexes at the surfaces of the cells with the Fab G2D12. For an accurate comparison of the geometric mean fluorescence intensity (gMFI) values measured for the different cells

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**Fig. 1.** Detection of pMHCs by recombinant Fabs with TCR-like specificity. TAP-deficient T2 cells (A, H), B-lymphoblastoid cells JY (B, I, K), SK melanoma cells (C), monocytes, B and T lymphocytes (E–G, respectively) and DCs (D, J, L) were incubated with the indicated peptides and analyzed with the Fabs G2D12 specific for complexes of KTWGQYWQV and HLA-A*0201 [KTW/A2] (A–G), with 4A9 for complexes of ILAKFLHWL and HLA-A*0201 [ILA/A2] (H–J) or with T3F2 for complexes of LLFGYPVYV and HLA-A*0201 [LLF/A2] (K, L). The cells were pulsed for 1.5 h at 26°C followed by 1.5 h at 37°C with 20 μg/ml cognate peptide for the respective Fab (underlined) or irrelevant HLA-A*0201-binding control peptide, or were left untreated. The panels M and N show a representative four-color flow cytometry analysis of mDCs with anti-CD83, -CD80, -HLA-DR and -CD86 antibodies.
at different peptide concentration used for pulsing, we calibrated the gMFI values with standard PE latex beads included in the same measurements. The results of these analyses are shown in Fig. 2(A and B) as numbers of PE molecules per cell. Since the valency of the binding of the tetramerized G2D12 to A2/KTW at the different cell surface densities of the complexes is not known, we could not exactly calculate the numbers of pMHCs per cell. Nonetheless, the comparison of the loading rates of the different cell types shows strong differences. DCs require about 100-fold higher peptide concentrations than JY cells for a comparable number of A2/KTW complexes at their surfaces. B and T cells as well as monocytes, on the other hand, take up and present less of KTW than DCs. The high numbers of A2/KTW complexes detected at the surfaces of T2 cells even at relatively low peptide concentrations are due to the largely reduced numbers of endogenous epitopes available for loading into the HLA molecules. In fact, the initial phase of the titration curve is paralleled by the increase of total surface HLA-A*0201 expression levels (Fig. 2A). The increase of pMHCs at high peptide concentrations probably reflects replacement of TAP-independent endogenous peptides by KTW. The different cell types differed greatly in the levels of HLA expression. To assess the fraction of the HLA-A*0201 molecules occupied by KTW, we quantified the amount of the HLA molecules by flow cytometry, calibrated the gMFI for HLA-A*0201 expression with calibration beads and calculated the ratio of calibrated gMFI values for the specific A2/KTW complexes and total HLA-A*0201 (Fig. 2C). This results in a measure for the fractional occupancy of the HLA molecules with the specific peptide. In the cases of the tumor cell lines JY and SK as well as the primary monocytes and CD4 T cells, the pulsing concentrations of KTW translate in similar degree of occupancy of their surface HLA molecules with the specific peptide. DCs, however, require far higher peptide concentration than the other cells for a comparable occupancy of their HLA molecules. B cells are less efficient in incorporating KTW into their HLA molecules than the other cell types but still far more efficient than DCs. A2/KTW complexes become detectable at the surface of DCs only at an ~100 times higher peptide pulsing concentration than in the cases of the other cell types. These observations are supported by time-course experiments (Fig. 2D), which show that the numbers of specific A2/KTW complexes on DCs saturate rapidly whereas they steadily increase on JY cells.

The results obtained with the KTW are reproduced with the ILA and LLF as shown for a comparison of the loading efficiencies for JY cells and DCs in Fig. 3(A and C). Of particular interest is a comparison of peptide handling by mDCs and iDCs. The iDCs appear to be even less efficient in peptide uptake and presentation than mDCs (Fig. 3D). The large difference in peptide loading, however, does not linearly translate into a corresponding difference in the fractional occupancy of the HLA molecules with KTW. Still mDCs are more

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**Fig. 2.** Kinetics of peptide loading onto HLA-A*0201 molecules. Melanoma cells (filled circles), T2 cells (open circles), mDCs (filled triangles), JY (open triangles), CD4+ T lymphocytes (filled squares), monocytes (open squares) and B lymphocytes (filled diamonds) were pulsed with serial 2-fold dilutions of the KTW starting with 320 μg ml⁻¹, washed and stained with the Fab G2D12 for the specific peptide complexes with HLA-A*0201 (Panels A, B). The gMFI values were calibrated with fluorescence rPE beads. Numbers of fluorochrome molecules per cell are shown. The peptide-dependent expression levels of HLA-A*0201 molecules on T2 cells are shown in Panel A (asterisks). The graphs shown are representative for two to three separate analyses done with independent cell preparations. The HLA-A*0201 expression of the cells was analyzed and quantified using Quantum Fluorescence Kit for determination of molecules of equivalent soluble fluorochrome units. In contrast to T2 cells, the expression of HLA-A*0201 is not influenced by the peptide (data not shown). In panel C, the fraction of total MHC molecules loaded with specific peptide, calculated as ratios of the calibrated G2D12 and BB7.2 fluorescence intensities, is plotted versus peptide concentrations used for pulsing the cells. Panel D: Time course of peptide binding to HLA-A*0201 molecules on DCs (closed circles) and JY cells (open circles). The cells were incubated for the indicated times with 320 μg ml⁻¹ peptide at 37°C in the presence of Brefeldin A.
efficient than iDCs, but only by 2-fold with respect to the peptide concentration needed to get a similar fractional occupancy (Fig. 3E). Among the cell types tested for the efficiency of peptide uptake and presentation, DCs are the least efficient.

Possible causes of the low efficiency of peptide presentation by DCs

In search of causes for the low efficiency of the processing of external peptide by DCs, we tested four possibilities. First, DCs might, more than other cells, degrade peptides. To test whether such effect could be responsible for the low rate of peptide binding by DCs, we incubated DCs and JY cells with KTW at concentrations of 160 or 5 μg/ml, then collected the supernatants, incubated them with T2 cells and quantified the resulting A2/KTW complexes with the Fab G2D12 as a measure for the peptide concentration in the supernatants (Fig. 4A). The T2 cells had been cultured overnight at 26°C to allow peptide-free HLA molecules to accumulate on these cells and, thus, increase sensitivity of detecting the specific peptide. As control, KTW was incubated in culture medium without cells. At the lower peptide concentration, significant reduction of KTW in the supernatant of JY cells and complete elimination of the peptide in the supernatant of DCs were found. At high peptide concentration, however, there was no significant difference between these cell types. The degradation of peptides by DCs reported by Amoscato et al. (13) is, thus, only detectable at low peptide concentration and cannot explain the large shift of the titration curves for peptide loading when DCs and other cell types are compared. Under the limiting peptide concentrations expected in vivo, however, different degrees of peptide degradation in the vicinity of different cell types may be important.

Second, we considered the possibility that DCs differ from the other cells in the synthesis and turnover rates of the MHC molecules or, third, that loading of exogenous peptides onto MHC molecules in different cell types occurs differently in different subcellular compartments. These two possibilities would depend on active transport of the MHC molecules to the cell surface or active recycling of these molecules. Therefore, we incubated JY cells and mDCs with peptide in the presence of either sodium azide to inhibit all energy-dependent processes including energy-dependent membrane and, with that, MHC turnover or brefeldin A to block export of newly synthesized HLA molecules and thereby decouple peptide loading from conventional MHC class I-specific antigen processing. With both reagents and both cell types, the efficiency of peptide loading is somewhat reduced compared with the controls without inhibitors (Fig. 4B). However, DCs are still less efficient than JY cells in peptide uptake and presentation. The relative efficient loading of the HLA with exogenous peptide in the presence of sodium azide suggests that a large fraction of the complexes are formed at the cell surface by exchange against peptides bound to the
Influence of peptides occupying the MHC
indicated concentration of peptides either without additional reagents (squares), in the presence of Brefeldin A (triangles) or sodium azide
B: Peptide pulsing in the presence of Brefeldin A or sodium azide. The mDCs (filled symbols) or JY cells (open symbols) were loaded with
/C176/C176 receptive for peptide. After incubation for 1.5 h at 26°C and 1.5 h at 37°C, the resulting KTW/A2 complexes were quantified with G2D12.
C: Influence of peptides occupying the MHC molecules on the binding of new peptide. T2 cells were incubated with media only (squares), with 320 µg ml⁻¹ of high- (gp100280–288, triangles) or low- (hTERT110–1116, circles) affinity HLA-A2-binding peptides. After removing excess peptide, the cells were pulsed with serial 2-fold dilutions of KTW beginning with 320 µg ml⁻¹. The amounts of the resulting HLA-A*0201/KTW complexes were then determined with G2D12.

Discussion
DCs, compared with other antigen-presenting cells, are described as superior in processing and cross-presenting antigens from other cells for cross-priming of CD8 T cells (1,14–17). In addition to whole or partially degraded antigens, peptides released from MHC molecules are potential antigen sources for cross-priming. To test this possibility, we investigated the extent of peptide uptake and presentation by DCs in comparison to various other cell types. These analyses were done with recently developed recombinant antibodies
with TCR-like specificities for defined MHC–peptide complexes that enabled us to detect and quantify the complexes directly and independent of T cell responses that, usually used for such analyses, are problematic as they reflect inseparably the effects of both antigen presentation and co-stimulation. Surprisingly, the studies revealed that for mDCs, far higher peptide concentrations are required for detectable pMHCs at the cell surfaces than for other cell types such as primary monocytes, B and T cells or tumor cells. Normalization of the densities of pMHCs with respect to the MHC expression levels of these cells further emphasizes the differences: except for DCs and TAP-deficient T2 cells, a given peptide concentration results in similar specific occupancies of the MHC molecules of all tumor and primary cell types analyzed. For DCs, 10 to 100 times higher peptide concentrations are required for the same cell surface densities of specific complexes.

The inefficiency in peptide handling by DCs could explain published observations that peptides released from MHC molecules do not cross-prime T cells (4). The differences in peptide handling by different cell types may be explained by differences in the availability of the peptides in their vicinity. We had found that supernatants from DCs incubated with peptide contain less peptide than supernatants from B-lymphoblastoid cells incubated with the same amount of the peptide. This reduction of the peptide concentration might be explained with a higher endocytotic activity of DCs with intracellular retention or intracellular degradation of the peptide. However, mDCs have a slower membrane turnover than other cell types, which exclude a major contribution of endocytosis to the removal of peptide from the cultures (11). Moreover, the peptide pulsing was done in a large volume of 1 ml that by far exceeds the volume of the cells and the volume that possibly can be processed through the cells in the time given. A more likely explanation would be that DCs, more than other cells, produce proteases that degrade peptide extracellularly. Such DCs-associated proteases have been described by Amoscato et al. (13, 18). However, we found detectable elimination of peptide from the supernatants of the cells only at low peptide concentrations. At higher concentrations, no difference in the peptide concentrations was seen; yet, the differences in peptide loading remained. While neither of the above two hypotheses can explain the differences in peptide loading in our experiments, at the likely low concentrations of MHC-released peptide in the local environments of tissues, the observed reduction of available peptide for reloading MHC molecules might be significant and could indeed account, at least in parts, for the failures to demonstrate cross-priming by MHC-released peptides.

Because of the above considerations, we believe that the different efficiencies of peptide presentation are due to differences in the generation and turnover of pMHC by the different cells. The total amount of pMHC at the surfaces of cells is, in general, determined by the rate of generation of these complexes and the rate of their elimination from the cell surface by decay or internalization of membrane. We had shown before that pMHC of mDCs are longer-lived than the same complexes on other cell types including iDCs (11). In fact, besides a fast initial decay aspect of specific complexes on macrophages, which is followed by a second phase with a very slow kinetic, pMHC on iDCs are the most short-lived of the cells analyzed. The different apparent stabilities of the pMHC on the different cells are in part explained by the different rates of membrane turnover and in part with a high intrinsic stability of the pMHC on mDCs. The half-life times of the cell surface pMHC of the other tested cell types are significantly lower than the half-life of the pMHC of mDCs but still higher than those of iDCs. With this background from our earlier studies (11), the apparent low efficiency of peptide loading onto the MHC molecules of iDCs seems to be due to a combination of both the instability of their pMHC and the high membrane turnover which prevents any accumulation of pMHC at their cell surfaces. As a consequence, iDCs appear to be inefficient in peptide–MHC loading although the actual initial loading rate might not be low. For mDCs, on the other hand, the high intrinsic stabilities of the pMHC make peptide exchange and, thereby, loading of external peptide onto their MHC molecules less likely. The low membrane turnover, however, allows the complexes that are formed to remain and accumulate at the cell surfaces. The half-life times of the pMHC and the membrane turnover rates of other cell types are somewhere between those of iDCs and mDCs. The
biological consequences of these differences in peptide handling would be that IDCs would constantly exchange the peptides presented by their MHC molecules. Once induced to mature, for example, by inflammatory cytokines in their environment or by TLR agonists, the cells would preserve the antigenicity of this environment. The resulting mDCs would retain and carry this information to the draining lymph nodes to induce T cells with specificity for the antigens in the inflamed tissue.

The importance of discriminating the antigen presentation and the co-stimulatory aspects of T cell induction is underlined by the comparison of the antigen dose–T cell response relationships for mDCs and JY cells as antigen-presenting cells. At low peptide concentrations, JY cells appear to be more efficiently stimulating T cells. At higher peptide concentrations this relationship is inverse. Only when the pMHCs are quantified at the surfaces of the cells, and related to the T cell response, is the superior stimulatory capacity of the DCS obvious in the shift of the dose–response curve to lower pMHC cell surface densities by about one order of magnitude.

The findings reported herein have important implications for the development of DC-based vaccines. A number of protocols use DCs specifically loaded with tumor-associated T cell epitopes as therapeutic vaccines for the treatment of cancer. Given the relatively low efficiency of peptide loading onto mDCs, some of these protocols may need revision. As it is done for tumor lysates containing unknown peptides at low concentrations, it should be considered to pulse monocytes or iDCs instead of mDCs with the vaccine peptides and only then induce maturation of the DCs.

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Abbreviations

APC  allophycocyanin
DC  dendritic cell
gMFI  geometric mean fluorescence intensity
hTERT  human telomerase reverse transcriptase
iDC  immature dendritic cell
ILA  peptide ILAKFLHWL
KTW  peptide KTWGQYWQV
LLF  peptide LLFGYPVYV
mDC  mature dendritic cell
2-ME  2-mercaptoethanol
pMHC  specific peptide–MHC complex
SK  SK-mel-24
TTL  peptide TTLTALEAA
YLEP  peptide YLEPGPVTV

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