

Dendritic cells up-regulate immunoproteasomes and the proteasome regulator PA28 during maturation

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Dendritic cells (DC) are highly specialized professional antigen presenting cells which are pivotal for the initiation and control of the cytotoxic T cell response. Upon stimulation by cytokines, bacteria, or CD40L DC undergo a maturation process from an antigen-receptive state to a state of optimal stimulation of T cells. We investigated the composition of proteasomes of DC derived from human peripheral blood monocytes before and after stimulation by CD40L, LPS, or proinflammatory cytokines (TNF- α +IL-6+IL-1 β). Immunoprecipitation of proteasomes and analysis on two-dimensional gels revealed that during maturation the inducible proteasome subunits LMP2, LMP7, and MECL-1 are up-regulated and that the neosynthesis of proteasomes is switched exclusively to the production of immunoproteasomes containing these subunits. The proteasome regulator PA28 is markedly up-regulated in mature DC and in addition a so – far unidentified 21-kDa protein co-precipitates with the proteasome in LPS – stimulated DC. These changes in proteasome composition may be functionally linked to special properties of DC like MHC class I up-regulation or cross-priming. Our findings imply that the spectrum of class I-bound peptides may change after DC maturation which could be relevant for the design of DC – based vaccines.

Key words: Antigen presentation / TNF- α / CD40 / Lipopolysaccharide / LMP2

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1 Introduction

Dendritic cells (DC) are highly specialized professional antigen-presenting cells which have unique morphological and molecular properties enabling their functions as ‘sentinels of the immune system’ and ‘adjuvants of nature’ [1]. DC are found in an immature differentiation state in non-lymphoid tissues preferentially in the skin and mucosa where they may encounter pathogens. Upon uptake of bacteria [2] or infection with viruses [3] as well as after stimulation with the bacterial cell wall component LPS or proinflammatory cytokines like TNF- α or IL-1 β [4] immature dendritic cells undergo a maturation process which induces them to migrate to the T cell areas of the spleen and lymph nodes. During

their migration DC switch their properties from a state of efficient antigen capture to a state of optimal antigen presentation by enhancing the cell surface expression of co-stimulatory molecules like CD80 and CD86 as well as MHC class I and class II molecules [2–4]. Activated T helper cells which express CD40 ligand (CD40L) can maintain the mature state of dendritic cells via CD40 stimulation.

The proteasome is the main proteolytic system that generates most of the peptides for presentation on MHC class I molecules [5]. It consists of the proteolytically active core particle called 20S proteasome and regulatory complexes like the PA28 and PA700 complexes. PA700 and the 20S core form together the 26S proteasome. Upon stimulation of cells with IFN- γ three subunits of the 20S proteasome, namely LMP2, LMP7, and MECL-1, are induced and incorporated in so called ‘immunoproteasomes’ by replacing their homologous constitutive counterparts designated delta, MB1, and Z, respectively, during proteasome assembly. This subunit

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Abbreviations: DC: Dendritic cells NEPHGE: Non equilibrium pH gradient gel electrophoresis

exchange alters the cleavage pattern of the proteasome and increases the efficiency of antigen presentation [5]. Conversely, the deletion of LMP7 or LMP2 reduces MHC class I cell surface expression and antigen presentation due to a lack of suitable peptide ligands. Likewise inducible with IFN- γ are the two subunits α and β of the proteasome regulator PA28. The two subunits form a heptameric ring that binds to the α endplates of 20S proteasomes thus rendering the *in vitro* processing of several nonameric MHC ligands more efficient. Moreover, the overexpression of PA28 enhanced the antigen presentation of at least two viral antigens, indicating a role for PA28 in antigen processing [5].

As the invariant chain processing and cell surface transport of MHC class II molecules changes dramatically during maturation of DC we wondered whether alterations may also occur in peptide processing for the MHC class I pathway. A first approach was to analyze the subunit composition of the proteasome in immature and mature DC. We found that during maturation of DC the assembly of proteasomes changed from the generation of roughly equal portions of constitutive proteasomes and immunoproteasomes to the exclusive production of immunoproteasomes. Moreover, the proteasome regulator PA28 became strongly up-regulated during maturation. These results imply that the spectrum of MHC class I bound peptides may differ between immature and mature DC, a finding which has important impli-

cations for T cell priming and stimulation in lymphoid tissues.

2 Results

2.1 Generation of DC and induction of maturation

DC were raised in primary culture from human peripheral blood monocyte precursors by magnetic sorting of CD14⁺ cells and cultivation with GM-CSF and IL-4 according to previously established protocols [4]. To test the quality of our preparations the cells were analysed by flow cytometry (Fig. 1). The generated DC expressed high levels of MHC class I and II as well as CD40 molecules. To exclude contaminations with other cell types antibodies against CD3 (expressed in T cells), CD14 (monocytes and macrophages), CD16 (natural killer cells and macrophages), and CD19 (B cells) were used and DC were found to be surface negative for these lineage markers (Fig. 1 B). Together with the typical DC morphology observed (not shown) these results made us confident in having properly generated immature DC.

The maturation of DC was achieved through three different stimuli consisting of LPS, of a cocktail of proinflammatory cytokines (TNF- α +IL-1 β +IL-6), and of CD40 stimulation through co-cultivation of immature DC with

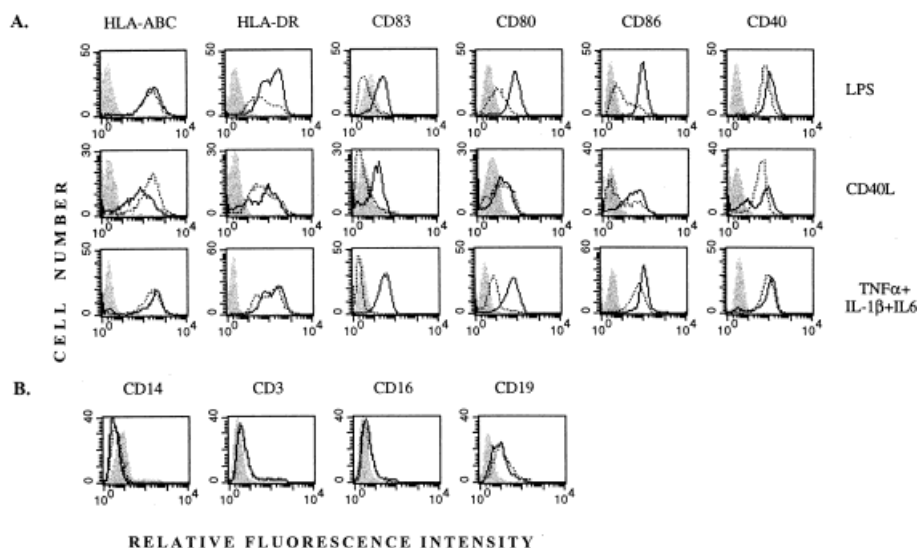


Figure 1. Flow cytometric characterization of DC by cell surface markers before and after maturation *in vitro*. A. Comparison of the maturation induced by different stimuli as indicated. B. Control stainings of LPS-treated cells to assess the purity of the DC population obtained. Shaded profiles are stainings with isotype control or secondary reagents only, the dotted lines represent stainings of unstimulated DC and the solid lines stainings of mature DC for the indicated surface markers.

CD40L-expressing cells. All stimuli resulted in an elevation of the costimulatory molecules CD80 and CD86 as well as CD83 (Fig. 1 A). Notably, the expression of MHC class I and II molecules did not change significantly during maturation except for an increase in class II expression on LPS-stimulated DC.

2.2 Western analysis of LMP2, LMP7, MECL-1, PA28 α , and PA28 β expression in immature and mature DC

As an initial approach to characterize the proteasome in DC before and after maturation we decided to assess the amount of immunoproteasomes containing the subunits LMP2, LMP7, and MECL-1 as well as the expression level of the α and β subunits of the proteasome regulator PA28. We have raised polyclonal antibodies against human LMP2, LMP7, and MECL-1. As shown in Fig. 2, each of the antisera recognized a single band of appropriate molecular weight in IFN- γ -treated HeLa cells but not in uninduced HeLa cells or LMP2/LMP7-deficient T2 cells indicating their specificity for the respective proteins. Interestingly, the subunits LMP2, LMP7, and MECL-1 as well as PA28 α and β were expressed already at high levels in immature DC. The MECL-1 antiserum

recognized two closely juxtaposed bands of 25 and 27 kDa in DC while only one band was detected in IFN- γ -treated HeLa cells. The identity of this second MECL-1 – reactive protein is unclear at present and deserves further investigations. The 29-kDa band seen in lysates of T2 cells corresponds most likely to the MECL-1 precursor (predicted M_r of 28.9) which is not incorporated into the 20S proteasome complex in the absence of LMP2. A comparison of immature and mature DC in Western analysis did not reveal an obvious increase of MECL-1, LMP2, LMP7, PA28 α , and PA28 β after 1 day of stimulation (Fig. 2). Even a careful quantitative Western analysis in the linear range of detection showed an enhancement of the signals for LMP2, LMP7, and MECL-1 of only 10–30 % in mature DC 2 days after stimulation with LPS (data not shown). As it is difficult to assess quantitative differences of a factor of less than two among different samples in Western analysis we decided to further investigate the quantitative expression of LMP2, LMP7, MECL-1, PA28 α , and PA28 β in DC by immunoprecipitation.

2.3 Analysis of proteasome composition of immature and mature DC by metabolic labeling and immunoprecipitation

Immunoprecipitation of proteasomes from DC and two-dimensional non equilibrium pH gradient gel electrophoresis (NEPHGE)/SDS-PAGE analysis was applied because this technique allows the monitoring of both the inducible subunits LMP2, LMP7, and MECL-1 as well as their constitutive homologues delta, MB1, and Z on the same gel. We employed the monoclonal antibody MCP444 for proteasome precipitation because this antibody is specific of the β -type subunit HN3 and precipitates native proteasome complexes [6]. The binding of this antibody should thus not interfere with docking of proteasome regulators to the α endplates of 20S proteasomes. We used rather mild hypotonic lysis and immunoprecipitation conditions in the absence of detergent and in the presence of glycerol and ATP in order to maintain the association of 20S proteasome with the PA700 or PA28 regulators. Two-dimensional gels of proteasome precipitates from immature and mature DC after stimulation with LPS (A), CD40L (B), and (TNF- α + IL-1 β + IL-6) (C) are shown in Fig. 3. In immature DC approximately equal amounts of immunoproteasomes and constitutive proteasomes are present. Interestingly, all three maturation stimuli abrogated the incorporation of the constitutive subunits delta, MB-1, and Z into the proteasome which were replaced by the inducible subunits LMP2, LMP7, and MECL-1, respectively. A quantitative analysis of radioactivity of the spots on two-dimensional gels revealed an increase in these subunits of a factor of 1.5–2 in mature DC. Also the PA28 α and β proteins

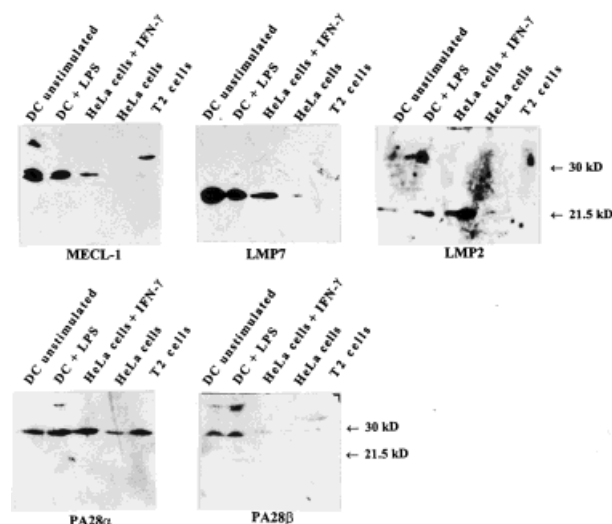


Figure 2. Western blot analysis of the expression of LMP2, LMP7, MECL-1, PA28 α , and PA28 β in immature and mature DC after 1 day of LPS stimulation. IFN- γ -treated HeLa cells were taken as positive control, uninduced HeLa cells and T2 cells as negative controls. Shown are X-ray films after exposure to enhanced chemiluminescence-treated Western blots.

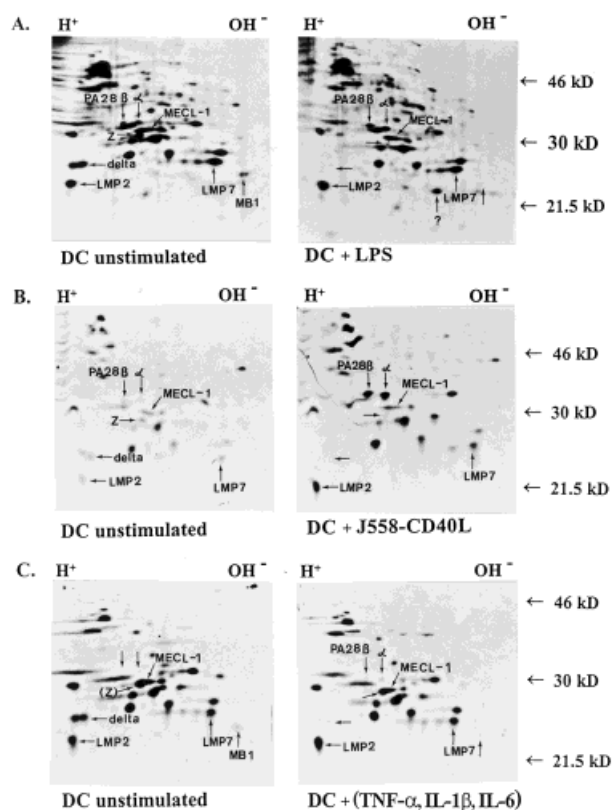


Figure 3. NEPHGE/SDS-PAGE analysis of proteasome subunits in DC before and after stimulation with LPS (A), CD40L (B), and TNF- α +IL-1 β +IL-6 (C). Proteasomes were metabolically labeled and immunoprecipitated with mAb MCP444 specific for proteasome subunit HN3. In panel A the question-mark indicates a so far unidentified protein of 21 kDa that is strongly induced in LPS-stimulated DC.

appeared to be up-regulated in mature DC as was particularly evident after stimulation with CD40L (Fig. 3, panel B). Curiously, the position of subunit delta was taken by two closely juxtaposed protein spots in immature DC. As both of them disappeared upon maturation of DC it is likely that they represent two so far uncharacterized modifications of the delta subunit. The overall pattern of proteins in proteasome immunoprecipitates was virtually identical in immature and mature DC except for a very prominent and reproducible induction of a 21-kDa protein after treatment of immature DC with LPS (labeled with a question mark in Fig. 3A). As we did not see an induction of this protein after stimulation of immature DC with CD40L or inflammatory cytokines this might not be linked to the maturation of DC in general. On the other hand, a similar experiment performed with a number of untreated and IFN- γ -treated cell lines or LPS-treated B cell lines did not reveal the induction of this protein suggesting that its expression is more specific for LPS-stimulated DC.

2.4 PA28 immunoprecipitations

On two-dimensional gels it appeared that PA28 was more prominent in proteasome precipitates from mature as compared to immature DC. Hence we decided to investigate whether this represents an up-regulation of the synthesis or is just due to a preferred association with immunoproteasomes. We used a PA28 α -specific antiserum (by courtesy of Dr. P.-M. Kloetzel) to immunoprecipitate PA28 from the same DC lysates used for proteasome precipitation above. Clearly, PA28 α was found to be strongly up-regulated in DC stimulated either with CD40L, TNF- α (Fig. 4A), or LPS (Fig. 4B). Again the same gentle lysis and precipitation conditions were chosen in order not to lose proteins which might be co-precipitated. PA28 β , which would be expected to co-precipitate with PA28 α under these conditions, was not visible as a separate band below PA28 α . As PA28 α and β have very similar apparent molecular weights of 28.7 kDa and 27.1 kDa, respectively, PA28 β may not have been separated from PA28 α in these experiments in spite of using a high resolution system for electrophoretic separation. Interestingly, a protein of about 33 kDa (labeled by an asterisk in Fig. 4) was reproducibly precipitated in immature DC which we did not see in mature DC. This might be due to a mere cross-reactivity of the PA28 α peptide antiserum with this unidentified protein but as it disappears upon DC maturation this issue deserves further investigation.

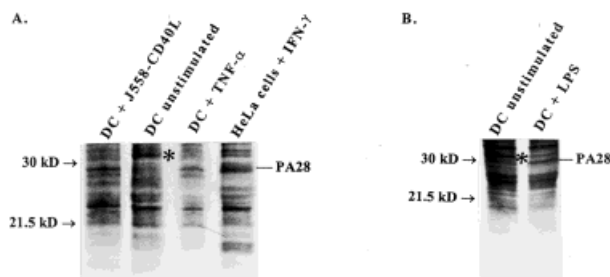


Figure 4. Analysis of PA28 expression by immunoprecipitation in immature and mature DC. PA28 – precipitates from metabolically labeled DC were separated on a 16 % (A) or on a 10 %–16 % two-phase (B) tricine-SDS gel and visualized by autoradiography. The asterisk indicates a PA28 – associated protein of 32 kDa in unstimulated DC that is down-regulated upon stimulation with CD40L, TNF- α (both on panel A), or LPS (panel B).

3 Discussion

In this study we analyzed for the first time the composition of proteasome complexes in immature and mature dendritic cells. We found that upon stimulation of immature DC with either LPS, CD40L, or $\text{TNF}\alpha + \text{IL-1}\beta + \text{IL-6}$ the proteasome synthesis and assembly switches from the formation of a mixed proteasome population consisting of approximately equal amounts of constitutive proteasomes and immunoproteasomes to the almost exclusive formation of immunoproteasomes containing the subunits LMP2, LMP7, and MECL-1. Moreover, the synthesis of the proteasome regulator PA28 is up-regulated and PA28 associates with the 20S proteasome.

An important issue is the *in vivo* relevance of our findings. The *in vitro* model for DC maturation used in this study has been validated by many laboratories and reflects the phenotypic and functional properties of immature and mature DC *in vivo* [4, 7]. In this system we found that upon maturation of DC the proteasome neo-synthesis is changed entirely to the production of immunoproteasomes (Fig. 3). However, since the half life of 20S proteasomes is in the order of 4 days it may take almost a week until the constitutive proteasomes are replaced by immunoproteasomes. Accordingly we could observe a significant increase in the steady-state amount of immunosubunits by a factor of 1.1 to 1.3 only 2 days after DC were induced with LPS in quantitative Western analysis. As it is probably the steady state composition of proteasomes which defines the spectrum of peptides produced for MHC class I-restricted presentation it is pertinent to consider the time period during which DC are maintained in the mature state *in vivo* in order to appreciate the biological effect of immunoproteasome induction.

DC in the spleens of mice which have been treated with LPS did not survive longer than 24 h unless a cognate antigen was co-injected leading to the rescue of DC from apoptosis [7]. Antigen-specific T helper cells apparently control the survival of those mature DC which present the respective antigen. As activated T helper cells express CD40L it can be expected that as long as the survival signal is provided by T cells DC will gradually replace their constitutive proteasomes with newly assembled immunoproteasomes. This will affect the spectrum of class I-presented peptides and shape the repertoire of cytotoxic T cells which are being activated.

A matter of current debate is the effect of DC maturation on the cell surface expression and half life of MHC class I molecules. In the present experiments we could not detect an elevation of HLA-ABC cell surface expression

of human monocyte-derived DC upon maturation in contrast to previous studies which demonstrated a significant increase in class I surface expression after stimulation with $\text{TNF}\alpha$ or LPS [4]. Also the infection of DC with influenza virus [3] or co-incubation with live bacteria [2] led to a higher cell surface expression and a threefold prolongation in the half life of MHC class I molecules. Therefore it would be interesting to assess whether the induction of immunoproteasomes and/or PA28 could contribute to an increase in MHC class I cell surface expression or even to a stabilization of class I molecules by producing more peptide ligands which bind with high affinity. The data obtained in this study would be consistent with such a function for LMP2, LMP7, and MECL-1. An alternative hypothesis for the function of immunoproteasomes, namely that a greater diversity in proteasome populations would increase the variety of peptides, is less compatible with our results because a maximal variety in peptide products would be expected if cells express about equal amounts of immunoproteasomes and constitutive proteasomes and this was already found in immature DC prior to maturation.

The enhanced expression of immunoproteasomes and PA28 in mature DC could be due to a transcriptional induction of the respective mRNA or to a posttranscriptional mechanism. A semi-quantitative RT-PCR analysis performed in our laboratory revealed that the mRNA levels of LMP2, LMP7, MECL-1, PA28 α , and PA28 β were enhanced in mature compared to immature DC (A.M., unpublished) which is noteworthy because an increase in these mRNA after stimulation by LPS has not been reported before. The increase in immunoproteasome content of mature DC could also be due to proteins which specifically promote the assembly of immunoproteasomes in maturing DC. We have observed a 19-kDa protein in mature DC which transiently associates with proteasomes during their assembly (not shown) as well as a 21-kDa protein which co-precipitates with mature 20S proteasomes of LPS-stimulated DC (Fig. 3). Whether these novel proteins are involved in the assembly of immunoproteasomes or promote other functional properties of DC will be the subject of further investigations.

4 Materials and methods

4.1 Generation of DC

Monocytes were isolated from PBMC by Ficoll-Paque® density centrifugation and CD14⁺ magnetic sorting and differentiated to immature DC by cultivation in GM-CSF and IL-4 as previously described [4]. Maturation was achieved by cultivation for 1 day with either 1.5 $\mu\text{g/ml}$ LPS (from *Salmonella*

abortus equi, Sigma, Buchs), or 50 ng/ml rTNF- α (R&D Systems, Abingdon, GB), or by addition of mitomycin C treated (50 μ g/ml for 50 min) J558 cells transfected with CD40L [4] at a ratio of 1xDC:5xJ558-CD40L. Alternatively, DC were generated from plastic-adherent PBMC by cultivation for 6 days in complete medium (CM): X-VIVO 15 (BioWhittaker, Verviers), 1 % heat-inactivated (56 °C for 1 h) human plasma, 2 mM L-glutamin, 100 U/ml penicillin/streptomycin, 75 ng/ml GM-CSF (Leukomax) and 1000 U/ml rIL-4 (R&D Systems). Maturation was induced within 3 days after addition of 10 ng/ml rTNF- α + 10 ng/ml rIL-1 β + 150 ng/ml rIL-6 (all from R&D Systems).

4.2 Flow cytometry

Cells were analyzed on a FACScan® flow cytometer after staining with mAb: anti-HLA-DR-FITC, anti-CD14-FITC, anti-CD80-PE, (Becton Dickinson), anti-CD83-FITC (Immunotech, Marseille), anti-CD86-FITC (PharMingen, Basel), anti-CD40-FITC, anti-CD3-FITC, anti-CD16-FITC, anti-CD19-FITC, anti-HLA-ABC (Serotec, Oxford).

4.3 Metabolic labeling and immunoprecipitation

DC were starved for 30 min in cysteine- and methionine-free RPMI 1640, 10 % dialysed FCS, 50 ng/ml GM-CSF, 1000 U/ml rIL-4 and pulsed for 4 h with 0.2 mCi/ml [³⁵S] methionine followed by a 16 h chase period. Cells were lysed in hypotonic lysis buffer (25 mM Tris-HCl, pH 7.6, 2 mM MgCl₂, 5 mM KCl, 17.4 % v/v glycerol, 1 mM PMSF, 4 μ M pepstatin, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 2 mM ATP, 1 mM dithiothreitol, 40 mM creatin-phosphate, 0.2 mg/ml creatin kinase) by sonication followed by a 30-min incubation at 37 °C. Protein A-Sepharose beads precoated with mAb MCP444 [6] or PA28 α rabbit antiserum were incubated with precleared lysates for 3 h at 4 °C and washed five times in lysis buffer (without creatin phosphate and creatin kinase).

4.4 NEPHGE/SDS-PAGE, tricine-SDS PAGE and Western blotting

NEPHGE/SDS-PAGE was performed as described [8]. Tricine-SDS PAGE and Western blotting was performed according to standard protocols.

4.5 Production of antisera

Polyclonal antibodies were raised in rabbits against KLH – coupled synthetic peptides: huLMP2 (CHRVILGNELPKFYDE), huLMP7 (CTDVSDLLHQYREANQ), and huMECL-1 (CTGAKLLRTLSSPTPEVK).

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