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Pronounced up-regulation of the PA28α/β proteasome regulator but little increase in the steady-state content of immunoproteasome during dendritic cell maturation

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Dendritic cells (DC) are professional antigen-presenting cells that activate CTL by presenting MHC class I-restricted peptides that are processed through the proteasome pathway. Previously, we reported that upon DC maturation the synthesis is switched towards the exclusive production of immunoproteasomes containing the active site subunits LMP2, LMP7 and MECL-1. In this study we investigated the mechanism by which proteasome assembly is regulated in mature DC. Quantitative analysis of mRNA expression showed very limited transcriptional induction of LMP7, MECL-1 and UMP1 in mature DC and a moderate mRNA increment for LMP2 and PA28α and β. We investigated a role of PA28α/β in regulating proteasome assembly in DC. PA28α/β coprecipitated with 13S/16S proteasome precursor complexes but associated with mature constitutive and immunoproteasomes to the same extent. Furthermore, we determined the steady-state proteasome subunit composition in DC. Replacement of constitutive proteasomes by immunoproteasomes in maturing DC was very slow and occurred only to a minor extent. Our data suggest that the limited turnover of 20S proteasomes in mature DC probably contributes little to recently reported marked differences in antigen presentation between immature and mature DC and that alternative mechanisms may be responsible for this phenomenon.

Key words: Dendritic cell / Proteasome / PA28 / Antigen processing / MHC class I

1 Introduction

DC are professional antigen-presenting cells that play a key role in the initiation and regulation of antigen-specific immune responses [1]. Pathogen-derived molecules trigger maturation of DC which results in up-regulation of costimulatory molecules CD80 and CD86 concomitant with the migration of DC to draining lymph nodes. There, they present MHC class I-bound peptides processed from antigens by the proteasome [2, 3]. The proteasome is a proteolytic system composed of the 20S catalytic core and regulatory complexes like PA28α/β [4, 5], PA28γ, PA700 or PI31. Substitution of active site subunits of the 20S core as well as association with regulatory complexes affect catalytic activity and proteolytic specificity of the proteasome, which results in marked differences in the peptide production from specific antigens [6–10].

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Abbreviations: DC: Dendritic cells **GAPDH:** Glyceraldehyde-3-phosphate dehydrogenase **NEPHGE**: Non-equilibrium pH gradient gel electrophoresis

In most cell types the catalytically active proteasome subunits are the constitutive subunits delta (β1), MB1 (β5) and Z (β2). Upon exposure to IFN-γ, the 'immunosubunits' LMP2 (β1i), LMP7 (β5i) and MECL-1 (β2i) are induced and replace their respective constitutive homologues during *de novo* assembly of proteasomes. In a previous study we have shown that immature DC synthesize about equal amounts of constitutive and immunoproteasomes, whereas in mature DC proteasome assembly was switched to the exclusive production of immunoproteasomes [11]. In the present study we investigated the mechanism accounting for the shift towards exclusive biogenesis of immunoproteasomes in mature DC. Quantitative analysis of mRNA of immunosubunits revealed only modest differences between immature and mature DC, suggesting that the regulation of proteasome synthesis in mature DC may occur on a post-transcriptional level.

Incorporation of subunits during proteasome assembly follows a specific order. Upon initial formation of the α ring, β–type subunits associate to form 16S-half proteasomes. Two half-proteasomes then combine, propeptides are cleaved off from β–subunit precursors, and the

mature catalytically active 20S complex is formed [12]. In yeast, UMP1 was described as a protein associated with proteasome precursor complexes which is required for the correct and efficient biogenesis of proteasomes [13]. More recently, murine and human homologues of UMP1 were found to interact with 16S complexes and were shown to be moderately up-regulated by IFN-γ [14–16]. When examining a potential involvement of UMP1 in assisting the production of proteasomes in DC, we merely detected an about twofold up-regulation of UMP1 mRNA in mature DC. Another potential candidate that could govern proteasome assembly in DC is PA28α/β, as PA28β$^{-/-}$ mice have been reported to display impaired immunoproteasome assembly [17]. Consistent with this potential role of PA28, we detected an association of PA28α/β with 13/16S proteasome precursors but the association of PA28α/β with mature 20S proteasomes was similar for constitutive and immunoproteasomes.

Recently, Morel et al. [18] identified an epitope poorly presented by mature DC but efficiently presented by immature DC. The authors suggested that this difference may be attributed to an inefficient generation of this epitope by immunoproteasomes in mature DC. Although this explanation would be in line with an exclusive biogenesis of immunoproteasomes in mature DC, the steady-state pool of proteasomes is more likely to shape epitope production. Given that the 20S proteasome is a long-lived protein complex with a half-life of several days [19], neosynthesis is likely to affect the steady-state subunit composition of proteasomes only gradually over an extended period of time, particularly in non-proliferating cells like mature DC. In agreement with this expectation, when we carefully investigated the kinetics of proteasome replacement in DC, we found that immunoproteasomes represent already half of the proteasome population in immature DC and that maturation induces a low extent replacement of constitutive proteasomes by immunoproteasomes during the course of several days. Hence, differences in antigen presentation between immature and mature DC can hardly be attributed to these minor changes in steady-state composition of 20S proteasomes, suggesting that other factors account for this phenomenon.

2 Results

2.1 IFN-γ treatment of DC does not induce complete phenotypic maturation but switches proteasome biogenesis to the exclusive production of immunoproteasomes

In a previous study we showed that treatment of DC with LPS, CD40L, or TNFα + IL-1 β + IL-6 induces the exclusive biogenesis of immunoproteasomes [11]. As in many cell lines IFN-γ induces assembly of immunoproteasomes as a consequence of LMP2, LMP7 and MECL-1 expression [20], an obvious question was whether IFN-γ treatment would have the same effect on proteasome composition in DC. Flow cytometric analysis of cell surface markers revealed that phenotypic maturation was only partially achieved when DC were treated with IFN-γ as compared to LPS stimulation which was evident from a lack of MHC-II and CD83 up-regulation as well as a weak enhancement of CD80 and CD86 (Fig. 1A). Despite the failure to phenotypically mature DC, IFN-γ had the same effect as LPS in inducing the exclusive production of immunoproteasomes, as evidenced by proteasome immunoprecipitates of metabolically labeled and IFN-γ-treated DC, where only the immunosubunits LMP2, LMP7 and MECL-1 can be detected (Fig. 1B). This indicates that with respect to proteasome synthesis, DC respond to IFN-γ in the same way as other cells.

2.2 Proteasome assembly in DC may be regulated on a post-transcriptional level: analysis of mRNA expression of active site proteasome subunits, PA28α/β and UMP1

The mRNA expression in DC was initially studied by Northern blotting (Fig. 2). While IFN-γ induced the expression of the immunosubunits in HeLa cells and markedly up-regulated mRNA of both PA28α and β, a comparison of LPS matured with immature DC in the same blot revealed no detectable up-regulation of LMP2, LMP7 and MECL-1 mRNA but only of PA28α and β.

Quantification was subsequently attempted by means of real time PCR, a more sensitive method. The numbers shown in Fig. 3A are ratios of specific mRNA quantities found in mature DC relative to the respective mRNA quantities in immature DC, after standardization on β-actin mRNA content. Except for the constitutive subunits, the values were obtained in three experiments based on three different DC preparations. Reproductions of results based on the same RNA samples gave a deviation of about 10%, indicating that quantification by real time PCR provides high precision. Although there was some variability between the ratios measured in different

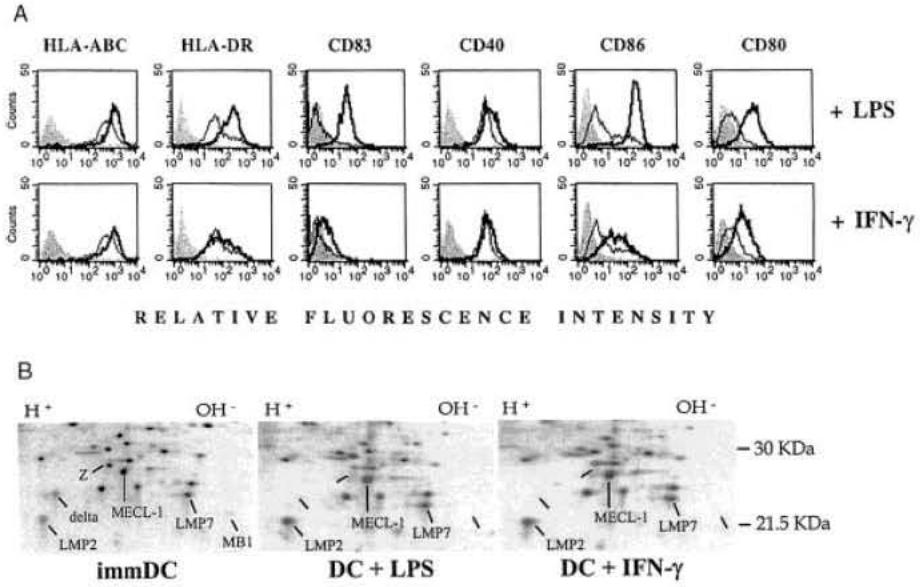


Fig. 1. (A) Flow cytometric analysis of cell surface markers of DC treated for 24 h with IFN-γ or LPS. Shaded profiles are stainings with isotype control, thin lines represent stainings for unstimulated DC, thick lines represent DC treated with LPS (1 μg/ml) or IFN-γ (100 U/ml). (B) NEPHGE/SDS-PAGE analysis of proteasome subunits. Proteasomes were metabolically labeled and immunoprecipitated with the mAb MCP21. The constitutive proteasome subunits delta, Z and MB1 as well as their respective inducible homologues LMP2, MECL-1 and LMP7 are labeled.

DC preparations the same trend of up-regulation was seen with all cDNA analyzed. In accordance with the Northern blot results (Fig. 2), PA28α and β were up-regulated with an increment for PA28β that was 2-fold higher than for α in all three independent experiments. Induction of LMP2 was more pronounced when compared to LMP7 and varied between ratios 3.2 and 6.7, while MECL-1 showed the least pronounced up-regulation with a ratio of lower than two. On the other hand, mRNA levels of the constitutive subunits were rather stable. Unexpectedly, comparatively low up-regulations were found in mature DC, given that in HeLa cells, a shift towards an exclusive production of immunosubunits occurred after an IFN-γ–mediated increase of immunosubunit mRNA in the order of 20-fold (data not shown). From these data, we conclude that the shift towards the production of immunoproteasomes in mature DC may at least in part be controlled on a post-transcriptional level.

Since the mammalian homologue of the proteasome assembly factor UMP1 was recently found to be up-regulated by IFN-γ, we investigated UMP1 expression in immature and mature DC. The transcriptional up-regulation was limited to a factor of 2–2.3, comparable to that reported for IFN-γ treated HeLa cells [14]. Attempts to perform Western analysis of UMP1 in DC were unsuccessful, but the low increment in UMP1 mRNA in mature DC renders a major impact of UMP1 on the induction of immunoproteasome assembly improbable.

Recent reports have shown that expression levels of the actin bundling protein fascin increases with DC maturation and that mature Langerhans cells display an enhanced expression of β- and γ-actin [21, 22]. Since we had chosen β-actin as an internal reference for real time RT-PCR, we investigated whether β-actin is up-regulated also in mature monocyte-derived DC. We amplified GAPDH from the same DC samples and found that in all

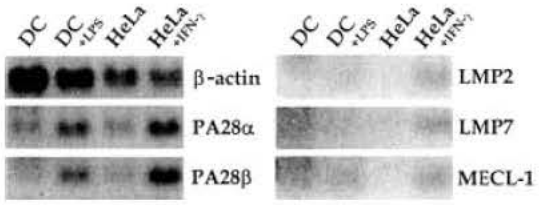


Fig. 2. Northern blot analysis of immunosubunits LMP2, LMP7 and MECL-1 and of PA28α and β in DC and HeLa cells. Alignots of 15 μg of total RNA from unstimulated and LPS (1 μg/ml, 24 h) -stimulated DC as well as untreated and IFN-γ (100 U/ml, 24 h) -treated HeLa cells were loaded. After hybridization with specific ^{32}P-labeled cDNA probes, blots were exposed to X-ray films. Hybridization with β-actin probe is shown for standardization.

A

REAL TIME PCR (Mat/Imm)

UMP1	2.0	delta	0.8	LMP2	3.2	Prep 1
	2.3		--		6.7	2
	2.0		--		5.5	3
PA28α	3.0	MB1	0.8	LMP7	1.6	Prep 1
	3.8		--		3.2	2
	4.5		--		2.7	3
PA28β	6.5	Z	0.8	MECL-1	1.2	Prep 1
	8.0		--		1.7	2
	8.0		--		2.0	3
β-actin	1.0	GAPDH	0.5			

B

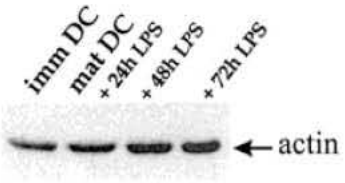


Fig. 3. (A) Quantification of mRNA by real time RT-PCR. cDNA were synthesized from RNA of immature and mature DC (LPS, 24 h) obtained in three independent preparations (Prep1–3). The numbers indicate the ratios of specific mRNA content in mature DC related to immature DC. For details see Materials and methods. (B) Western blot analysis of actin content in immature and mature DC after 24, 48 and 72 h of LPS stimulation.

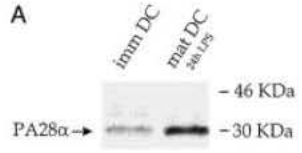
preparations GAPDH was lower in mature than in immature cells. Thus, a pertinent question is: which of the two should be taken as reference for standardization, β-actin or GAPDH? In principle it is conceivable that GAPDH is down-regulated upon DC maturation but if GAPDH was indeed constant, our results would imply a twofold up-regulation of β-actin during DC maturation. Western blot analysis on total lysates of DC (Fig. 3B) showed that, during the time course of maturation, the actin expression becomes more prominent. Therefore, for future studies on mRNA in monocyte-derived DC we propose GAPDH rather than β-actin as a more appropriate internal reference.

2.3 PA28α/β associates with proteasome precursors but binding occurs equally well to constitutive as compared to immunoproteasomes

A recent analysis of PA28β$^{-/-}$ mice suggested a specific requirement of PA28α/β for the assembly of immunoproteasomes [17]. Given that PA28α and β showed a high transcriptional induction in mature DC, we explored a possible role of PA28α/β in immunoproteasome assembly in DC. By quantification of PA28α immunoprecipitates (Fig. 4A), we first determined that PA28α protein neosynthesis was about 3.5 times enhanced in mature DC. Next, we asked whether PA28α/β had higher affinity for immunoproteasomes compared to constitutive proteasomes and could therefore influence the assembly. PA28α/β was precipitated from immature DC under conditions that were previously determined to preserve interactions with the proteasome [23]. The pattern of proteasomes coprecipitated with PA28α/β was not different from that precipitated with the proteasome-specific antibody (Fig. 4B). If PA28α/β did have a higher affinity for the immunoproteasome, the ratio of immunosubunits versus constitutive subunits would be expected to increase in PA28α/β coprecipitates, but this was not the case, as exemplified by the pair of homologous subunits delta and LMP2. A comparison of the other pairs of homologous subunits (Z/MECL-1, MB1/LMP7) is not so informative, because MECL-1 is not well resolved from another subunit and because of poor metabolic labeling of MB1. Taken together, these data suggest that PA28α/β does not preferentially associate with immunoproteasomes.

To our knowledge a direct interaction of PA28α/β with proteasome precursor complexes has not yet been demonstrated; therefore, we examined such a possible inter-

A



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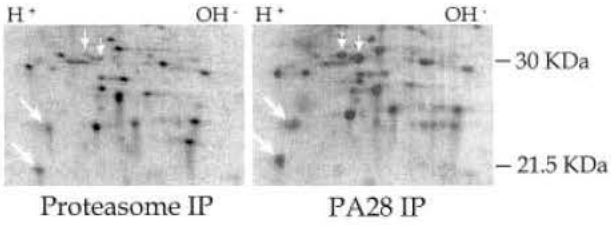

Fig. 4. (A) Analysis of PA28 expression in immature and mature (LPS, 24 h) DC. Cells were metabolically labeled and PA28α was immunoprecipitated. Quantitative analysis determined that PA28 is 3.5 times more prominent in mature compared to immature DC. (B) NEPHGE/SDS-PAGE analysis of PA28-proteasome co-immunoprecipitates. Immunoprecipitation from metabolically labeled immature DC was performed with the proteasome-specific mAb MCP21 or with a PA28α/β–specific antibody. Big diagonal arrows indicate delta (24 kDa) and LMP2 (21 kDa) for comparison of constitutive and immunoproteasome content. Positions of PA28α (right) and β (left) are indicated by small arrows.

action. 13/16S precursor complexes were separated from mature 20S proteasomes by gel filtration, as evidenced by the presence of the 30-kDa MECL-1 precursor in fractions 31–35 and of the two forms of mature MECL-1 (25 and 27 kDa) previously described in DC [11] in fractions 15–21. Subsequently, fractions 33–35 were pooled and immunoprecipitation of PA28α/β was performed to determine whether assembling 13S/16S proteasome complexes were coprecipitating. Immunoprecipitation from fractions 16–18, containing mature 20S proteasomes, was performed in parallel as positive control. Proteasome subunit XAPC-7 (α4) was detected by Western blotting at the expected position in two-dimensional gels, that is in precipitates from both, mature proteasome-containing fractions and proteasome precursor-containing fractions (Fig. 5B). The specificity of the PA28α/β immunoprecipitation and XAPC-7 detection was confirmed in control experiments using beads precoated with preimmune serum that gave no signal (not shown). From these results it is evident that PA28α/β associates with proteasome precursors during the assembly.

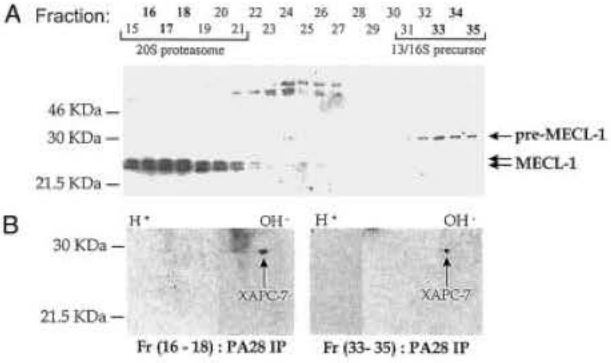

Fig. 5. Analysis of PA28-proteasome interactions. (A) Lysate of mature DC was separated by gel filtration and fractions were analyzed by Western blotting for detection of precursor and mature forms of proteasome subunit MECL-1 as indicated. (B) Precipitation of PA28α/β was performed from fractions 33–35 (containing 13/16S proteasomes) or 16–18 (containing 20S proteasomes). Precipitates were resolved by NEPHGE/SDS-PAGE and Western blotted for the detection of the proteasome α subunit XAPC-7.

2.4 The steady-state subunit composition of proteasomes changes only very gradually and to a low extent after DC maturation

Western blot analysis was performed to assess the consequences of the increments in PA28α/β and proteasome immunosubunit mRNA on the steady-state proteasome composition in mature DC. The total content of proteasomes was the same in immature and mature DC, as shown by the comparable intensity of reaction against the constitutively expressed α-type subunit HC2 (α6) (Fig. 6). In mature DC, the enhancement of the immunosubunits LMP2, LMP7 and MECL-1 on one hand, and the decrease of the constitutive subunits delta and Z on the other hand, were rather slow and became apparent just after several days, suggesting that the composition of the proteasome population does not change dramatically. Unfortunately, our Western analysis was of limited use for the detection of subtle changes in the amounts of proteasome subunits so that further conclusions were precluded. Noteworthy, the antiserum against MECL-1 recognized two forms of mature MECL-1 in DC as previously described [11] and mAb against delta visualized

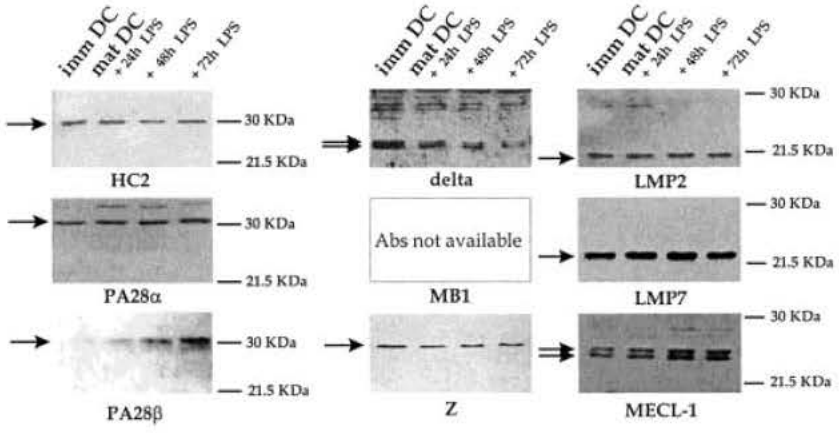

Fig. 6. Western blot analysis of PA28α/β and proteasome subunits in immature and mature (LPS, 24, 48, 72 h) DC. Detection of HC2 subunits is shown for standardization of total proteasome content in different samples.

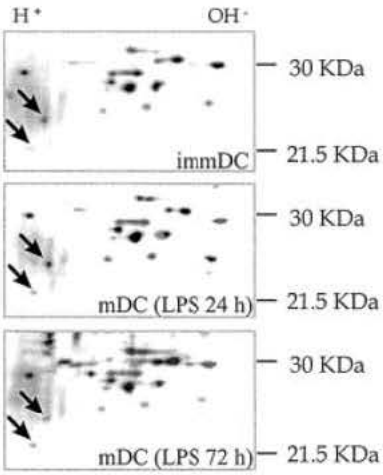

Fig. 7. Steady-state analysis of subunit composition of proteasomes from immature and mature (LPS, 24 h and 72 h) DC on silver-stained NEPHGE/SDS-PAGE. Upper and lower arrows indicate the positions of subunits delta and LMP2, respectively.

two bands in lysate of DC, but not of HeLa cells (not shown). The exact nature of these additional forms of subunits is not known but it is likely to result from post-translational modifications. In agreement with increased neosynthesis, Western analysis revealed higher content of PA28α/β in mature DC, more obvious for PA28β than α, possibly pointing at a specific function of this subunit.

To complement the Western analysis, which assesses only changes of single components, and to directly compare the levels of constitutive and immunosubunits under steady-state conditions, we silver-stained proteasome immunoprecipitates from immature and mature DC. Immature DC contained approximately equal amounts of constitutive and immunoproteasomes (compare delta with LMP2 in Fig. 7). With LPS stimulation, the increment of LMP2 was very little after 24 h, but more appreciable after 72 h, indicating a low rate replacement of constitutive proteasomes by immunoproteasomes. Therefore, together with Western blot analysis, these data show only small differences in steady-state proteasome population between immature and mature DC.

3 Discussion

IFN-γ induces a rapid response of the innate immune system at the site of infection through activation of NK cells and macrophages, but to what extent IFN-γ can influence DC in their antigen-presenting function is still a matter of debate. Initially, it was reported that only type I IFN activate human DC generated from $CD34^+$ precursors, whereas IFN-γ did not lead to phenotypic maturation [24]. In contrast, a $CD11c^+$ subset of human peripheral blood DC was recently shown to undergo similar phenotypic maturation after treatment with either IFN-α or IFN-γ [25]. In this study we investigated the effect of IFN-γ on monocyte-derived DC, which down-regulate surface expression of IFN-γR during their maturation [26]. Although phenotypic maturation was only partially induced, DC fully responded to IFN-γ in terms of proteasome biogenesis, with a shift towards immunoproteasomes. Hence, it appears that like other cell types, DC respond to IFN-γ favoring immunoproteasome assembly and may, therefore, present the same peptides that are produced in the inflamed tissues to the adaptive immune system for the induction of antigen-specific responses.

We investigated the mechanism by which immunoproteasome assembly is regulated in DC and, in particular, whether maturation stimuli would enhance mRNA expression of immunosubunits leading to a strongly elevated production of precursors for the immunosubunits. This may suffice to outcompete constitutive subunits from proteasome assembly without the need for other factors. A vigorous transcriptional induction of immunosubunits by IFN-γ occurs in HeLa cells, which in the absence of IFN-γ and TNF-α express very low levels of LMP2, LMP7 and MECL-1. In DC, however, the picture looks quite different. We found constitutive expression of immunosubunits in immature DC and limited up-regulation during maturation. Interestingly, the promotor for LMP2 reacted more strongly to LPS stimulation of DC and up-regulation of LMP2 mRNA was more pronounced compared to that of other immunosubunits. This suggests that LMP2 may serve as a pacemaker for immunoproteasome assembly in DC, in particular in view of its role in facilitating the incorporation of MECL-1 [27, 28]. One may argue that the observed up-regulation of mRNA for immunosubunits could suffice to prevent the neosynthesis of constitutive proteasomes, but given that this is 5–10-fold stronger in IFN-γ-stimulated HeLa cells (data not shown), it may be that additional factors could bias the assembly process in favor of immunoproteasomes in mature DC.

In search for such proteins, we investigated a potential role of PA28α/β, as was suggested by Preckel et al. [17] in their report on $PA28\beta^{-/-}$ mice. Consistent with a role of PA28α/β in proteasome assembly, we show that PA28α/β associates in DC with proteasomes at the stage of assembly (Fig. 5B), but whether such an association favors the maturation of immunoproteasomes remains to be determined. We investigated whether PA28α/β facilitates the assembly of immunoproteasomes by preferentially binding to mature 20S immunoproteasomes. However, in coimmunoprecipitation experi-

ments (Fig. 4B) we found that PA28α/β equally associated with constitutive and immunoproteasomes arguing against this possibility. Our data are in agreement with previous investigations [29, 30], which failed to provide evidence for a preferential association of immunoproteasomes with recombinant or purified PA28 proteins *in vitro* using proteasome activation kinetics rather than physical association in cell lysates as a read out. It would be interesting to test an involvement of PA28α/β simply by examining proteasome synthesis in immature and mature DC from PA28α/β deficient mice. If PA28α/β is actually an assembly regulator, the expectation would be that immunoproteasomes should not be found in immature DC and that maturation stimuli would not be able to switch proteasome assembly to immunoproteasomes.

An important goal of our study was to determine the steady-state subunit composition of proteasomes in immature and mature DC. Morel et al. [18] recently reported that the $RU1_{34–42}$ epitope from the RU1 antigen of renal carcinoma was presented by immature but not by mature human DC. IFN-γ treatment of renal carcinoma cells as well as overexpression of LMP2, LMP7 and MECL-1 down-regulated $RU1_{34–42}$ presentation suggesting that immunoproteasomes interfered with an efficient processing of this epitope. Given that other tumor epitopes like Melan-$A_{26–35}$ or $gp100_{209–217}$ were shown to be poorly presented after IFN-γ stimulation of cells [18], the role of immunoproteasome induction by IFN-γ in tumor cells and by maturation stimuli in DC could be of major importance for tumor immunology and vaccination. Hence, we determined the expression level and the changes in the relative ratios of constitutive and inducible proteasome subunits during DC maturation. We found that in immature DC, immunoproteasomes represent already a consistent part of the proteasome population and that, despite the observed shift towards immunoproteasome synthesis [11] and in contrast to previous assumptions [31], proteasome replacement during maturation occurred only at a very low degree on the steady-state level (Fig. 7). According to recent investigations [32, 33], it is reasonable to assume that 72 h after pathogen-mediated stimulation, DC will have reached the lymph nodes and present MHC-I-restricted peptides to CTL in order to mount a specific immune response, so this should be an appropriate time point to look at proteasome composition. When we stimulated DC for an extended period of 6 days with LPS or, alternatively, for 24 h with LPS and then for 48 h with CD40L, the replacement of constitutive proteasomes by immunoproteasomes did not exceed the level which we found after 3 days of LPS stimulation (not shown). Given that the life span of mature DC in the lymph node is limited [33, 34] it seems unlikely that a greater exchange of proteasomes in DC will occur *in vivo* than observed on the steady-state level after 3 days of LPS stimulation *in vitro*.

We should point out that in the study by Morel et al. [18] monocyte-derived DC were matured with a cocktail of TNF-α + IL-1β + IL-6 + PGE2, whereas in this work LPS stimulation was used. However, we previously showed that these two maturation protocols promote the biogenesis of immunoproteasomes to a similar extent [11]. Therefore, we feel that the minor replacement of constitutive proteasomes by immunoproteasomes which we detected on steady-state level in mature DC can hardly explain the difference in $RU1_{34–42}$ presentation [18]. Obviously, we can not rule out that a threshold level exists for the content of immunoproteasomes in DC beyond which the production of $RU1_{34–42}$ is too inefficient for presentation and that this threshold is passed in mature DC. However, no evidence for the existence of such a threshold could be obtained when the cellular content of immunoproteasomes was increased from 40% to 60% in inducible transfectants using the presentation of an adenovirus derived epitope as a read out system [7]. Taken together, our data suggest that mechanisms other than changes in 20S proteasome composition may be responsible for abrogating $RU1_{34–42}$ presentation in mature DC.

One possibility would be that proteolytic activity of proteasomes could be differently affected by binding of regulatory complexes in immature and mature DC. We showed that enhanced expression of PA28α and β in mature DC results in PA28α/β complex formation and association with 20S proteasomes [11]. This may lead to a change in antigen presentation as PA28α/β has been shown to alter polypeptide fragmentation *in vitro* [35] and as the overexpression of PA28α/β can enhance antigen presentation in cells without affecting the subunit composition of proteasomes [9, 10]. Although in the particular case of the $RU1_{34–42}$ epitope the overexpression of proteasome immunosubunits sufficed to abrogate presentation of the epitope, PA28α/β may still influence the generation of this and other epitopes in mature DC. Another intriguing hypothesis would be that the activity of constitutive proteasomes but not immunoproteasomes is specifically silenced by selective proteasome regulators or inhibitors in mature but not immature DC. This hypothesis, which would be analogous to the regulation of cathepsin S activity by cystatin C in the MHC class II-processing pathway of DC, deserves further investigations.

4 Materials and methods

4.1 DC preparation

Monocytes were isolated from PBMC by Ficoll-Paque density centrifugation and magnetic sorting of $CD14^+$ cells. Differentiation to immature DC was achieved by cultivation for 5 days in GM-CSF and IL-4 as described [36]. Immature cells were treated with 100 U/ml IFN-γ (Roche, Basel) or 1 μg/ml LPS (from *Salmonella abortus equi*, Sigma, Buchs).

4.2 Flow cytometry

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

4.3 Metabolic labeling and immunoprecipitation

DC were starved for 30 min and pulsed for 4–8 h with 0.2–0.4 mCi/ml [^{35}S]methionine/cysteine followed by a 16-h chase. Cells were lysed in hypotonic lysis buffer (25 mM Tris-HCl, pH 7.6, 2 mM $MgCl_2$, 5 mM KCl, 17.4% v/v glycerol, 2 mM ATP, 1 mM DTT) by sonication followed by a 1–2-h incubation on ice. For proteasome immunoprecipitation and coimmunoprecipitation, protein A-Sepharose beads (Pharmacia, Zürich) precoated with mAb MCP21 [37] or a rabbit antiserum raised against hPA28α/β [38] were incubated with precleared lysates for 4 h or overnight at 4°C and washed five times in lysis buffer. Alternatively, cells were lysed in 20 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 2% Triton X-100, 150 mM NaCl for 1–2 h on ice, with occasional vortexing. Beads were precoated with a rabbit antiserum raised against the KLH-conjugated PA28α peptide RVQPEAQAKVDVFRED or with a rabbit antiserum raised against purified 20S proteasomes from human erythrocytes in combination with mAb MCP21. The beads were incubated for 3 h or overnight to immunoprecipitate PA28α/β and the proteasome, respectively, and washed three times in 650 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5% Triton X-100 and two times in the same buffer but containing 150 mM NaCl.

4.4 Gel filtration

DC (10^7 cells) were lysed in 200 μl of hypotonic lysis buffer (25 mM Tris-HCl, pH 7.6, 2 mM $MgCl_2$, 5 mM KCl, 17.4% v/v glycerol, 2 mM ATP, 1 mM DTT) by sonication followed by 2-h incubation on ice. The lysate was centrifuged at 20,000×g for 10 min before loading onto Superose® 6 HR 10/30 (Pharmacia) equilibrated in lysis buffer. The sample was eluted in lysis buffer at a flow rate of 0.25 ml/min and 50 fractions of 0.25 ml eluate were collected from 7 to 19.5 ml.

4.5 NEPHGE/SDS-PAGE, Western blotting and silver staining

NEPHGE/SDS-PAGE was performed as previously described [35]. Silver staining and Western blotting with enhanced chemiluminescence detection were performed according to standard protocols. Polyclonal antibodies used were: goat anti-h actin (C-terminal peptide, Santa Cruz Biotech.), rabbit antiserum against hLMP2, hLMP7, hMECL-1 [11], hPA28α (see above) and hPA28β (kindly provided by Dr. George DeMartino). The following mAb against proteasome subunits were used: anti-hdelta (MCP421), hZ (MCP168) (kindly provided by Dr. Klavs Hendil, [37]), hHC2 (MCP20) and hXAPC-7 (MCP34) (Affiniti Ltd., Mamhead, GB).

4.6 Northern blot

Total RNA was extracted from cells using TRIzol® reagent (GIBCO, Basel) according to the manufacturers' instructions, separated on agarose formaldehyde gels, transferred to a membrane and probed as described elsewhere [39]. ^{32}P-labeled probes were generated by PCR amplification on cDNA templates using the same specific primers as in the section below.

4.7 Real time RT-PCR

Total RNA was extracted using TRIzol reagent. cDNA was synthesized from 4 μg RNA using 2 mM dT15, 2 mM dNTPs, 0.6 U/μl RNasin (Promega), 200 U M-MLV RT (Promega, Wallisellen, Switzerland) per reaction (1 h at 42°C). After cDNA synthesis, the nucleic acids were precipitated and resuspended in H_2O. Real time PCR was performed with the Light Cycler (Roche) using the DNA Master SYBR Green I reaction mix (Roche) and 0.5 pmol/μl of each primer in 2.25 mM $MgCl_2$. Sense and antisense primers used for PCR amplification were: hβ-actin: 5'-TCATCACCATTGGCAATGAG-3' and 5'-CACTGTGTTGGCGTACAGG-3'; hGAPDH: 5'-ATGACCCCTTCATTGACC-3' and 5'-TGAGTCCTTCCACGATACC-3'; hPA28α: 5'-TGTACCAAGACAGAGAACCTGC-3' and 5'-AGGAAGGCTCCTGCTGTTACTC-3'; hPA28β: 5'-GCAAACAGGTGGAGGTCTTCAGGCAGAATC-3' and 5'-TAAAGCTCAGCATAGAAGGCCCTCAGGTCC-3'; hUMP1: 5'-GAAACATTCAGGGTCTATTTGCTCC-3' and 5'-GACTATAAACAAGATGCAGCCCTCG-3'; hdelta: 5'-CATTCACTCCAGACTGGGAAAGC-3' and 5'-CTCTACCCCTGACTCTGCAATGG-3'; hMB1: 5'-GGAGTCTCAGTGATGGTCTGAGC-3' and 5'-TCCGTATTACCAATGACAGTCACC-3'; hZ: 5'-TCTCTTTTGATAACTGCCGCAGG-3' and 5'-TGCAGTAGTCCCTTTCTCACACC-3'; hLMP2: 5'-CGTTGTGATGGGTTCTGATTCC-3' and 5'-GTTCATTGCCCAAGATGACTCG-3'; hLMP7: 5'-AATGCAGGCTGTACTATCTGCG-3' and 5'-TGCAGCAGGTCACTGACATCTG-3'; hMECL-1: 5'-CCTTCGAGAACTGCCAAAGAAATGC-3' and 5'-CAAGCTCTAAGCCTCAGCTTACTCC-3'. All runs were programmed as follows. Denaturation: 1 min at 95°C; amplification (50 cycles, with

readings of the fluorescence at the end of each cycle): 10 s at 95°C, 30 s at 60°C, 30 s at 72°C; analysis of the products (reading of the fluorescence in continuous mode): 0 s at 95°C, 55 to 95°C transition with 0.1°C increment/s. Amplifications for different products were performed in separate runs. In each run, samples from mature DC were compared to samples from immature DC. The value calculated by the quantification analysis (Light Cycler Software 3) for the mature DC was always within the range covered by three concentrations of sample from immature DC that were taken as arbitrary units to construct the standard curve for linear regression with r=1.0 and $e<0.0001$. In a first set of runs, the amounts of templates were standardized to yield the same amounts of β-actin RT-PCR product. Specificity of the amplification was checked with melting curve analysis of the products as well as analysis on agarose gels.

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