A proteasome-dependent, TAP-independent pathway for cross-presentation of phagocytosed antigen

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Major histocompatibility complex (MHC) class I cross-presentation is thought to involve two pathways, one of which depends on both the TAP transporters and the proteasome and the other on neither. We found that preincubation of TAP-deficient dendritic cells at low temperature increases the density of MHC class I at the surface and fully restores cross-presentation of phagocytosed antigen, but not of soluble antigen internalized through receptors. Restoration of cross-presentation by TAP-deficient cells requires antigen degradation by the proteasome. Thus, TAP might mainly be required for recycling cell surface class I molecules during cross-presentation of phagocytosed antigens. Furthermore, phagosomes—but not endosomes—seem to have a TAP-independent mechanism to import peptides generated by cytosolic proteasome complexes.

Keywords: antigen presentation; cross-presentation; transporters associated with antigen processing; proteasome; MHC class I

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INTRODUCTION

Presentation of exogenous antigen (Ag) by major histocompatibility complex (MHC) class I molecules, referred to as cross-presentation, is important in priming of CD8⁺ T-cell responses to a variety of pathogens and to tumors as well as in immune tolerance to self and in autoimmunity (Rock & Shen, 2005). The cellular pathways mediating cross-presentation are generally divided according to their dependence on the TAP transporters and the proteasome (Rock & Shen, 2005). A pathway referred to as vacuolar and generally considered minor overlaps with the MHC class II Ag processing pathway and uses endolysosomal proteases to degrade internalized bacteria and other, frequently particulate Ag, the degradation products of which are loaded on recycling class I

molecules (Ramachandra *et al*, 2009). Given that Ags are degraded in the compartments also containing loadable class I molecules, a transport step between the cytosol and vacuoles is not required.

The bulk of cross-presentation is thought to involve proteasome and TAP-dependent pathways and thus implies a link between the endocytic pathway and the endoplasmic reticulum (ER; Monu & Trombetta, 2007). This concept is based on both cell biological and functional evidence. Recruitment of ER membranes and/or components including TAP to phagosomes (reviewed in Monu & Trombetta (2007)) and more recently to endosomes (Burgdorf *et al*, 2008) has been described. However, at least the former finding remains controversial, so that a main role of the perinuclear ER in cross-presentation remains conceivable (Touret *et al*, 2005). Evidence in favour of the concept derives from Ag presentation assays, in which TAP deficiency and proteasome inhibitors reduce cross-presentation strongly. However, interpretation of the latter observations is complicated by several considerations. Proteasome inhibition interferes with a large number of cellular processes, including signalling pathways, cell cycle, regulation of transcription and protein transporting in the endocytic pathway (Goldberg, 2007). Moreover, incubation with proteasome inhibitors depletes cellular stocks of free ubiquitin, which in turn inhibits ubiquitination of endosomal proteins required for their intracellular targeting (Lee *et al*, 2005). TAP deficiency also has a side effect with the potential to affect cross-presentation, namely, scarcity of cell surface class I molecules able to recycle through endosomes (Van Kaer *et al*, 1992). This side effect has been shown to affect vacuolar cross-presentation; however, its potential effect on the proteasome-dependent pathway is unknown (Chefalo *et al*, 2003). As the source of class I molecules loaded in this pathway remains unclear, it is possible that TAP deficiency affects cross-presentation by reducing the number of recycling class I molecules. Considering this, we set out to re-examine the role of TAP in proteasome-dependent cross-presentation of phagocytosed and soluble receptor-targeted Ag.

RESULTS AND DISCUSSION

MHC class I expression on TAP-deficient DCs

We speculated that the decreased cell surface expression of MHC class I molecules might contribute to reduced cross-presentation by TAP-deficient dendritic cells (DCs). To examine

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this hypothesis, we took advantage of the phenomenon, previously shown for RMA-S lymphoma cells (Ljunggren *et al*, 1990), that incubation of murine TAP-deficient cells at 26 °C results in increased cell surface export of MHC class I heavy/light chain complexes. Overnight DC incubation at 26 °C increased expression of cell surface K^b molecules recognized by the conformation-independent antibody B8.24.3 fourfold (Fig 1A), whereas the complexes recognized by the conformation-dependent antibody AF6 increased by <30%. Thus in murine DCs, as in previously studied lymphoma cells, low-temperature incubation results in the increased presence at the cell surface of class I molecules, most of which have not acquired a fully native conformation.

To further characterize the nature of class I molecules exported by TAP-deficient DCs at 26 °C, we studied DCs expressing H-2L^d, a class I allomorph for which antibodies recognizing free heavy chains (64.3.7) and fully conformed complexes (30.5.7) are available (Hansen *et al*, 2005). Acid treatment or 'stripping' is known to induce dissociation of cell surface MHC class I complexes (Wang *et al*, 1998). A short treatment with acid converted most L^d complexes to free heavy chains, which could rapidly be removed by trypsin digestion, while fully conformed L^d complexes were not sensitive to trypsin (Fig 1B,C). Similar results were obtained with wild-type (wt) DCs and TAP knockout (ko) DCs preincubated at 26 °C (Fig 1B,C). Fully conformed peptide-K^b complexes expressed by both wt and TAP ko DCs were insensitive to trypsin digestion. Acid treatment rendered molecules recognized by the non-conformational antibody B8.24.3 sensitive to trypsinization, suggesting that free K^b heavy chains can be removed by trypsin digestion similar to free L^d heavy chains. Importantly, K^b molecules expressed by TAP ko DCs preincubated at 26 °C were insensitive to trypsinization, suggesting that low-temperature preincubation results in export of peptide-MHC complexes but not free heavy chains.

Previous studies concluded that TAP-deficient RMA-S lymphoma cells export normal amounts of MHC class I molecules to the cell surface, which however show reduced peptide-binding capacity (Day *et al*, 1995). While these molecules are internalized and rapidly degraded at physiologic temperature, incubation at 26 °C would promote stabilization by exogenous β_2 -microglobulin (β_2 m) contained in fetal calf serum (FCS; Rock *et al*, 1991; Day *et al*, 1995). We used protocols published by these authors to examine intracellular and surface class I molecules in DCs. TAP-deficient, but not wt, DCs harboured significant amounts of intracellular peptide-receptive class I molecules (Fig 1D). However, in contrast to RMA lymphoma cells (Day *et al*, 1995), DCs expressed very low numbers of peptide-receptive class I molecules on the surface that were only slightly increased by 26 °C incubation and whose numbers were not affected by TAP deficiency. We also studied the requirement of exogenous β_2 m for class I stabilization at 26 °C (Fig 1E). When DCs were incubated in IMDM, a protein-free medium not designed to be used without serum, addition of FCS (or 2.5 μ g/ml β_2 m, not shown) greatly enhanced surface class I expression. However, in the presence of AIM-V, a medium devoid of β_2 m but designed to sustain cell growth, addition of FCS had no effect on class I expression (Fig 1E). Thus, at least for DCs, the requirement for exogenous β_2 m is limited to conditions of cell starvation, where the amount of endogenously synthesized β_2 m might be insufficient. Consequently, increased surface class I expression by TAP ko DCs

incubated at 26 °C does not seem to be due to stabilization by exogenous β_2 m, but might rather result from reduced internalization and degradation. In conclusion, incubation of DCs at 26 °C results in stabilization at the cell surface of incompletely folded, mainly endogenously synthesized class I heavy chain/ β_2 m complexes, most of which are not peptide-receptive.

Cross-presentation after preincubation at 26 °C

We studied the effect of low-temperature preincubation on Ag presentation by DCs. As expected, TAP deficiency compromised presentation of vaccinia-expressed full-length ovalbumin (OVA) but not that of S8L targeted to the ER by a signal peptide (Fig 2A). Preincubation at low temperature did not restore endogenous presentation of full-length OVA (Fig 2A). Next we examined cross-presentation of phagocytosed Ags by DCs. For this, we used two particulate Ags latex beads coated with OVA and recombinant *Saccharomyces cerevisiae* produced according to a published strategy (Howland & Wittrup, 2008) and expressing OVA covalently linked to the cell surface. Cross-presentation was read out as interleukin (IL)-2 secretion by naive T-cell antigen receptor-transgenic OT-I T cells recognizing the H-2 K^b-restricted peptide S8L. As expected, TAP deficiency compromised cross-presentation of both Ag forms at physiologic temperature (Fig 2B). However, 26 °C preincubation had a striking effect on TAP ko DCs and normalized cross-presentation of both Ags. The kinetics of the effect of low-temperature preincubation on cross-presentation paralleled that on K^b expression; first small effects were notable after 2–4 h, while 16 h was required for full normalization (Fig 2C). Conversely, when DCs preincubated overnight at low temperature were shifted to 37 °C, incubation of >2 h was sufficient to reverse the effect of low-temperature incubation on cross-presentation capacity and on class I expression (Fig 2D).

Role of recycling MHC class I in cross-presentation

Constitutive recycling of class I molecules through endocytic compartments has been described in a variety of cell types including DCs (Gromme *et al*, 1999; Basha *et al*, 2008). Recycling class I molecules have been reported to be required for cross-presentation of soluble OVA and for cross-presentation of bacterial Ags through the vacuolar pathway (Chefalo *et al*, 2003; Basha *et al*, 2008). To find out whether cell surface class I molecules were implicated in restored cross-presentation by TAP ko DCs preincubated at 26 °C, we treated DCs with acid and trypsin before adding yeast cells (Fig 3A). Removal of surface class I molecules compromised cross-presentation by TAP ko DCs, suggesting that cell surface class I molecules are recruited to present exogenous Ags in these cells. The same treatment had a much smaller but reproducible effect on wt DCs.

Primaquine is a weak base that accumulates in endosomes, neutralizes the endosomal pH and inhibits recycling of some, but not all, class I molecules to the plasma membrane (Reid & Watts, 1990; van Weert *et al*, 2000). Surprisingly, primaquine increased cross-presentation of yeast cells in a dose-dependent manner (Fig 3B). This effect was more pronounced after acid treatment and trypsinization, particularly for TAP ko DCs. Considering that this might be due to an effect of primaquine on MHC class I accumulation in phagosomes, we monitored phagosomal MHC class I concentrations (Fig 3C). Primaquine did not affect peak MHC class I concentrations observed at 10 min of phagosome maturation, but increased class I concentra-

Fig 1 | Effect of temperature on cell surface expression of MHC class I molecules on TAP-deficient DCs. (A) Wt and ko DCs were incubated overnight at 37 °C or 26 °C and analysed for expression of H2K^b using antibodies AF6 and B8.24.3. The numbers indicate mean fluorescence intensities (MFIs). (B) Cell surface molecules on Balb/c, B6 wt or TAP ko (left to right) BM-DCs were subjected to trypsin digestion either directly or after acid stripping for 90 s, followed by flow cytometric analysis for native L^d or K^b molecules, as indicated. (C) Experiment analogous to that in (B), but staining was for L^d free heavy chains (antibody 64.3.7) or total K^b molecules, as indicated. Background staining values of acid-trypsin-treated cells using an isotype control antibody were 10–20. (D) Wt or TAP ko DCs were preincubated at 26 °C or 37 °C overnight, incubated directly (right panel) or after fixation and permeabilization (left panel) with FITC-labelled peptide S8L for 10 min in the presence or absence of a 100-fold excess of unlabelled peptide S8L, and analysed by flow cytometry. (E) TAP ko DCs were incubated for 48 h in IMDM or AIM-V medium with or without FCS, with addition of 10 µg/ml human β₂m during the last hour where indicated. Total K^b expression was analysed using antibody Y3. BM, bone marrow; β₂m, β₂-microglobulin; DC, dendritic cell; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; ko, knockout; MHC, major histocompatibility complex; wt, wild type.

Fig 2 | Effect of temperature on direct and cross-presentation of phagocytosed antigens by TAP-deficient DCs. All results of antigen presentation experiments correspond to means of duplicate or triplicate wells. In (A), BM-DCs were infected with vaccinia viruses encoding epitope S8L preceded by a signal peptide (left-hand panel), or full-length OVA. (B–D) In all cross-presentation experiments shown, wt and TAP ko BM-DC preincubated overnight at 37 °C or 26 °C were pulsed for 8 h with antigen, fixed, and added for 24 h to naive OT-I T cells. (B) The left-hand and center panels show representative experiments using OVA-coated beads or yeast cells expressing OVA at the surface, respectively. The right-hand panel shows the percentage stimulation relative to wt BM-DCs preincubated at 37 °C, set at 100, in 10 experiments (mean + s.e.m.). In (C), TAP ko BM-DCs cultured at 37 °C were shifted for different periods to 26 °C, followed by analysis of H-2 K^b expression (left-hand panel) and of cross-presentation capacity (right-hand panel). Wt cells were tested as reference. The panels in (D) show the inverse experiments, in which TAP ko BM-DCs were first incubated for 16 h at 26 °C before shifting for different periods to 37 °C, followed by analysis of K^b expression and cross-presentation. BM, bone marrow; DC, dendritic cell; IL, interleukin; ko, knockout; OD, optical density; OVA, ovalbumin; wt, wild type.

tions at later time points in TAP-deficient cells. Thus, blocking the recycling of endosomal MHC class I molecules to the plasma membrane enhances cross-presentation of phagocytosed Ag, possibly through prolonged retention of class I molecules in phagosomes. We

speculate that enlarged endosomes induced by primaquine, containing large amounts of internalized class I molecules unable to recycle to the surface, might fuse with phagosomes, providing an abundant MHC class I pool for loading with exogenous Ags.

Fig 3 | Distinct pathways for cross-presentation of phagocytosed and soluble receptor-targeted antigen. In (A), cells were treated with acid and/or trypsin before addition of OVA-expressing yeast cells. (B) BM-DCs were pretreated with acid followed by trypsinization or not before pulsing with OVA-expressing yeast cells in the presence of primaquine at indicated concentrations. (C) Phagosomes were prepared from wt or TAP ko BM-DCs preincubated at 37 °C or 26 °C, respectively, that had phagocytosed polystyrene beads in the presence or absence of 0.04 mM primaquine. Phagosomes were fixed, stained with a cocktail of antibody recognizing K^b and D^b, and analysed by flow cytometry. (D) BM-DCs preincubated overnight at 37 °C or 26 °C were pulsed with graded amounts of CD11c-targeted OVA fusion protein. In (E), BM-DCs were pulsed similarly in the presence or absence of 0.04 mM primaquine with CD11c-targeted OVA fusion protein, at a concentration equivalent to 3 µg/ml OVA before adding to OT-I T cells for 36 h. Cells fixed before antigen addition were negative controls. BM, bone marrow; DC, dendritic cell; IL, interleukin; ko, knockout; MFI, mean fluorescence intensity; OD, optical density; OVA, ovalbumin; wt, wild type.

Complete TAP-dependence of receptor-targeted Ag

Next to particular Ags, DCs also cross-present soluble Ags that are internalized on binding to cell surface receptors. We examined cross-presentation of fusion proteins developed by us, which readily form complexes with a large variety of targeting Abs and are cross-presented with high efficiency (Kratzer *et al*, 2010). The effect of TAP deficiency on cross-presentation of OVA targeted by a specific antibody to the CD11c receptor was strikingly different from that on presentation of phagocytosed Ag (Fig 3D). First, whereas, at physiologic temperature, cross-presentation of phagocytosed Ag by TAP ko DCs was reduced but not abolished (Fig 2B), cross-presentation of receptor-targeted soluble Ag was completely undetectable. Second, exposure to low temperature had no effect at all on cross-presentation of OVA targeted to CD11c. Thus, while cross-presentation of phagocytosed Ag is only partially TAP dependent and can be fully rescued by DC exposure to low temperature, cross-presentation of soluble receptor-targeted Ag is completely TAP dependent and cannot be rescued.

We also studied cross-presentation of receptor-targeted Ag in the presence of primaquine. Primaquine reduced presentation of CD11c-targeted OVA by about 65% (Fig 3E), suggesting that cell surface export of recycling MHC class I molecules had a main role in cross-presentation of this Ag. In conclusion, cross-

presentation of phagocytosed and soluble receptor-targeted Ags showed striking differences both with respect to the requirement of the TAP transporters and to the export pathway of MHC class I molecules.

Proteases degrading cross-presented Ag in TAP ko DCs

Next we sought to define the proteases involved in cross-presenting the different OVA forms in TAP-deficient DCs, using pharmacological protease inhibition. At the concentrations chosen, the inhibitors were free of non-specific effects and did not inhibit presentation of synthetic or endogenously expressed, minigene-encoded epitope S8L by DCs (supplementary Fig S1 online). Proteasome inhibitors did not change the number or the peptide-receptive state of DC surface class I molecules (not shown). Surprisingly, cross-presentation of phagocytosed Ag by TAP ko DCs was strongly reduced by both proteasome inhibitors (Fig 4A). In seven experiments using epoxomicin and in four using MG132, the extent of reduction did not differ between wt and TAP ko DCs preincubated at 37 °C or 26 °C (71–79%). Bafilomycin, which inhibits phagosome acidification, consistently increased cross-presentation (six experiments). An inhibitor of cathepsins B, L and S (LHVS) and a general cathepsin inhibitor (Z-FA-fmk) both reduced cross-presentation by an average of 80% and about 45%,

Fig 4 | Effect of protease inhibitors on cross-presentation of phagocytosed and receptor-targeted Ag by TAP-deficient DCs. (A) Wt and TAP ko BM-DCs preincubated at 37 °C or 26 °C were incubated for 8 h with OVA-expressing yeast cells in the presence of the inhibitors indicated. (B) Wt BM-DCs were incubated with CD11c-targeted OVA fusion proteins in the presence of protease inhibitors. (C) Wt and TAP ko BM-DCs preincubated as indicated were incubated for 8 h with yeast cells carrying epitope S8L at the cell surface in the presence of the drugs indicated. In (D), different concentrations of S8L-carrying yeast cells were used for a cross-presentation experiment in the presence of proteasome and cathepsin inhibitors. BM, bone marrow; DC, dendritic cell; IL, interleukin; ko, knockout; OD, optical density; OVA, ovalbumin; wt, wild type.

respectively. Thus cross-presented phagocytosed Ag is processed in TAP ko DCs preincubated at 26 °C and in wt DCs at 37 °C by the same proteolytic pathway, which requires both the proteasome and cathepsins. Presumably, initial Ag cleavage in endocytic compartments is followed by export to the cytosol for degradation by the proteasome, consistent with other cross-presentation studies (Singh & Cresswell, 2010). The effect of bafilomycin indicates that the cathepsin involved is active at neutral pH, a feature characterizing cathepsin S but not B and L. Enhanced cross-presentation of particulate Ag upon blocking phagosome acidification is also consistent with previous observations (Savina et al, 2006).

We asked whether receptor-targeted soluble Ag was processed using the same set of proteases. As shown in Fig 5B, presentation

of OVA fusion protein targeted to CD11c was strongly dependent on the proteasome and partly on cathepsins as well as favored by reduced endosome acidification, as observed for particulate Ag. However, LHSV had little effect, indicating that cathepsin S was not involved in processing of this Ag. Thus, the cross-presentation pathways for particulate and soluble receptor-targeted Ags use a similar though not entirely overlapping combination of endosomal proteases and the proteasome.

Proteasome requirement is limited to full-length Ag

We considered that the effect of proteasome inhibitors might reflect a requirement of the ubiquitin–proteasome system for a process unrelated to Ag degradation, such as phagosome maturation, or cytosolic tail ubiquitination required for transport-

Fig 5 | An alternative model of cross-presentation pathways. The model depicts possible pathways for cross-presentation of phagocytosed antigen (left panel) and soluble receptor-targeted antigen (right panel) in TAP-sufficient cells, and of phagocytosed antigen in TAP-deficient cells (center panel). See text for details. Ag, antigen; DC, dendritic cell; ko, knockout; MHC, major histocompatibility complex; PQ, primaquine; wt, wild type.

ing in the endocytic pathway of molecules involved in MHC class I loading (Lee *et al*, 2005). To examine this hypothesis, we produced yeast cells carrying a precursor of peptide S8L covalently linked to the surface, in which the epitope was preceded by tandem cleavage sites for cathepsin S. Cross-presentation of these yeast cells was strongly inhibited by cytochalasin D, showing that the yeast cells were devoid of free peptide S8L and that presentation required phagocytosis (Fig 4C). Presentation of phagocytosed yeast carrying the S8L precursor was independent of the proteasome in both wt and TAP ko DCs preincubated at 26 °C and 37 °C. This was not because of saturating Ag amounts as the same was observed at limiting Ag concentration (Fig 4D). In contrast, both the specific and the general cathepsin inhibitor reduced presentation, indicating that processing of S8L-carrying yeast required acid proteases but not the proteasome; that is, it was entirely ‘vacuolar’. This in turn suggests that Ags cross-presented in a proteasome inhibitor-sensitive manner (OVA–yeast) were indeed degraded by cytosolic proteasome complexes in both wt and TAP ko cells.

Model for cross-presentation of phagocytosed Ag

The surprising finding that a TAP-independent, proteasome-dependent highly efficient pathway is shown by preincubation of TAP ko DCs at low temperature is difficult to reconcile with the standard model of cross-presentation pathways. We propose an alternative model according to which the principal or partial role of TAP in proteasome-dependent cross-presentation of phagocytosed Ags is to provide recycling cell surface class I molecules (Fig 5). We speculate that phagocytosed Ags are translocated into the cytosol for degradation by the proteasome and then reimported by an unknown TAP-independent mechanism into a compartment distinct from the ER, where recycling class I molecules are loaded before export to the surface using a primaquine-resistant pathway. In TAP-deficient cells, a dearth of surface class I molecules, caused by insufficient export or by rapid internalization of unstable class I molecules into a degradation pathway reduces class I loading with phagocytosed Ags. The reduction of cross-presentation is incomplete and proportional to

cell surface class I density. Complete TAP-dependence of receptor-targeted Ag cross-presentation suggests that the unknown transport mechanism is not accessible to these Ags, highlighting fundamental differences between the pathways handling particular and soluble receptor-internalized Ags. By showing that TAP and proteasome dependence can be dissociated in cross-presentation of phagocytosed Ags, our observations question the validity of the current model of a TAP- and proteasome-dependent pathway for cross-presentation of phagocytosed Ags, and call for new concepts and experimental advances in this field.

METHODS

Mice and reagents and detailed methods. These are described in supplementary information online.

Antigen presentation assays. Bone marrow DCs were prepared by culture for 6 to 7 days in media containing granulocyte–macrophage colony-stimulating factor. Where indicated, DCs were acid-stripped using 300 mM glycine, pH 2.8, in PBS, neutralized and trypsinized. ‘Prefixed’ cells were treated with 0.002% glutaraldehyde before assays. Cells preincubated overnight at 26 °C were shifted to 37 °C at the time of Ag addition. Ultraviolet-irradiated yeast cells carrying covalently linked OVA or S8L at the surface, or polystyrene beads incubated overnight with 50 mg/ml OVA, were used as particulate Ag, and OVA fusion protein (Kratzer *et al*, 2010) in an equimolar complex with anti-CD11c antibody was used as receptor-targeted Ag. DCs were incubated for 8 h (particulate Ag) or overnight (fusion protein) with Ag, fixed, added to T cells purified from the lymph nodes from RAG ko OT-I mice at a ratio of 1:1, and incubated for 24–36 h. OT-I T-cell stimulation was evaluated by commercial IL-2 enzyme-linked immunosorbent assay. Background IL-2 secretion elicited by bone marrow DCs fixed before addition of protein Ags was between optical density 0.12 and 0.18 in all experiments and is subtracted in all figures except Fig 4B. All experiments shown were performed at least three times.

Flow cytometry. Bone marrow DCs were stained with anti-CD11c antibodies together with anti-MHC class I antibodies and analysed on FACSCalibur equipment. Binding of fluorescein

isothiocyanate-labelled S8L to surface K^b, and stabilization of surface K^b by exogenous β_2m , were measured according to published protocols (Rock *et al*, 1991; Day *et al*, 1995). MHC class I recruitment to phagosomes was performed exactly as described (Savina *et al*, 2009).

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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Author contributions: N.M. performed most experiments. R.K. produced fusion proteins. L.S. co-designed and co-supervised the project and performed some experiments. P.v.E. designed and supervised the project and wrote the paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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