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ORIGINAL RESEARCH

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Exosome-driven transfer of tumor-associated Pioneer Translation Products (TA-PTPs) for the MHC class I cross-presentation pathway

Emilie Duvallet[a,*], Mathilde Boulpicante[a,*], Takahiro Yamazaki[a], Chrysoula Daskalogianni[b], Rodrigo Prado Martins[b], Sonia Baconnais[c], Bénédicte Manoury[d], Robin Fahraeus[b,*], and Sébastien Apcher[a,*]

[a]Institut Gustave Roussy, Université Paris Sud, Université Paris Saclay, Unité 1015 département d'immunologie, Villejuif, France; [b]Equipe Labellisée la Ligue Contre le Cancer, Inserm UMR1162, Université Paris 7, Institut de Génétique Moléculaire, Paris, France and RECAMO, Masaryk Memorial Cancer Institute, Brno, Czech Republic; [c]Signalisations, Noyaux et Innovations en Cancérologie, CNRS UMR8126, Université Paris Sud, Université Paris Saclay, Villejuif, France; [d]INEM, U1151-CNRS UMR8253, Paris, France and Université Paris Descartes, Sorbonne Paris Cité, Faculté de medicine, Paris, France

ABSTRACT

Cellular immune reactions against non-self-epitopes require activation of cytotoxic $CD8^+$ T-cells via cross-presentation of MHC class I-restricted peptides by professional antigen presenting cells (pAPCs), with the consequent detection and elimination of cells expressing the same antigens via the endogenous (direct) pathway. The source of peptides for the endogenous pathway is constituted of alternative mRNA translation products; however, it is still unclear which source of peptides is used for cross-presentation. Furthermore, the presentation of non-canonical translation products, produced during a non-conventional translation event, on class I molecules of tumor cells has been reported but how these peptides are generated, presented to pAPCs, and their capacity to stimulate $CD8^+$ T cells is still not known. Here, we report that pioneer translation peptides (PTPs) derived from intron or exon pre-mRNAs can serve as tumor-associated antigens (TA-PTPs) and are delivered from the producing tumor cells to pAPCs via exosomes where they are processed by the cytosolic pathway. Injection of TA-PTPs and tumor-derived exosomes efficiently induce $CD8^+$ T-cell proliferation and prevent tumor growth in mice. Our results show that TA-PTPs represent an efficient source of antigenic peptides for $CD8^+$ T cell activation and that full-length proteins are not required for cross-presentation. These findings can have interesting implications for generating tolerance and for designing vectors to generate vaccines.

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The major histocompatibility complex (MHC) class I antigen presentation pathway allows the immune system to detect and eliminate damaged or infected cells.[1] This requires the activation of naive $CD8^+$ T cells to become effector cytotoxic T lymphocytes (CTLs) by professional antigen-presenting cells (pAPCs).[2-4] pAPCs take up peptide material from the external environment to present it on their own MHC class I molecules via a process called cross-presentation. Although various types of pAPCs are capable to cross-present antigens *in vitro*, most studies have shown that DCs are the main cross-presenting cells.[5] There are two major pathways proposed for the processing of exogenous antigens: the cytosolic and the vacuolar pathways. The cytosolic pathway requires internalization of antigens into the cytosol where they are processed by the proteasomes and then delivered either to the phagosomes or to the endoplasmic reticulum (ER) to be loaded on class I molecules via the transporter associated with antigen processing (TAP).[3,6,7] In the vacuolar pathway, the peptides are processed within the phagosome itself and do not require TAP.[3] The mechanisms showing how exogenous antigens are cross-presented in pAPCs are well defined but less is known regarding the natural source of peptides for cross-presentation. Full length proteins delivered to pAPCs either directly or on coated beads have been estimated to be 10^5 less efficient as sources for cross-presentation compared to endogenous antigens.[8,9] Synthetic long peptides (SLP) of 22 to 24 amino acids of the Ovalbumin or HIV-1 Gag proteins are a better source of peptides for the cross-presentation pathway as compared to full-length proteins.[10-12] Together with the fact that antigenic peptides derived from non-canonical mRNA translation such as intron or alternative open reading frames have been detected on MHC class I molecules highlights the need to investigate the role of alternative sources of peptides for $CD8^+$ T cell activation against tumor cells.[13-15] Recent studies have shown that alternative translation products such as DRiPs (defective ribosomal products) [16] or pioneer translation products (PTPs) constitute an important source of peptides for the endogenous class I pathway.[13,17,18] PTPs are derived from non-spliced mRNAs and do not require the corresponding full-length protein to trigger a specific $CD8^+$ T cell reaction. However, if these

CONTACT Sébastien Apcher sebastien.apcher@inserm.fr Institut Gustave roussy, B2M, salle 218, 114 rue edouard Vaillant, 94800, Villejuif.
*These authors contributed equally to this work.

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alternative peptide sources are also used for cross-presentation and activation of $CD8^+$ T cells is still not known.

Here, we addressed PTPs as a source of peptides for cross-presentation and their role as tumor-associated antigens (TA-PTPs) in triggering $CD8^+$ T cell activation in animal models. The results show that PTPs are delivered from the producing tumor cells to the pAPCs via exosomes and that vaccination of mice with tumor-derived PTPs efficiently suppresses the growth of tumor cells. This sheds light on the role of alternative sources of peptides for $CD8^+$ T cell activation and serves as a starting point for developing PTP-exosomes-based cancer vaccines.

Results

Pioneer translation products (PTPs) promote tumor cell rejection

PTPs is a major source of peptides for the endogenous MHC class I pathway *in vitro*.[13,17] To precisely define the immunogenicity of PTPs and their roles as tumor antigens (TA-PTPs) to elicit a $CD8^+$ T cell response *in vivo*, we inoculated C57BL/6 mice with two different tumor cells: the MCA205 sarcoma and the B16F10 melanoma. Each cell line stably express the SIINFEKL (SL8) epitope either from an intron (Globin-intron-SL8), from an exon (Glob-exon-SL8) in the β-Globin gene, or from its natural context within the open reading of the chicken ovalbumin (Ova) (Fig. 1A and Tables S1and S2). For both tumor models, the adoptive transfer of the SL8-specific TCR-transgenic OT-1 $CD8^+$ T cells few days after inoculation prevented the development of each tumor expressing the Globin-intron-SL8 (Figs. 1B and D) or Glob-exon-SL8 (Figs. 1C and E). We observed a difference in the growth of MCA205 and B16F10 cell lines expressing the SL8 epitope from the intron without the presence of OT-1 T cell in mice, indicating that the presence of the intron-SL8 epitope alone triggers a mild immune reaction *in vivo* (Figs. 1B and D). Parallel experiments using MCA205 and B16F10 cells stably expressing Ova cDNA construct showed similar tumor development after adoptive transfer of OT-1 $CD8^+$ T (Figs. S1A and B) than what we observed with the cell lines stably expressing the SL8 epitope either from an intron or an exon.

To test if PTPs have the capacity to trigger a specific $CD8^+$ T cell proliferation and an antitumor response we injected human HEK-293 cells expressing the foregoing expression constructs (Table S3) into mice that had received OT-1 T cells stained with CFSE 3 h earlier. HEK-293 cells lack the K^b molecule and cannot present antigens directly to the murine OT-1 T cells. Fig. 1F shows a diminution of the CFSE fluorescence in the OT-1 T cells from the animals injected with HEK-293 cells expressing the different constructs, as compared to empty vector. These results demonstrate that PTPs are a source of tumor-associated antigens that induce an antigen-specific suppression of tumor growth and specific $CD8^+$ T cell proliferation.

PTPs as a source of peptides for cross-presentation

These data indicate that PTPs constitute a source of peptides for $CD8^+$ T cells activation and to determine the pathways by which DCs process and present PTPs, murine bone marrow-derived dendritic cells (BMDCs) were incubated for 24 h with HEK-293 cell expressing the SL8 epitope either from an exon or intron within the β-Globin gene constructs (Fig. S2A). The cross-presentation of the PTPs by BMDCs was evaluated using the SL8 epitope-specific B3Z T cell hybridoma [19] or the OT-1 T cells and revealed a specific and similar $CD8^+$ T cell activation if the SL8 was expressed from an intron or exon (Figs. 2A and B). In parallel adding free SL8 showed a further 4- to 10-fold increase in T cell activation, demonstrating that the T-cells assays were conducted under non-saturated conditions (Figs. S2B, left and right panels). In order to minimize the possibility that the PTP cross-presentation data could be restricted to the SL8 epitope, the K^b molecule or the BMDCs, we determined whether PTPs containing the MBP(79–87) epitope, which is derived from the Myelin Basic Protein (MBP) and presented on K^k molecules can be cross-presented by mouse LK35.2 B cells and fibroblast L929 cells [20-22] (Fig. 1A). Using the specific MBP $CD8^+$ T cell hybridoma, [23] we could observe cross-presentation of the MBP(79–87) PTP epitope expressed in HEK-293 cells by both LK35.2 and L929 cells (Fig. 2C and Figs. S2B) under non-saturated conditions (Figs. S2B, bottom panel). Hence, cross-presentation of PTPs can be mediated by different types of cells and is independent of class I molecule or of the epitope.

Cross-presentation of PTPs requires the proteasome and TAP systems in the pAPCs

To address which of the cytosolic and vacuolar pathways is used to cross-present PTPs, we incubated HEK-293 cells expressing intron- or exon-derived SL8 epitopes with BMDCs treated, or not, with the specific proteasome inhibitor epoxomicin. Four hour epoxomicin treatment results in a complete suppression of $CD8^+$ T cell activation, independent of which construct used, demonstrating the need for proteasome activity in the pAPCs to process the cross-presented PTPs (Figs. 3A and B). In line with this, we observed that BMDCs transfected with Globin-intron-SL8 or Globin-exon-SL8 constructs were not capable of directly activating B3Z cells after 4 h of epoxomicin treatment (Figs. S3A).

We next tested the need of the BMDCs TAP system for PTPs cross-presentation. The human cytomegalovirus (HCMV)-encoded US6 protein and the herpes simplex virus (HSV)-encoded ICP47 protein both block TAP activity.[24-27] Co-transfection of the HCMV-encoded US6 constructs in BMDCs prevented cross-presentation of the SL8 epitopes expressed in HEK-293 cells (Figs. 3C and D). When BMDCs expressing the SL8 epitope were co-transfected with these viral vectors this also resulted in a sharp decrease in endogenous peptide presentation (Figs. S3B and C). In line with this, TAP knockout BMDCs (TAP-KO BMDCs) transfected with Globin-exon-SL8 or Ova cDNA constructs were not able to activate B3Z cells (Figs. S3D).

Together, these results support the notion that processing of antigenic peptides for cross-presentation by BMDCs is mediated via the cytosolic pathway, regardless if the epitope is derived from an exon- or intron-derived PTP.

Suppressing TAP and proteasome in the producing cells stimulate cross-presentation of PTPs

To address the need of the proteasomal system in the producing cells, we instead treated the PTP-producing HEK-293 cells

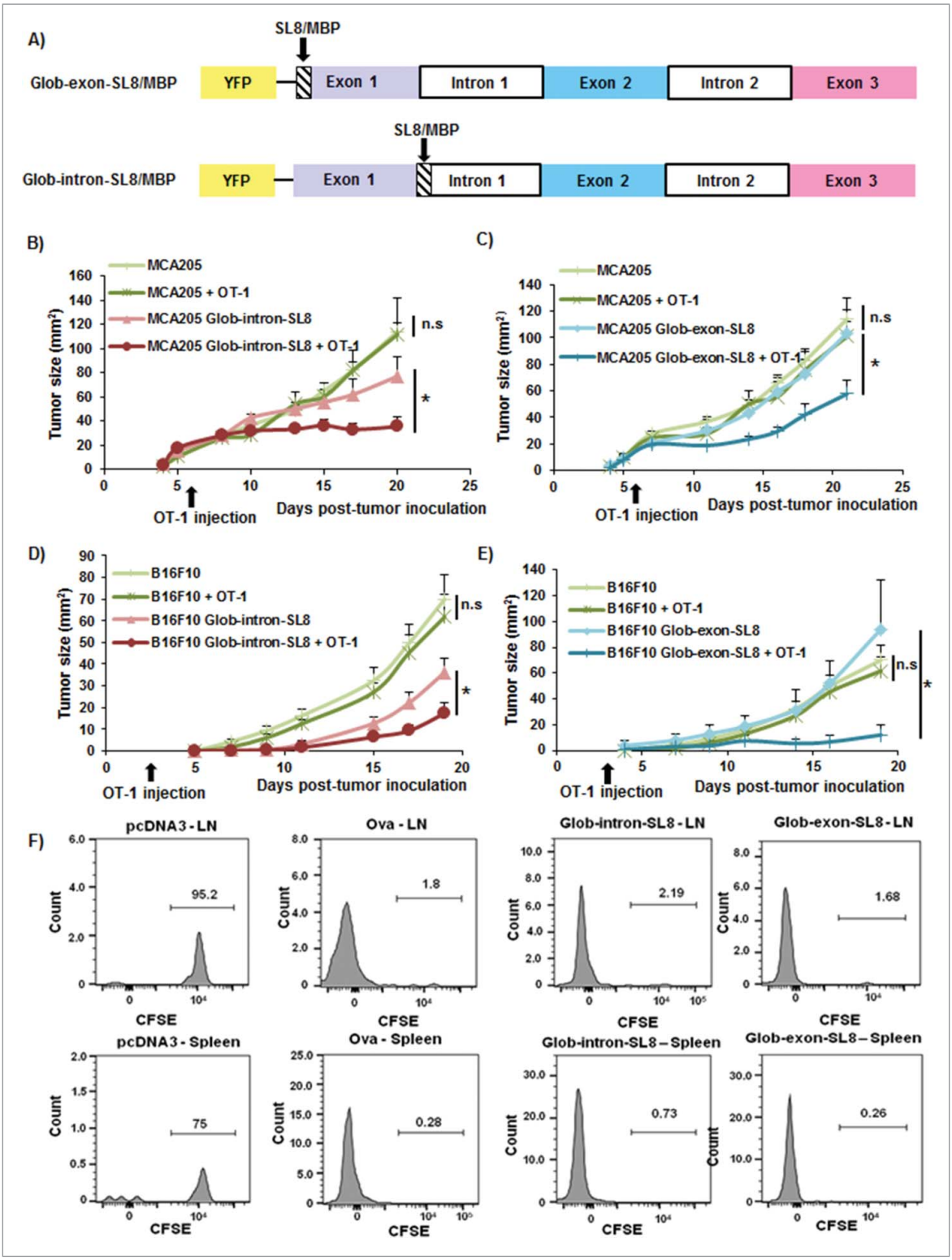

Figure 1. Pioneer Translation Products (PTPs) promote tumor cell rejection. (A) Cartoon illustrating the different positions of the SL8 and MBP antigenic epitopes in the exon or intron sequences of the β-Globin gene. (B and C) Mice were injected subcutaneously with either 1×10^5 of MCA205 or MCA205 tumor cells expressing stably the different constructs. Half of the mice from each group received 1×10^5 OT-1 T cells intravenously at day 6. Tumor sizes were assessed through time. (D and E) Mice were injected subcutaneously with 1×10^5 B16F10 or B16F10 tumor cells expressing stably the different constructs. At Day 3, half of the mice from each group received 2×10^5 OT-1 T cells intravenously. Tumor sizes were assessed through time until day 19. (F) CD45.1 congenic C57Bl/6 mice were injected intravenously with 2×10^6 CD45.2 positive OT-1 T cells stained with CFSE. After 3 h, 5×10^6 HEK-293 cells or HEK-293 cells expressing the different constructs were injected intraperitoneally. After 3 d, cells from the lymph nodes and the spleens were collected and the CFSE expression in $CD8^+$ T cells was analyzed. Data are given as mean $\pm$ SEM. Data are representative of four independent experiments performed with three mice for each group. $^*p < 0.05$, n.s: not significant (unpaired t test).

with epoxomicin for 4 h, before cells were presented to BMDCs and we observed an increase in B3Z and OT-1 T cells activation, as compared to non-treated cells (Figs. 3E and F). As expected, treating HEK-293 cells stably expressing the MHC class I K^b molecule (HEK-293-K^b) and transiently expressing SL8-PTP constructs with epoxomicin suppressed endogenous

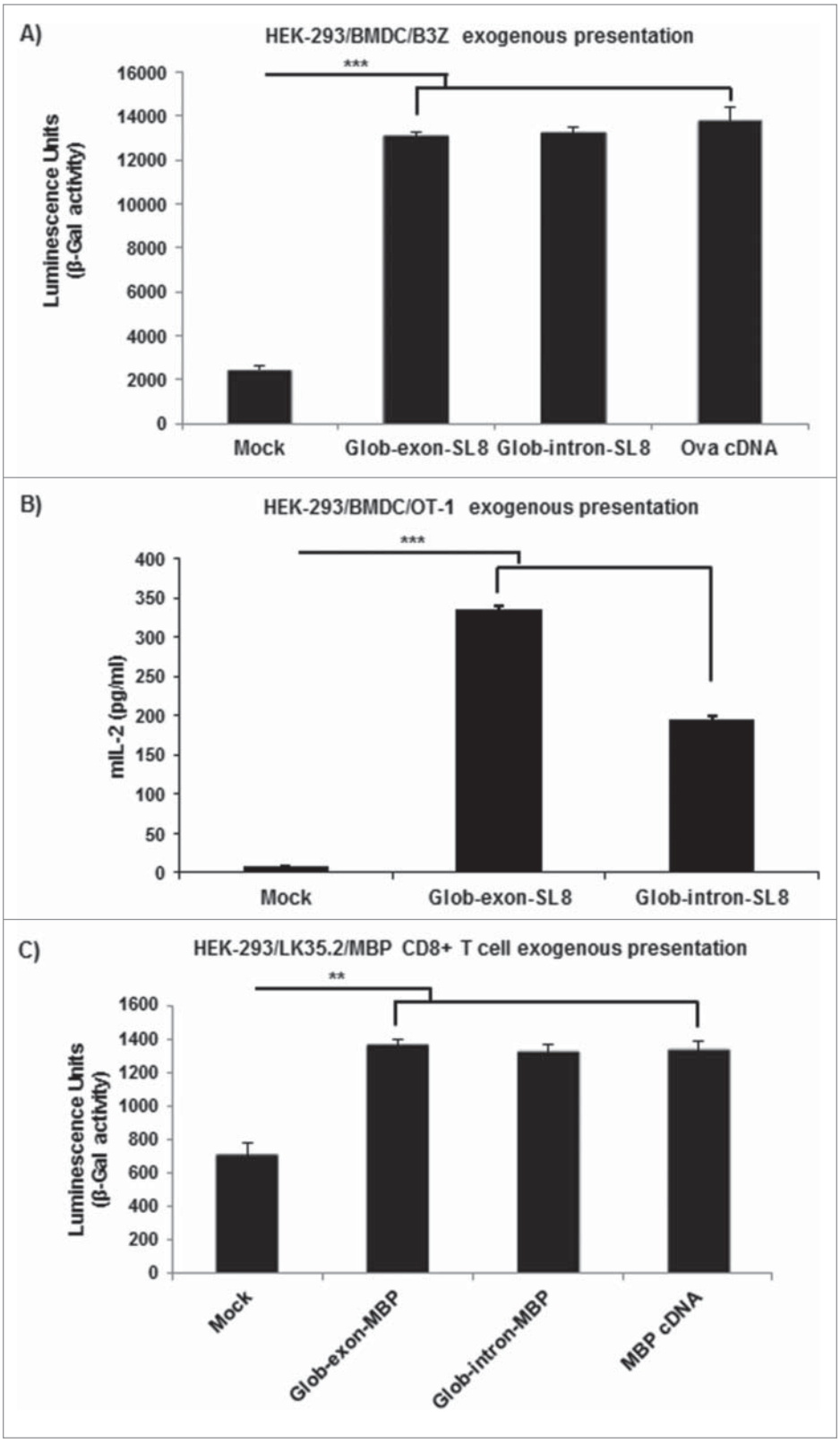

Figure 2. PTPs as a source of peptides for the cross-presentation pathway. (A) HEK-293 cells or HEK-293 cells expressing the different constructs (see Fig. 1A) were cultured with BMDCs for 24 h. The BMDCs were then co-culture with the SL8-specific $CD8^+$ T-cell hybridoma (B3Z)[19] for 16 h and T-cell activation was estimated by measuring β-galactosidase. (B) HEK-293 cells or HEK-293 cells expressing the different constructs (see Fig. 1A) were cultured with BMDCs. After 24 h, the BMDCs were collected and put in presence of the OT-1 T cells during 18 h. T-cell activation was estimated by measuring mIL-2 production. (C) HEK-293 cells or HEK-293 cells expressing the different constructs (see Fig. 1A) were cultured with B cells (LK35.2) for 24 h. The B cells were then co-cultured with the MBP-specific $CD8^+$ T-cell hybridoma for 16 h and T-cell activation was estimated by measuring β-galactosidase expression. The data show the average of at least three independent experiments. Data are given as mean ± SEM. $^{***}p < 0.001$, $^{**}p < 0.01$ (unpaired t test).

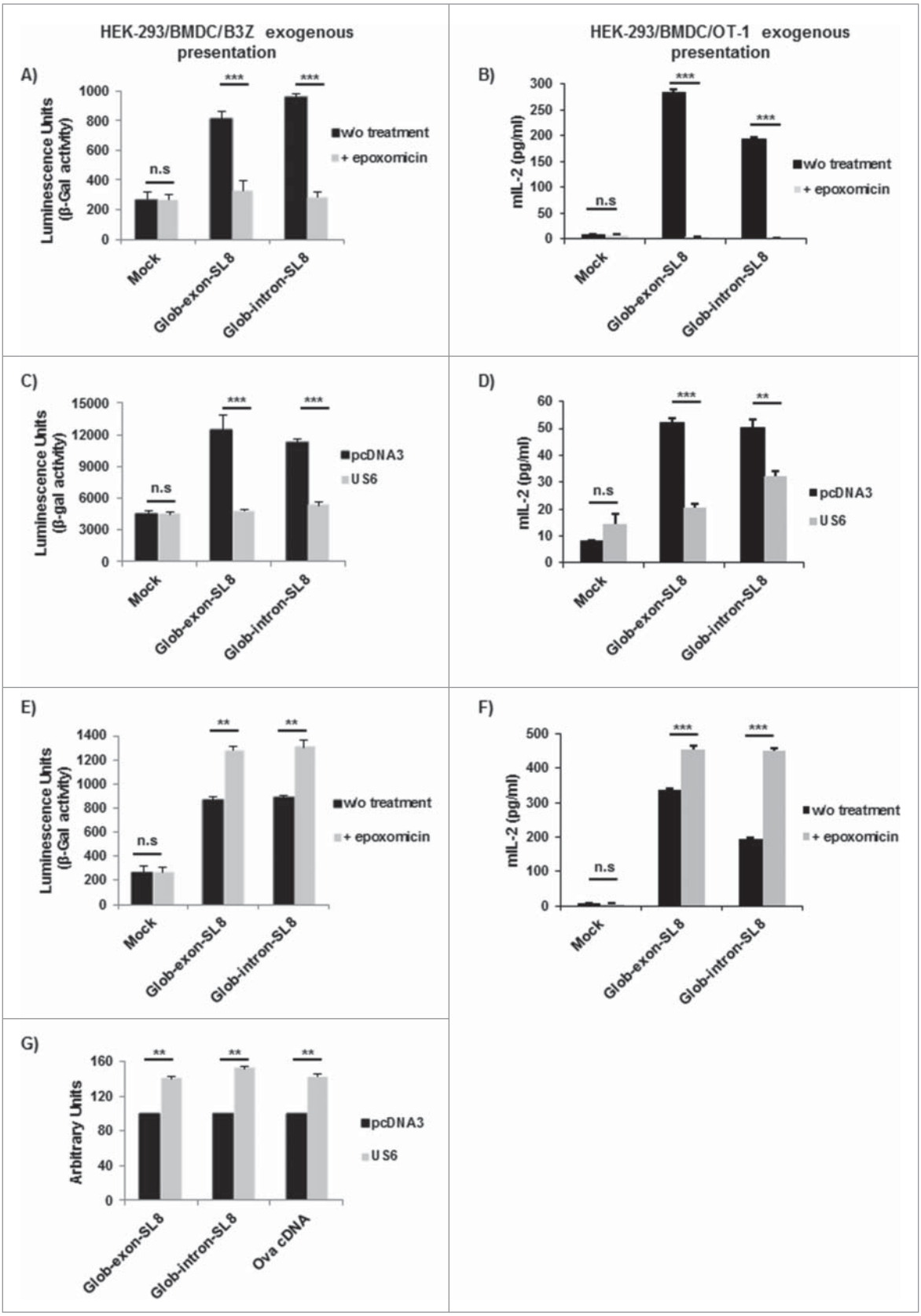

Figure 3. Requirement of the proteasome and TAP systems in the producing and presenting cells for the PTPs cross presentation pathway. HEK-293 cells expressing the different constructs were cultured with BMDCs, pre-treated or not with epoxomicin (a proteasome inhibitor) at 250 nM for 4 h before the addition of B3Z (A) or OT-1 T cells (B). In the same manner, BMDCs were transfected with the human cytomegalovirus (HCMV)-encoded US6 expression plasmid. They were then co-cultured with HEK-293 cells expressing the different constructs. After 24 h, the BMDCs were cultured with B3Z (C) or OT-1 T cells (D). T-cell activation was estimated by measuring β-galactosidase expression and mIL-2 production, respectively. To test the need of the proteasome and TAP systems in the producing cells, HEK-293 cells expressing the different constructs were treated or not with epoxomicin (250 nM for 4 h). Then the untreated or treated HEK-293 cells were co-cultured with BMDCs for 24 h before the addition of B3Z (E) or OT-1 T cells (F). T-cell activation was estimated by measuring β-galactosidase expression and mIL-2 production; respectively. (G) BMDCs were co-cultured with the different HEK-293 cells co-transfected with the different constructs and the TAP inhibitors US6 expression plasmid. BMDCs were then cultured with B3Z and T-cell activation was estimated by measuring β-galactosidase expression. The data show the average of at least three independent experiments. All the data are expressed as mean $\pm$ SEM. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ (unpaired t test).

SL8 presentation (Figs. S3E). Finally, when we suppressed TAP activity in HEK-293 cells using US6 or ICP47 we observed an increase in the capacity of the BMDCs to cross-present PTPs (Fig. 3G and Figs. S3F).

Together these results show that preventing the processing or delivery of PTPs in the producing cells enhance their availability for the exogenous pathway.

Cross-presented PTPs are stable as compared to direct presented PTPs

The presentation of the SL8 epitope on K^b transfected human cells has previously been shown to result in a high K^b turnover rate of approximately 10–15 min and has been used to demonstrate that PTPs have a short half-life for the endogenous pathway.[17] This has raised the question how suitable PTPs are for cross-presentation and if they would survive the transport from the producing cell to the presenting pAPCs. To address the stability of PTPs for cross-presentation, we respectively transfected HEK-293-K^b cells with *in vitro* transcribed capped Glob-intron-SL8 and Ova mRNAs. After cell fixation at indicated time points, the cells were exposed to B3Z to determine direct presentation of the SL8 epitope from the transfected mRNAs. In parallel, HEK-293 cells were respectively transfected with the same mRNAs, fixed and exposed to mouse BMDCs before incubation with B3Z cells to test for cross-presentation. The presentation of the SL8 epitope for the endogenous pathway from either of these mRNAs showed a similar rapid increase of presentation peaking at approximately 2 h after which the presence of the SL8 epitope rapidly diminished (Fig. 4A). For the exogenous presentation, we observed a parallel rapid increase but the pool of PTP substrates available for cross-presentation by the BMDCs remained more or less unchanged for up to 6 h post-transfection (Fig. 4B). Importantly, there was little, or no, difference for the endogenous or the cross-presentation pathways using cells transfected with either the capped Ova or Glob-intron-SL8 mRNA.

It has been suggested that exosome-delivered mRNAs can be engaged with the ribosomes [28] and one possibility for the observed difference in endogenous *vs.* cross-presentation (Fig. 4C) could be that the mRNAs from the producing cells are taken up by the pAPCs where they would be engaged in a pioneer round of translation and produce their own PTPs. However, treating the fixed HEK-293 cells transfected with capped Glob-intron-SL8 mRNA with RNAse after fixation resulted in a 75% loss of mRNA levels as determined using RT-qPCR, but importantly, had no effect on PTPs cross-presentation (Fig. 4D).

These results indicate that PTPs for endogenous and cross-presentation are produced by the same translation event and that the two pathways then diverge quickly. Once the peptide material has been destined for the exogenous pathway and becomes a source for cross-presentation it is protected from degradation but has no longer access to the endogenous pathway.

Delivery of PTPs for cross-presentation

We next set out to investigate how PTPs are delivered from the producing cells to the BMDCs and we first tested if a direct contact between the producing and presenting cells is needed. For that purpose, BMDCs were co-cultured with the HEK-293 cells expressing the Glob-exon-SL8 or Glob-intron-SL8 constructs or with conditioned media from HEK-293 cells expressing the same constructs for 24 h. Interestingly, we could observe a significant activation of OT-1 T cells even if the cells were not in direct contact (Fig. 5A). The HEK-293 cells were then separated from the BMDCs by transwells of different sizes spanning from 3.0 to 0.4 μm and we observed the same level of OT-1 T cell activation from Glob-intron-SL8 expressing cells grown in either transwells. However, there was a 50% less activation of T cells grown in 0.4 μm transwells, as compared to the 3.0 μm, using the Glob-exon-SL8 construct (Fig. 5B). Subcellular fractions smaller than 0.4 μm are considered either microvesicles or exosomes. The latter have been shown to be a major cargo for tumor cell-derived antigens.[29,30] Electron micrographs of the isolated vesicles smaller than 0.4 μm revealed the typical rounded exosome structures of approximately 100 nm (Fig. 5C). Furthermore, FACS analysis of these vesicles showed the presence of surface proteins CD63 and CD81 for the HEK-293-derived vesicles and of the CD9 and CD81 for the MCA205-derived vesicles, confirming they are exosomes (Fig. 5D). We could observe a significant increase in mIL-2 production by the OT-1 T cells when BMDCs were pulsed with purified exosome fractions from 200 mL of supernatant from HEK-293 or MCA205 cells expressing the Globin-intron-SL8 (Fig. 5E). Since the MCA205 mouse cells express the MHC class I K^b molecules, we were concerned that exosomes from this cell line could directly induce an activation of OT-1 T cells. However, MCA205-derived exosomes pulsed on OT-1 T cells did not result in activation (Figs. S4A) and FACS analysis using the SL8-MHC-K^b specific antibody 25-D1, did not show this complex on the exosomes derived from the same cells (Figs. S4B). Hence, exosomes from MCA205 cells cannot activate the reporter OT-1 T cells without their cargo first being processed and presented by the BMDCs.

Exosomes are known to contain microRNAs and mRNAs and despite the fact that RNase treatment of PTP-producing cells showed little impact on PTP cross-presentation, we were wondering if exosomes from transfected cells could contain SL8 carrying mRNAs. We used RT-qPCR to look for the presence of mRNAs derived from the transfected construct and for mRNAs known to be present in exosomes.[28,31] We could indeed detect the presence of the *FTL* (ferrin light chain) mRNA taking *FTH1* (ferritin heavy chain) as the internal reference gene but we were not able to detect transfected β-globin mRNAs (Fig. 5F).

As we could not detect SL8-carrying mRNAs in exosomes, we next tested if we could detect the corresponding PTPs. We expressed a construct in MCA205 cells in which the 6xHis-tag was inserted next to the SL8 epitope in the intron (Figs. S4C). We then enriched the PTPs from 250 mg of purified and sonicated exosomes using nickel agarose beads and subjected these fractions to LC-MS/MS mass spectrometry analysis. Table 1 shows different peptide fragments carrying, or not, the SL8 epitope. It is worth pointing out that the enrichment step was required in order to yield a sufficient enough concentration of intron-derived PTPs to be detected by MS analysis. This is in line with previous observations showing that despite being an excellent substrate for the endogenous pathway, the PTPs are rare products.[13]

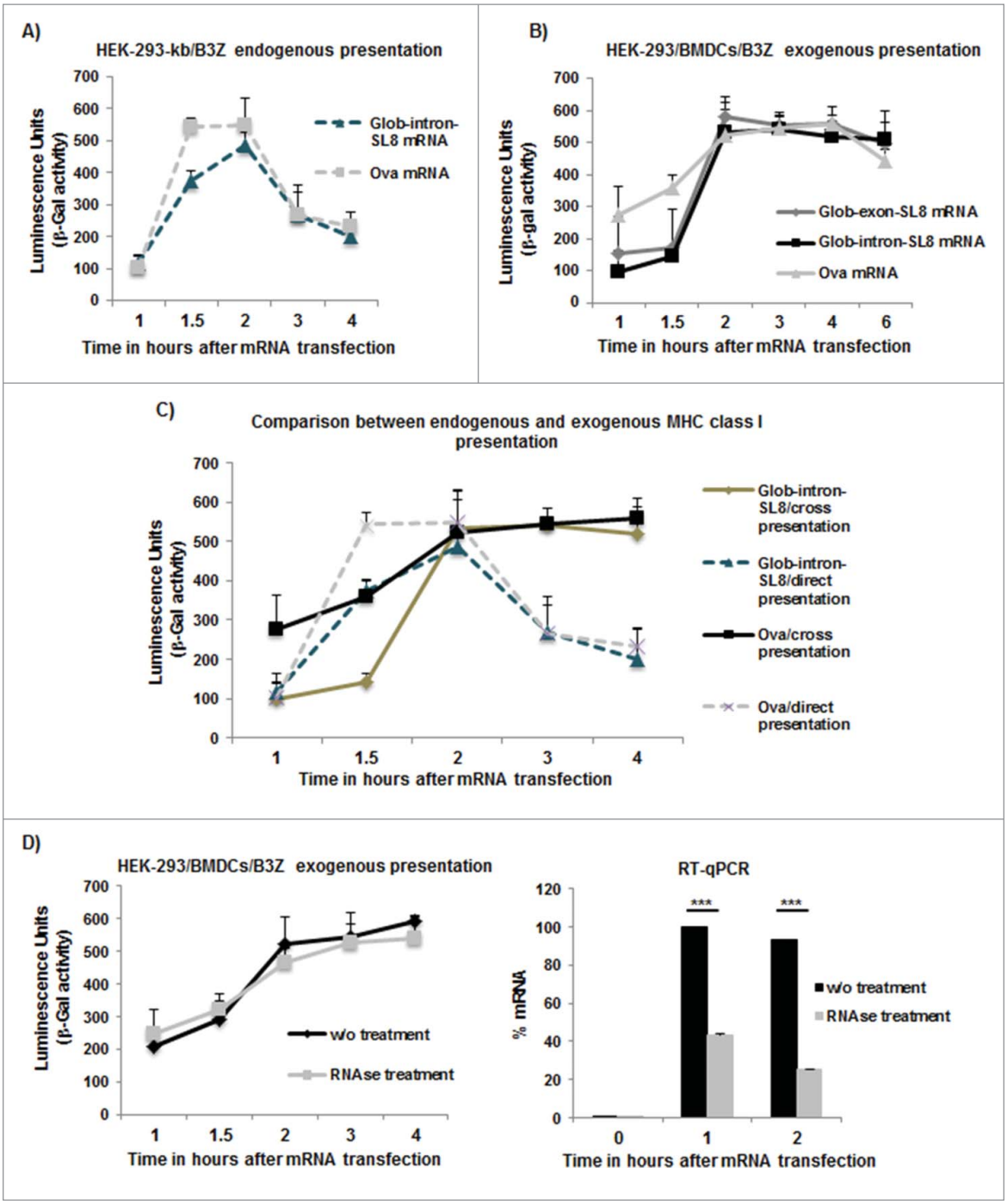

Figure 4. Cross-presented PTPs are stable as compared to direct presented PTPs. (A) Antigen presentation after transfection of *in vitro* transcribed capped Glob-intron-SL8 or Ova mRNAs in HEK-293 cells expressing the exogenous mouse K^b MHC class I molecules was determined on fixed cells using the B3Z hybridoma at the indicated time points. (B) HEK-293 cells were transfected with *in vitro* transcribed Glob-intron-SL8 or Ova mRNAs. At the indicated time points the cells were fixed and incubated with 5×10^5 BMDCs for 24 h. Then 16 h later the presentation of the SL8 epitope was determined using the B3Z hybridoma. The data show the average of at least three independent experiments with SD minus the values from mock-transfected cells. Free SL8 peptide was added to cells to ensure that T-cell assays were carried out at non-saturated conditions and that the expression of MHC class I molecules was not affected. (C) Comparison between the exogenous (graph 4A) and the endogenous MHC class I presentation (graph 4B). (D) (Left panel) HEK-293 cells were transfected with indicated *in vitro* transcribed capped Glob-intron-SL8 mRNA. 30 min before being fixed and incubated with 5×10^5 BMDCs for 24 h, HEK-293 cells were treated or not with RNAse. The presentation of the SL8 epitope was determined on fixed cells using the B3Z hybridoma at the indicated time points. (Right panel) levels of capped Glob-intron-SL8 mRNA in HEK-293 cells following RNA transfection before and after RNase treatment were determined using RT-qPCR. The arbitrary value of 100% was given at 1 h. The data show the average of at least three independent experiments. All the data are expressed as mean ± SEM.

PTP-carrying exosomes from tumor cells trigger $CD8^+$ T cell response

The fact that small amounts of TA-PTPs expressed in tumor cells efficiently activate $CD8^+$ T cells implies that the source of peptides and the context of how they are produced and presented to the cross-presenting cells, are important factors for triggering an efficient $CD8^+$ T cell activation. To test this idea further, we wanted to know if TA-PTPs alone can induce a $CD8^+$ T cell response in mice. Enriched TA-PTPs from

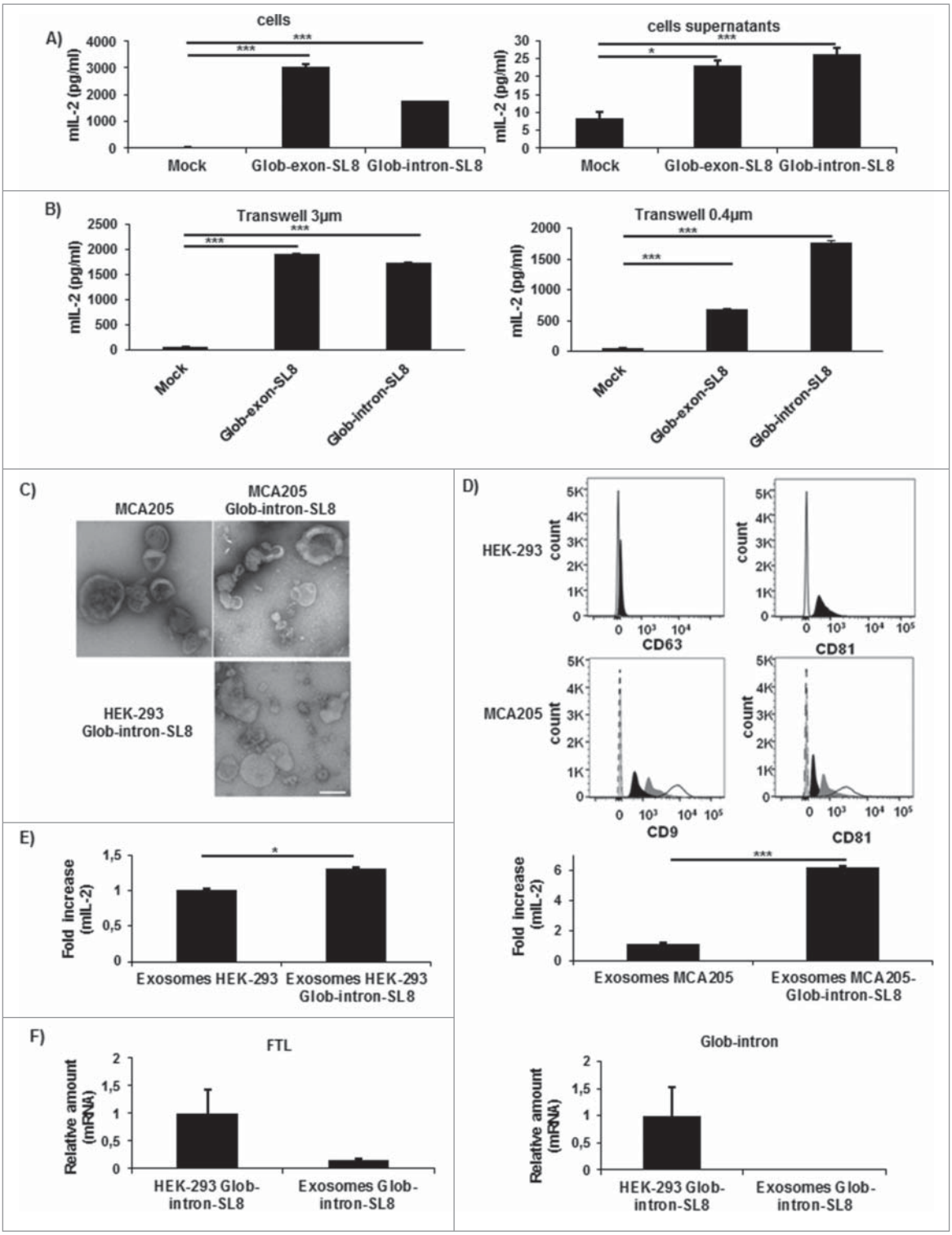

Figure 5. Delivery of PTPs for cross-presentation. (A) HEK-293 cells expressing the different constructs (left panel) or their supernatants (right panel) were incubated with BMDCs. After 24 h, the BMDCs were co-cultured with OT-1 T cells for 16 h. T-cell activation was estimated by measuring mIL-2 production. (B) BMDCs were plated in the bottom of a 12-wells plate. Transwells 3.0 μm (left panel) or 0.4 μm (right panel) were added, HEK-293 cells transfected with the different constructs were added to the upper chamber. After 24 h, the BMDCs were put in presence of OT-1 T cells during at least 18 h. A mIL-2 ELISA was performed on the supernatant to estimate T-cell activation. (C) Exosomes purified from the different cell lines were analyzed by Transmission Electron Microscopy (TEM) with a negative staining. Exosomes are characterized by a frequent plate-like morphology. Scale = 100 nm. (D) (Upper panels) Analysis of the expression of CD63 and CD81 in exosomes purified from HEK-293 cells expressing the Globin-intron-SL8 construct by FACS. In white the unstained exosomes, in black the Glob-intron-SL8 exosomes. (Lower panels) FACS analysis of the expression of CD9 and CD81 in exosomes purified from MCA205 cells expressing Globin-intron-SL8 construct. In pale gray, the unstained exosomes, in dashed line the unstained MCA205 Glob-intron-SL8 cells (negative control), in black exosomes from MCA205 cells expressing the Glob-intron-SL8 construct, in dark gray exosomes from MCA205 wild-type cells and, in white the MCA205 cells expressing the Glob-intron-SL8 construct. (E) BMDCs were pulsed by exosomes purified from HEK-293 or HEK-293 expressing the Glob-intron-SL8 construct. After 24 h, the BMDC were cultured with OT-1 T cells (left panel). BMDCs were pulsed by exosomes purified from MCA205 or MCA205 expressing the Glob-intron-SL8 gene. They were then cultured with OT-1 T cells. An ELISA to detect mIL-2 was performed (right panel). Data are given as mean ± SEM. (F) FTL and Globin transcripts were analyzed in exosomes purified from HEK-293 cells expressing the Glob-intron-SL8 gene taking FTH1 as the internal reference gene. The data show the average of at least three independent experiments. All the data are expressed as mean ± SEM. $^{***}p < 0.001$, $^{*}p < 0.05$ (unpaired t test).

Table 1. Mass spectrometry analysis of peptides derived from exosomes produced by MCA205 cells expressing the Globin-intron-SL8 construct. The peptide corresponding to the SIINFEKL peptide derived from an intron sequence is highlighted.

Peptide sequence	Peptide length, a.a.	Peptide origin
VNVDEVGGEALGR	13	YFP-globin
SAMPEGYVQER	11	YFP-globin
FEGDTLVNR	9	YFP-globin
FSVSGEGEGDATYGK	15	YFP-globin
SIINFEK	7	Chicken Ovalbumin
LEYNYNSHNVYIMADK	16	YFP-globin
GEELFTGVVPILVELDGDVNGHK	23	YFP-globin

MCA205 cells expressing the Glob-intron-SL8-His construct and commercially purified full-length chicken ovalbumin proteins were incubated directly with BMDCs for 24 h before the addition of OT-1 T cells. It should be pointed out that despite being enriched using the His-tag, not all purified polypeptides can be expected to contain the intact SL8 peptide (Table 1) whereas the Ova protein was considered 95% pure. Nevertheless, BMDCs that had been in contact with 10 or 20 μg of enriched TA-PTP-fractions triggered a 4.5-fold higher OT-1 T cell activation, as compared with 10 or 20 μg of Ova protein (Fig. 6A). Furthermore, when we compared BMDC-mediated cross-presentation using enriched exosome-derived PTPs with the corresponding amount of intact exosomes from 200 mL medium of MCA205 cells expressing Glob-intron-SL8 construct, we observed a similar proteasome-dependent activation of the OT-1 T cells (Fig. 6B).

To test this idea further *in vivo*, we purified intron-derived SL8-PTPs from MCA205 cells and inoculated mice with a total of 128, 64, and 32 μg/animal subcutaneously in both flanks and footpads. Two weeks post-vaccination, 5×10^5 MCA205 cells stably expressing Ova (MCA205-Ova) and MCA205 wild-type tumor cells (MCA205) were subcutaneously injected in the right and left flanks of the same mice. Fig. 6C, left panel shows a TA-PTP dose-dependent suppression in the growth of MCA205-Ova expressing cells with a complete suppression when 128 μg of PTPs had been used. In comparison, the vaccination with TA-PTPs did not affect the growth of the wild-type MCA205 tumor cells (Fig. 6C, right panel).

We next wanted to estimate if the exosomes themselves can play a role in TA-PTP-mediated suppression of tumor growth. We enriched PTPs from MCA205 cells lysates and the corresponding exosomes from the culture medium of the same cells. When we injected 64 μg of intron-derived TA-PTPs alone, we observed a mild suppression in the growth of MCA205-Ova expressing cells injected 14 d post-vaccination, as compared to control animals vaccinated with CpG+Poly I:C. When we instead injected 15 μg of exosomes purified from the same cells we observed a similar mild suppression in MCA205-Ova tumor growth. However, when we combined 15 μg of exosomes with 64 μg or 32 μg of purified PTPs we observed a sharp suppression in tumor growth (Fig. 6D). This indicates that the presence of the exosomes makes TA-PTPs more potent in triggering a $CD8^+$ T cell memory against PTP products.

Discussion

The cellular factors that govern the processing and loading of peptides for the endogenous and cross-presentation pathways have been the focus of intense studies but less is known about the importance of the source of peptides for the activation of a $CD8^+$ T cell-mediated immune response. Studies have shown that additional sources of peptide substrates can be processed and presented for the endogenous MHC class I-restricted pathway.[13,17,32,33] but how alternative sources of peptides are used in pAPC-dependent cross-presentation pathway is not known. PTPs derived from introns or nonsense-mediated decay mRNAs have previously been shown to be efficiently selected for the endogenous processing pathway.[13,17] We now show that this relatively rare source of peptide material is also a major source for the MHC class I cross-presentation pathway. In hindsight, it can seem logic that the same peptide material used for the endogenous pathway also provides peptide substrates for the exogenous pathway. If not, different epitopes could be presented for the endogenous *vs*. the cross-presentation pathways, running the risk to activate the immune system against peptides that are not presented for the endogenous pathway or *vice versa*, the presence of viral epitopes on infected cells would go unnoticed. The PTPs are the first peptide products to emerge from an mRNA which ensures that epitopes are rapidly presented for the endogenous pathway and also for the activation of $CD8^+$ T cells by the pAPCs. This helps to ensure a rapid detection of infected and transformed cells. These observations show that full-length proteins are not required to activate $CD8^+$ T cells and helps to explain the proliferation of specific tumor-infiltrating T lymphocytes (TILs) from non-conventional peptide sources.[14,15,34-38]

The fact that PTPs for the cross-presentation pathways are more stable as compared to the endogenous pathway, suggests that once they are destined for the cross-presentation compartment there is no way back to the endogenous pathway. Since blocking proteasomal degradation and TAP transporter in the producing cells leads to an increase in cross-presentation suggests that the storage of peptides for cross-presentation is outside the ER. It has been proposed that chaperone molecules could act as delivery vehicles between the producing and the presenting cells but we have no evidence that they play a role in delivering PTPs.[21,39-41]

The results showing that BMDCs, B cells, and fibroblasts can cross-present PTPs composed of different epitopes support the idea that PTPs cross-presentation is not epitope and cell specific. Nevertheless, in the last decade the question of DC subtypes capable to cross-present MHC class I antigen better than others has arisen and it has been suggested that the majority of cross-presentation in mice is carried out by the $CD8^+$ DCs,[5] whereas all DC subsets have been demonstrated to cross-present MHC class I antigens in human.[42] Most cross-presentation studies have been carried out using full-length proteins as the source of peptides but a few recent studies report that SLP can be cross-presented in mice by different types of DCs from BMDCs to pDCs.[10,12] It is possible that the uptake and processing of antigenic peptide substrates by the pAPCs for cross-presentation is specialized toward a certain type, and context, of peptide material and it will be interesting to compare if cross-presentation by different types of pAPCs depends on the nature of the antigen. In view of this, it is interesting that we detect PTP products

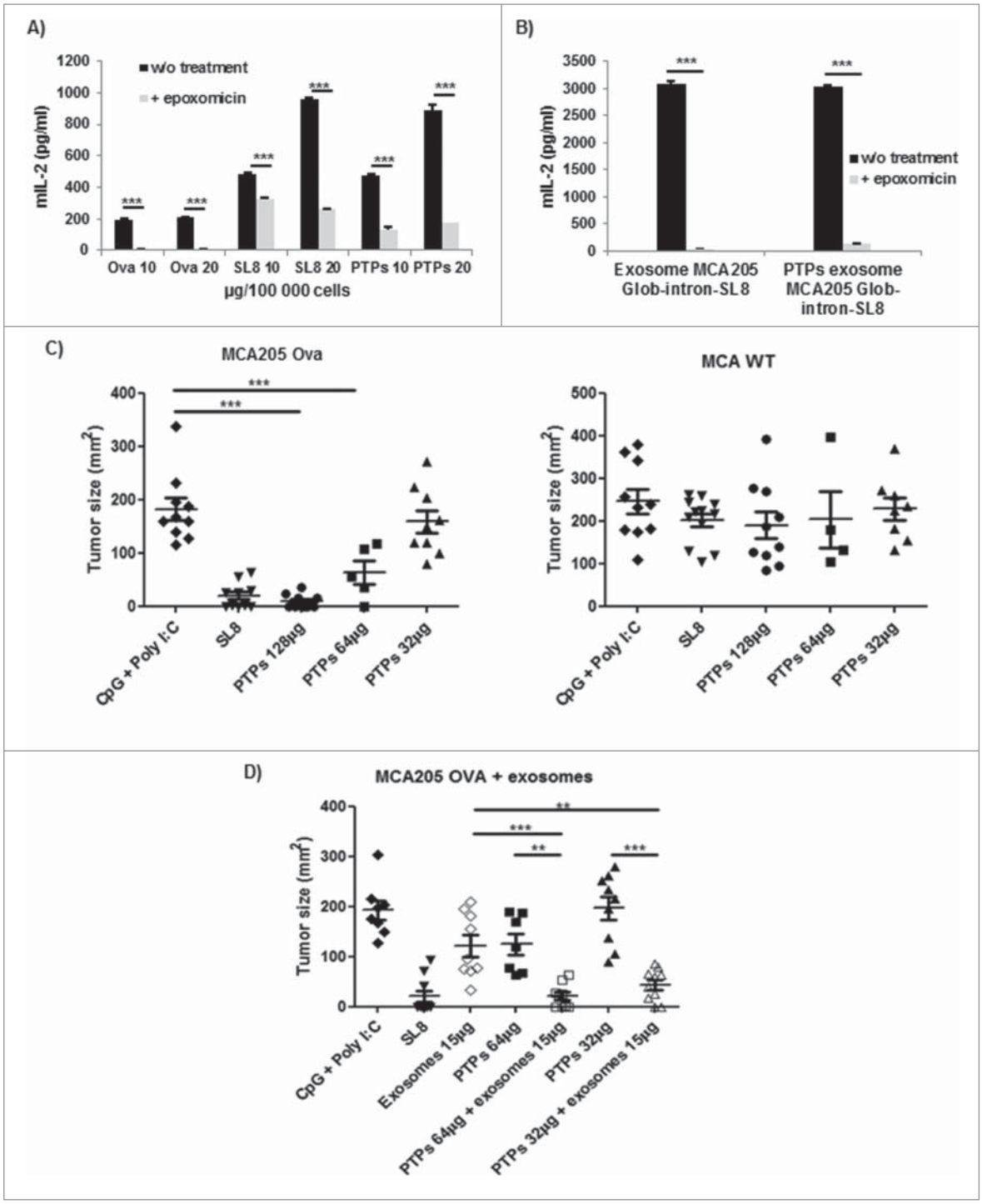

Figure 6. Tumor-derived PTP-carrying exosomes trigger $CD8^+$ T cell response. (A) BMDCs treated or not with epoxomicin were pulsed with different doses of PTPs purified from HEK-293 cells expressing the Glob-intron-SL8 construct. As a positive control, BMDCs treated or not with epoxomicin were pulsed with different doses of Ovalbumin protein. The BMDCs were then cultured with OT-1 T cells. The quantity of mIL-2 produced in the supernatant after at least 18 h was evaluated by ELISA. The value for the negative control, (BSA) was subtracted to the values of the other samples. (B) BMDCs were pulsed with 20 μg exosomes purified from MCA205 cells expressing the Glob-intron-SL8 construct or with 20 μg of PTPs purified from these exosomes. BMDCs were then cultured with OT-1 T cells. The quantity of mIL-2 produced in the supernatant was measured by ELISA after at least 18 h. (C) Mice were vaccinated with 128, 64, or 32 μg of purified tumor intron-derived PTP or with 20 μg (SL8 1/5) of SIINFEKL epitope emulsified in CpG+ poly I:C and 15 d later the mice were challenged s.c. with 5×10^5 live MCA205 expressing the Ovalbumin in the right flank (left panel) and with 5×10^5 live MCA205 WT in the left flank (right panel). Then every 7 d the tumor growth was measured for each tumor cell lines. The graph represents the tumor growth of each group at day 28. (D) Mice were vaccinated with 64 μg or 32 μg of tumor intron-derived PTPs, or with 64 or 32 μg of tumor intron-derived PTPs combine with 15 μg of tumor-derived exosomes containing PTPs, or as positive control 20 μg (SL8 1/5) of SIINFEKL epitope emulsified in CpG+ poly I:C. 15 d later the mice were challenged subcutaneously with 5×10^5 live MCA205-Ova in the right flank. Then every 7 d the tumor growth was measured for each tumor cell lines. The graph represents the tumor growth of each group at day 28. The data show the average of at least three independent experiments. All the data are expressed as mean ± SEM. $^{***}p < 0.001$, $^{**}p < 0.01$ (unpaired t test).

in the exosomes of tumor-derived cell lines and that PTP-loaded exosomes alone are sufficient to trigger an efficient cross-presentation by BMDCs.

One of the difficulties with this study has been to estimate the amount of PTPs produced by the producing cells or in exosomes. We know that PTPs are rare translation products and we had to enrich them from introns using nickel-agarose beads in order to have a sufficient concentration to detect them by mass spectrometry analysis. It is thus difficult to directly compare how efficient PTPs are cross-presented as compared to full-length proteins. But based on these data and previous work, plus the estimation that there are in general a 1:2800 ratio mRNA:proteins[43] and if the scanning of the pre-spliced mRNA takes place once, it is safe to say that PTPs represent a fraction of the amount of full-length proteins produced by the canonical mRNA translation machinery. Hence, the observations that an enriched but mixed pool of PTPs is approximately 4.5 times more efficient to activate $CD8^+$ T cells as compared to the same amount of pure ovalbumin proteins, it is probably a very low estimation and the actual value is most likely much higher. Further studies will be carried out to enrich PTPs that only include the antigenic epitope in order to more precisely determine their relative efficacy in provoking an immune response.

The observation that mice pre-injected with TA-PTPs in the presence of exosomes trigger a more efficient immune reaction toward tumor cells expressing chicken ovalbumin, as compared to mice inoculated with TA-PTPs, or exosomes, alone supports the idea that the context of how the antigenic peptide material is presented to the pAPCs plays a role. It is possible that the presence of exosomes forms a signal to ensure that its content is destined for the MHC class I pathway and not end up in lysosomal compartments. If so, one can speculate that the inefficient cross-presentation observed using full-length proteins to pAPCs simply represent a spill-over from a non-physiological delivery and uptake pathway. However, this notion needs to be tested.

Furthermore several reports have shown that different class I binding synthetic epitopes derived from cancers can be used as single peptide-based vaccines in pre-clinical trials but without beneficial clinical responses for the patients,[44,45] presumably due to the fact that short peptides bind directly to MHC class I molecules on numerous types of cells. In contrary, the use of PTPs as multiple peptides-based vaccines makes more sense since, unlike short peptides, PTPs need processing by the proteasome in the pAPCs to be presented at the cell surface and cannot bind directly the MHC class I molecules.

These findings support a concept whereby TA-PTPs in the context of exosomes are being cross-presented by professional APCs without the need of the expression of the corresponding full-length proteins. This can have interesting implications for designing vectors to generate cancer vaccines.

Materials and methods

Animal studies

C57Bl/6J mice were obtained from Envigo. OT-1 and CD45.1 C57Bl/6J mice were generously provided by the CERFE (C. Daviaud) and bred at Gustave Roussy animal facility. Eight weeks C57BL/6J mice were inoculated with 1×10^5 MCA-205 or B16F10 tumor cells subcutaneously in the right flank. For MCA205, 1×10^5 OT-1 T cells were injected intravenously 6 d after tumor inoculation. In the B16F10 model, 2×10^5 OT-1 T cells were inoculated intravenously 3 d after the tumor inoculation. All animal experiments were carried out in compliance with French and European laws and regulations.

Plasmid constructions

All plasmids were generated using standard procedures. Restriction enzymes, T4 DNA ligase, and calf intestinal alkaline phosphatase were obtained from New England Biolabs. Purified synthetic oligonucleotides were obtained from Sigma Aldrich. Routine plasmid maintenance was carried out in DH5α and TOP10 bacteria strains. All the constructs have been described previously.[13,17]

Cell line maintenance and transfection

MCA205 (syngeneic from C57BL/6J mice) were cultured at 37°C under 5% CO_2 in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 IU/mL penicillin/streptomycin, 1 mM sodium pyruvate, and MEM non-essential amino acids (Invitrogen). B3Z and MBP-$CD8^+$ T cell hybridomas were cultured at 37°C under 5% CO_2 in RPMI containing 10% FCS, 2 mM L-glutamine, 100 IU/mL penicillin/streptomycin and 50 nM β-mercaptoethanol. HEK293 and B16F10 (syngeneic from C57BL/6J mice) were cultured at 37°C under 5% CO_2 in DMEM containing 10% FCS, 2 mM L-glutamine, and 100 IU/mL penicillin/streptomycin.

For DNA transfection, MCA205, B16F10, and HEK-293 were transfected using JetPrime according to the manufacturer's protocol (Polyplus) during 48 h. For the inhibitor of TAP activity, the HCMV-encoded US6 protein and the HSV-encoded ICP47 were a gift of Peter Creswell (Department of Immunobiology, Yale University, New haven). For the transfection of TAP inhibitors in HEK-293 cells, the cells were transfected with 1 μg of the plasmids pcDNA3, Glob-intron-SL8 or Glob-exon-SL8 and with, respectively, 1 μg of plasmids coding for the different TAP inhibitors US6 or ICP47 for 24 h.

BMDC generation and transfection

The BMDCs were generated from C57Bl/6 bone marrow precursors. Briefly, mouse femurs were dissected out, sterilized with 70% ethanol, and washed in sterile PBS and PBS/5% fetal bovine serum (FBS; Life Technology) successively, to remove soft tissues; both ends of the femurs were cut off and the bone marrow cells were flushed out with PBS/5% FBS then cultured at 0.5×10^6 cells/mL in 145-mm Petri dishes for 6 d in IMDM (Sigma) supplemented with 10% heat-inactivated FCS, 0.5% penicillin/streptomycin, 1% L-glutamin, β-mercaptoethanol (50 nM, Life Technology), and enriched with 50 ng/mL J558 supernatant. After 3 d of culture, the medium was replaced.

For the transfection, on day 4, 3×10^6 BMDCs were transfected with 2 μg of pcDNA3, US6, or ICP47 using the Amaxa mouse dendritic cells nucleofactor kit according to the manufacturer's protocol during 48 h (Lonza). The BMDCs were harvested at Day 6.

OT-1 T cells purification

Single-cell suspensions were prepared from OT-1 mouse (TCR-transgenic $CD8^+$T cells specific for K^b-OVA257-264) lymph nodes and spleen. Lymph nodes and spleens were dilacerated and filtered through a 70 μm mesh filter. Red blood cells were eliminated with a standard red blood cell lysis (ACK) buffer and cells were washed twice before subsequent studies. The OT-1 T cells were then purified by negative selection with magnetic beads (Miltenyi Biotec) according to the manufacturer's protocol.

Exosome purification, transmission electron microscopy (TEM), and exosomes-derived PTPs purification

Exosomes from MCA205 and HEK-293 cells expressing or not the Glob-intron-SL8 construct were purified as described previously.[46] For negative staining of exosomes,[47] five microliters of exosome fractions were adsorbed onto a 300 mesh copper grid coated with a collodion film covered by a thin carbon film, activated by glow-discharge. After 1 min, grids were washed with aqueous 2% (w/vol) uranyl acetate (Merck, France) and then dried with ashless filter paper (VWR, France). TEM observations were carried out on a Zeiss 912AB transmission electron microscope in filtered low loss mode. Electron micrographs were obtained using a ProScan 1024 HSC digital camera and Soft Imaging Software system (Eloise). For exosome-derived PTPs purification, exosomes from MCA-205 cells expressing the Glob-intron-SL8-His construct were purified as previously. Then the exosomes were sonicated and the exosome lysates were then incubated with nickel-agarose beads. Then the exosome-derived PTPs-His were purified as previously described.[13]

BMDC cross presentation

On day 6 of BMDCs differentiation, 0.3×10^6 BMDCs were put in contact respectively with either 0.3×10^6 transfected HEK-293 cells, HEK-293 cells culture media, 60 μg of exosomes or purified PTPs-His (from 30 to 60 μg), in 2 mL of medium. As a positive control, Ova protein was added to the BMDCs. They were then treated or not with epoxomicin (250nM) (Merck). After 24 h, the BMDCs were washed in medium and cultured with 3×10^5 OT-1 T cells during at least 18 h in 24-wells plate. As a negative control, OT-1 T cells were also cultured with 60 μg of exosomes during 18 h.

OT-1 proliferation experiments OT-1 T cells were labeled with CFSE (1.,7 mM for 10 min) and injected via the tail vein (2×10^6/mouse) into CD45.1 C57BL/6 mice. 5×10^6 HEK293 cells transfected with pcDNA3, Ova, Glob-intron-SL8, or Glob-exon-SL8 were injected i.p. 3 h after injection of OT-1 T cells. Spleens were harvested ~72 h after OT-1 T cells injection and then the proliferation of the OT-1 T cells was determined by staining for $CD8^+$ and CD45.1 and gating for CFSE-labeled $CD8^+CD45.1^+$ cells using a CANTO II flow cytometer. A total of 1×10^5 events were normally acquired and analyzed with FlowJo software (Tree Star).

Transwell

On day 6 of the BMDCs differentiation, they were collected, 1×10^6 BMDC were put in the bottom of a 12-wells culture microplate, 2 mL of BMDC medium was added. 3 μm or 0.4 μm transwell (BD falcon) were added in the well by merging the bottom of the insert into the medium in the lower compartment. To the upper compartment, 1×10^6 of transfected HEK293 cells in 1 mL of medium was added. The cells were incubated 18 h, at 37°C under 5% CO_2. The BMDCs were then collected, washed in medium and cultured with 0.3×10^6 OT-1 cells during at least 18 h in 24-well plate.

Mouse anti-IL-2 ELISA

On the supernatant of the OT-1 cells activated by the BMDC, the production of murine IL-2 was measured by ELISA. IL-2 was quantified using the Mouse IL-2 ELISA MAX™ Standard from Biolegend. All ELISA procedures were performed according to the manufacturer's protocol.

T-cell assay

T-cell assays in mouse and human cell lines were carried out as described previously.[48]

Flow cytometry analysis

Exosomes from MCA 205 were labeled with the anti-mouse-CD81-PE, the anti-mouse-CD9-PE purchased from BD Biosciences (San Diego), the anti-mouse-MHCI(H-$2K^b$)-APC and the anti-mouse OVA257-264 (SIINFEKL) peptide bound to H-$2K^b$ PE from ebioscience (ebioscience). Exosomes from HEK293 were labeled with the anti-human-CD81-PE from BD Biosciences and the anti-human-CD63-PE from Beckman Coulter. Labeling was performed at 4°C using PBS containing 1% FBS during 30 min. Events were acquired using a FACS CANTO and analyzed using FACS Diva (version 6.1.3) followed by FlowJo (version X.0.7).

Quantitative RT-PCR for the analysis of gene transcription in transfected cells

The total RNA from the transfected cells was extracted by the RNeasy Mini Kit following the manufacturer's protocol (Qiagen). 1 μg of RNA sample was primed with oligo-dT and reverse transcribed into a volume of 20 μL with iScript reverse transcriptase (Biorad). Quantitative PCR reactions were performed on 100 ng of cDNA using the StepOne real time PCR system (Applied BioSystem). The reaction was performed with the PowerSYBR Green PCR Master mix (Applied BioSystem) and specific primers pairs for each gene of interest for Globin exon1-exon2: forward, 5′-GATGAAGTTGGTGGTGAGGC-3′ (exon 1); reverse, 5′-CAGCT- TGTCACAGTGCAGCTC-3′ (exon 2), for Globin intron: forward, 5′-GTATCAAGGTTAC-

AAGACAG-3′; reverse, 5′-GGGAAAATAGACCAATAGGC-3′; for Ova: forward 5′-GA-GGAGGCTTGGAACCT-AT-3′; reverse 5′-CAGTTTGAGAATCCACGGAC-3′. GAPDH was used as a reference gene using the following primers forward, 5′-TGGCAAAGTGGAG-ATTGTTGCC-3′; reverse, 5′-AAG ATGGTGATGGGCTTCCCG 3′.

Levels of capped Ova mRNA in HEK293 cells following RNA transfection

HEK293 cells were transfected by mRNA as described previously (Apcher et al. 2011). The quantity of mRNA was determined by qRT-PCR.

Western blot

HEK293 cells transfected were lysed 30 min on ice by RIPA lysis buffer. Samples were separated on a 12% SDS-PAGE and transferred to nitrocellulose blotting membrane (Amersham). 70 μg of proteins were loaded. After saturation of the membrane with PBS containing 5% non-fat milk, membranes were incubated overnight with an anti-Globin mAb (Sigma). After washing with TBS-Tween, bound antibodies were detected using a rabbit anti-mouse secondary antibody conjugated to horseradish peroxidase (1:2000; 1 h at room temperature) and then revealed with ECL (Thermo scientific).

Quantitative RT-PCR for the analysis of gene transcription in exosomes

Previously to total RNA extraction, the exosomes were treated with 500 U of RNase 1 (Invitrogen) in 100 μL of PBS. 1 mL of Tri-reagent (Euromedex) was added and the samples were incubated 5 min at room temperature. We added 200 μL of chloroform and incubated 15 min at room temperature followed by a centrifugation of the samples 15 min at 12000 g at 4°C. The transparent upper phase was transferred to a new tube, 300 μL of ethanol was added and the mix was transferred to a RNeasy Mini spin column (Qiagen). We span down 1min at 4°C, 10000 g and then discarded flow-through. 350 μL of buffer RW1 were added to the column and span down 1 min at 10000 g. 10 μL of Qiagen DNase I were mixed to 70 μL of buffer RDD and added to the column during 30 min at room temperature. 350 μL of RW1 were added to the column and centrifuged 1 min at 800 g. 500 μL of buffer RPE were added and span down 1 min. The same step was repeated. The column was then dried by centrifugation at 14000 g, at 4°C for 2 min. For RNA elution, the column was placed in a new eppendorf tube, 30 μL of RNase-free water was directly added to the membrane, incubated 5 min at RT and then span down for 1 min at 12000 g. 1 μg of RNA sample was primed with oligo-dT and reverse transcribed into a volume of 20 μL with iScript reverse transcriptase (Biorad). Quantitative PCR reactions were performed on 100 ng of cDNA using the StepOne real time PCR system (Applied BioSystem). The reaction was performed with the PowerSYBR Green PCR Master mix (Applied BioSystem) and specific primers pairs for the gene of interest, Globin intron. We used as well primers for FTL (Ferritin, light polypeptide): forward, 5′-GCTACGAGCG-TCTCCTGAAG-3′; reverse, 5′-GGCCTGGTTC-AGCTTTTTTCT-3′ and FTH1 (Ferritin heavy chain): forward, 5′-CCAGTTTGTGCAGTTCCAGT-3′; reverse, 5′- AGGTGGCC-GAATCTTCCTT-3′. GAPDH was used as reference.

PTPs-His purification and mice vaccination

PTPs-His were purified as previously described.[13] Vaccines were prepared according to what has been previously described.[2] Briefly, vaccines were prepared according to the following groups: PTPs-His 128 μg, PTPs-His 64 μg −/+ exosomes, PTPs-His 32 μg −/+ exosomes (15 μg), exosomes (15 μg) (purified from MCA205 transfected cells), CpG (20 μg) (Invivogen) and Poly(I:C)(50 μg) (Invivogen), PBS (up to 300 μL). Vaccines were prepared 2 h before injection and keeped on ice. Prior to vaccination, C57BL/6 mice were anesthetized with 3% isoflurane. The vaccines were injected subcutaneously in the paws (150 μL/leg) and in footpads (50 μL/foot). Two weeks later, subcutaneous injections of 5×10^4 MCA205 cells (right flank) and MCA205-OVA cells (left flank) are given. Once a week, tumors size was measured until they reached ~300 mm^2.

Statistical analysis

Results are expressed as the mean ± SEM and represent at least three independent experiments unless stated. Differences between means were compared using the unpaired student t-test. Differences were considered significant for $p < 0.05$.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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