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Antigen Presentation by Exosomes Released from Peptide-Pulsed Dendritic Cells Is not Suppressed by the Presence of Active CTL


Despite the potency of dendritic cells (DCs) as a vaccine carrier, they are short-lived and sensitive to CTL-mediated elimination. Thus, it is believed that the longevity of Ag presentation by peptide-pulsed DC is limited in vivo. Surprisingly, however, we found that although the majority of injected DCs disappeared from the draining lymph nodes within 7 days, Ag presentation persisted for at least 14 days following DC immunization. This prolonged Ag presentation was not mediated by the remaining injected DCs or through Ag transfer to endogenous APCs. We provide evidence that exosomes released by DCs might be responsible for the persistence of Ag presentation. Functional exosomes could be recovered from the draining lymph nodes of C57BL/6 mice following DC vaccination and, in contrast to DCs, T cell stimulation by exosomes in vivo was not affected by the presence of CTL. Our findings demonstrate that Ag presentation following delivery of DC vaccines persists for longer than expected and indicate that the exosome may play a previously unrecognized role in Ag presentation following DC vaccination. Furthermore, our study reinforces the application of exosomes as a vaccination platform and suggests that exosome-based vaccines may be advantageous for booster immunizations due to their resistance to CTL.


Materials and Methods

Mice and cell cultures

Six- to 8-wk-old C57BL/6 (H-2b) female mice were purchased from Charles River Breeding Laboratories. OT-1 transgenic mice (expressing a Vα2Vβ4 TCR specific for the peptide OVA257–264) and P14 transgenic mice (expressing a Vα2Vβ8 TCR specific for lymphocytic choriomeningitis virus (LCMV)-gp33–41) were bred in the Central Animal Facility, McMaster University (Hamilton, Ontario, Canada) (19, 20). B6.C-H-2b

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Peptides and Abs
The OVA\textsubscript{323-342} peptide (SIINFEKL) and the LCMV-\textsubscript{GP}33-41 (KAVYNFATM) were purchased from Dalton Chemical Laboratories. All flow cytometry Abs were purchased from BD Pharmingen. The mAb 25-D1.16 (specific for K\textsuperscript{\*}SIINFEKL complex) was provided by Yewdell and colleagues (22).

Preparation of DC-based vaccines
Bone marrow cells prepared from C57BL/6 mice or b61 mice were cultured in the presence of 20 ng/ml recombinant murine GM-CSF as previously described (23). On day 7 of culture, DCs were pulsed for 4 h in culture medium with 500 ng/ml SIINFEKL or KAVYNFATM or mixtures of these. We did not further manipulate cells before injection or exosome preparation.

Exosome preparation
On day 6 of culture, DCs were incubated with the peptides for 4 h as described and then washed three times with PBS to remove excess peptide. DCs were resuspended in fresh culture medium with 10% exosome-free PBS prepared by overnight ultracentrifugation at 100,000 \(\times g\), and incubated for an additional day to allow for exosome secretion. DC supernatants were harvested and centrifuged at 300 \(\times g\) (10 min), 1200 \(\times g\) (20 min), and 10,000 \(\times g\) (30 min) to remove cell debris (24). Exosomes were pelleted at 100,000 \(\times g\) for 1 h and resuspended in PBS for subsequent studies. For some experiments, the exosome pellet was additionally purified by floatation on a sucrose density cushion (1.0–1.3 g/ml), according to methods described by Raposo (24). The exosomes were quantified by protein content using a Bradford assay (Bio-Rad). To prepare DC-derived exosomes (DC\textsubscript{ex}) from the draining LN, C57BL/6 mice were injected with wild-type DCs (DC\textsubscript{WT}) pulsed with SIINFEKL into the footpads (1 \(\times 10^6\) cells/footpad). Nonpulsed DC\textsubscript{b6} or bm1 DCs (DC\textsubscript{bm1}) pulsed with SIINFEKL were similarly injected in control mice. Three days later, axillary LN and popliteal LN were harvested and digested with collagenase \(\times 5000\). All secondary Abs were conjugated to HRP. Blots were exposed to Hyperfilm (Kodak). In vivo CTL
C57BL/6 mice were s.c. immunized with 1 \(\times 10^8\) SIINFEKL-pulsed DC and cytolytic activity against SIINFEKL-pulsed target cells were measured in vivo at various times according to our published method (25).

Induction of Ag-specific immunity by DC\textsubscript{ex}
C57BL/6 mice were immunized s.c. with 1 \(\times 10^8\) SIINFEKL-pulsed DC to establish a SIINFEKL-specific CTL response. Seven days later, mice were immunized via footpad injection with 1 \(\times 10^8\) DC pulsed with both SIINFEKL and KAVYNFATM or \(-1\) \(\mu\)g exosomes derived from pulsed DCs. Three days after the secondary immunization, KAVYNFATM-specific immune response was determined by proliferation of adoptively transferred P14 DC\textsuperscript{8} cells as described in “Ag presentation assays”. Alternatively, mice were challenged with 10\(^6\) F10-gp33 tumor cells injected s.c. into the flank. Mice were monitored for tumor growth twice a week for 8 wk.

Results
Ag presentation persists beyond the peak of the CTL response following immunization with DC pulsed with SIINFEKL peptide
To understand the relationship between DC persistence and Ag presentation during the activation of CTL, we set out to establish the kinetics of CTL activation and DC clearance following immunization with DC pulsed with SIINFEKL peptide (DC/SIINFEKL). As shown in Fig. 1A, using an in vivo CTL assay we demonstrated that Ag-specific cytotoxicity was observed within the spleen and local LN (data not shown) as early as 3 days following immunization. Cytotoxic activity continued to increase, reaching its peak at day 7 followed by a gradual loss of activity over the subsequent 2 wk. To examine how the survival of Ag-loaded DC was affected by the appearance of activated CTL, we injected equal quantities of CFSE-labeled, nonpulsed and PKH-26-labeled, SIINFEKL-pulsed DC into the footpads (0.5 \(\times 10^6\) cells/footpad) of C57BL/6 mice to monitor the persistence of injected DCs in the draining popliteal LN over a period of 14 days. DC accumulation in the LN reached a maximum around 24–36 h.
postinjection, at which time ~2000 injected DCs could be recovered from the popliteal LN (Fig. 1B). By day 3, a reduction in the number of SIINFEKL-pulsed DC was detected and 90% disappeared by day 7, consistent with the peak of the CD8 T cell response observed in Fig. 1A and other reports (9, 10). Together with the fact that the disappearance of coinjected nonpulsed DC was much slower (Fig. 1B), our results confirmed that CTL played a role in eliminating Ag-presenting DC. However, a small number of Ag-pulsed DC (40 ± 8) were consistently detected until day 11, indicating that some DCs escaped CTL-mediated elimination.

This latter observation prompted us to reassess the durability of Ag presentation during CTL activation. To this end, we transferred CFSE-labeled OT-I CD8^+ T cells to mice at various times postimmunization with DCs, and the presence of Ag presentation was determined by proliferation of transferred transgenic T cells (Fig. 1, C–E). Surprisingly, although the majority of Ag-pulsed DC were cleared from the draining LN within 7 days, proliferation of OT-I CD8^+ T cells was detected up to 14 days after immunization; 1 wk beyond the peak CTL response (Fig. 1F). No proliferation was observed in mice immunized with unpulsed DC (data not shown) or PBS (Fig. 1H). This result suggested that Ag presentation following DC immunization persisted through a mechanism resistant to CTL-mediated clearance.

**Prolonged Ag presentation is not mediated by residual DCs present beyond day 7**

Because a small number of Ag-pulsed DC could be detected from the draining LN up to 11 days postinjection, we first sought to determine whether residual DCs from the injection were responsible for prolonged Ag presentation. To address this possibility, we immunized mice with graded doses of DC/SIINFEKL, ranging from 1 × 10^4 to 1 × 10^6 cells per mouse, to determine the minimal number of injected DCs required to stimulate naive transgenic T cell proliferation. Consistent with the results in Fig. 1B, an injection of 1 × 10^6 DCs per mouse resulted in ~1500–2000 DCs in the draining LN after 24 h, the peak time of DC accumulation, and recovery of migrating DCs was observed in a dose-dependent manner at the same time (Fig. 2A). When naive OT-I CD8^+ T cells were adoptively transferred into immunized mice, we found that doses of 1–5 × 10^6 DCs per mouse failed to stimulate T cell proliferation (Fig. 2, B and C), even though >100 DCs could be detected in the LN after injection with 5 × 10^6 DCs, which was comparable to the number of residual DCs observed between day 7 and 11 following injection of 5 × 10^5 DCs in Fig. 1B. Therefore, the Ag presentation observed in Fig. 1C does not appear to be a result of residual injected DCs.

**Peptide-pulsed DC vaccine does not transfer Ag to endogenous APC**

Another possibility is that injected peptide-loaded DC may transfer Ag to host APCs following lysis by CTL. To test this hypothesis, we generated bone marrow-derived DC from mice carrying the bm1 mutation of Kb. Previous studies have demonstrated that the SIINFEKL peptide can bind to Kb^bm1 with relatively high affinity but these complexes are unable to properly engage the TCR of CD8^+ T lymphocytes (22). Thus, DC^Kbm1 pulsed with SIINFEKL would not be able to directly activate Ag-specific T cell responses. To avoid the allogenic response against mutated Kb, we injected SIINFEKL-pulsed DC into (bm1 × B6) F1 mice and subsequently measured the proliferation of adoptively transferred OT-I cells and in vivo CTL activity. Whereas DCs from wild-type mice (DC^Kbm1) induced OT-I proliferation (Fig. 3A), DC^Kbm1 failed to elicit measurable OT-I responses (Fig. 3B), suggesting that Ag transfer to endogenous APC was not likely the case. This result is consistent with previous reports that only protein- but not peptide-loaded donor cells can transfer Ag to host APCs for cross-priming (22, 26). The lack of Ag transfer by peptide-pulsed DC was further confirmed through an in vivo CTL assay in which no Ag-specific killing was seen in mice immunized with DC^Kbm1 (Fig. 3E). In contrast, SIINFEKL-coated DC^Kbm1 elicited a strong CTL response (Fig. 3D).

**Ag presentation by DCex**

Because neither the injected nor the endogenous DCs appeared to be responsible for the prolonged Ag presentation, we investigated other possible mechanisms that could trigger proliferation of naive OT-I cells in our model. Recent studies have demonstrated that Ag-pulsed DC can release small membrane-bound vesicles called
exosomes that contain MHC-peptide complexes and costimulatory molecules, and thus possess the necessary ligands to elicit Ag-specific T cell responses (27, 28). Using electron microscopy, we were able to visualize these small particles (50–100 nm) in the supernatant of DCs culture (Fig. 4A). They were stained positive for CD11c, MHC class II, and CD86, reflecting their DC origin (Fig. 4, B–D). More specifically, the K\textsuperscript{b}/SIINFEKL complexes could be stained with a specific mAb, 25-D1.16 (Fig. 4E), indicating that exosomes indeed carried exogenously loaded Ag. We also confirmed in this study by Western blot analysis that exosome-associated proteins including LAMP-1, Alix/AIP1, TSG101, and MHC class II were abundant in both DC lysates and exosome samples (Fig. 4F) (29–31). In contrast, calnexin, a protein found in the endoplasmic reticulum (32), was only detectable in DC lysates but essentially absent in the exosome samples indicating that the exosome preparations were not contaminated with other vesicles (Fig. 4F).

Exosomes derived from SIINFEKL-pulsed wild-type DCs provoked robust OT-I proliferation confirming that DC\textsubscript{ex} possess a capacity for direct Ag presentation (Fig. 5A). We ruled out the possibility that proliferation might be stimulated by free-peptides carried over from DC supernatants or contaminating endogenous DCs from positive T cell selection (i.e., CD8\textsuperscript{+} DC) because similarly prepared exosomes from SIINFEKL-pulsed DC\textsuperscript{bm1} failed to trigger a measurable T cell response (data not shown). Furthermore, T cell proliferation was completely blocked by addition of 25-D1.16 but not an irrelevant control Ab, indicating that engagement of OT-I CD8\textsuperscript{+} T cells by exosomes was Ag-specific (Fig. 5A). Finally, the ability of DC\textsubscript{ex} to present Ag and stimulate immune responses in vivo was evaluated by immunizing mice with exosomes derived from SIINFEKL-pulsed DC that resulted in strong proliferation of adoptively transferred naïve OT-I CD8\textsuperscript{+} T cells (Fig. 5C).

Ag-carrying DC\textsubscript{ex} can be recovered from the draining LN after DC/SIINFEKL immunization

Although our data and results reported by others have suggested a role of DC\textsubscript{ex} in the generation of CD8\textsuperscript{+} T cell responses, the evidence that peptide-loaded DC could release exosomes in vivo has not been established. We next evaluated the presence of Ag-specific exosomes in the draining LN following DC immunization. Mice were injected with 1 × 10\textsuperscript{5} SIINFEKL-pulsed or unpulsed DC per footpad and 3 days later the draining popliteal LN were harvested and processed. The exosomes were purified and characterized using the same methods described for in vitro studies. Examination of the exosome preparations by electron microscopy revealed that exosomes carrying the K\textsuperscript{b}-SIINFEKL complexes could be found in mice immunized with Ag-pulsed DC\textsuperscript{bm1} (Fig. 6A) but not DC\textsuperscript{bm1} (data not shown) or unpulsed DC\textsuperscript{bm1} (Fig. 6B). Furthermore, Western blot analysis indicated that in vivo recovered exosomes (Fig. 6C) had a very similar protein composition to exosome fractions prepared in vitro (Fig. 4F), confirming their proposed origin as exosomes and not apoptotic blebs.

To assess the ability of in vivo recovered exosomes to stimulate T cells, OT-I CD8\textsuperscript{+} T lymphocytes were incubated with either LN-derived exosomes (10 μg/well) or DCs (1/100 ratio) purified...
from the same LN. Fig. 6D demonstrates that both CD11c+ DCs and exosomes from mice that received peptide-pulsed wild-type DCs were able to stimulate OT-I proliferation, whereas neither component prepared from mice injected with peptide-pulsed DCs nor nonpulsed DCs elicited any responses. Furthermore, proliferation of OT-I T cells elicited by exosomes prepared from the LN was completely abrogated after addition of the Kb-SIINFEKL specific blocking Ab, confirming that stimulation was Ag-specific (Fig. 6D).

Pre-existing CTL affects DC- but not DCex-mediated Ag presentation

Because exosomes lack a nucleus and do not appear to be metabolically active, they would not be expected to be sensitive to either perforin- or fas-mediated lysis. In that regard, the release of Ag-loaded exosomes following injection of DCs may explain the observation of prolonged Ag presentation shown in Fig. 1C. To demonstrate that Ag presentation by exosomes is resistant to CTL-mediated clearance, we pulsed DCs with two different peptides: SIINFEKL (presented on Kb) and KAVYNFATM (LCMV-gp33 peptide presented on Db). Using this model, we can monitor Ag presentation to P14 CD8+/H11001 T cells (responsive to KAVYNFATM) in the presence of SIINFEKL-specific CTL. To verify that DCs and exosomes pulsed with two peptides were able to present both antigenic epitopes, CFSE-labeled OT-I or CFSE-labeled P14 transgenic T cells were adoptively transferred into mice concomitant with DCs or exosome injection. DCs and DCex were both able to stimulate OT-I and P14 CD8+/H11001 T cell proliferation confirming that both epitopes are presented using this approach (Fig. 7A–D).

When DC pulsed with KAVYNFATM and SIINFEKL were injected into mice that had been immunized with DC/SIINFEKL 7 days earlier, no P14 CD8+/H11001 T cell proliferation was observed (Fig. 7E), consistent with previous observations by Hermans et al. (9). To directly examine the impact of pre-existing CTL on DC survival, we injected equal numbers of DC/SIINFEKL, labeled with PKH-26 and nonpulsed DC, labeled with CFSE into naive mice or mice preimmunized with DC/SIINFEKL for 7 days. Two days later, the recovery of injected DCs from the draining LN was analyzed. As shown in Fig. 7H, both Ag-pulsed and nonpulsed DC...
reached the local LN with the same efficiency in naive animals, whereas the recovered Ag-loaded DCs were only 10% of the number of nonpulsed DC in preimmunized mice, indicating that DC are specifically eliminated by pre-existing CTL. In contrast, P14 CD8+/H11001 T cell stimulation by exosomes from DC pulsed with both peptides was not affected by pre-existing immunity (Fig. 7 F). This result supports the notion that Ag presentation by exosomes is not influenced by the presence of activated CTL.

To determine whether T cells activated by exosomes become functional effectors, we examined the antitumor activity of CD8+ T cells elicited by exosomes. For these studies, we examined the response of endogenous polyclonal T cells rather than T cells from TCR transgenic mice. Mice were first immunized with DC loaded with SIINFEKL, to induce SIINFEKL-specific CTL responses, and 7 days later mice were immunized with DCs or exosomes pulsed with KAVYNFATM and SIINFEKL. Seven days later, mice received equal numbers of CFSE-labeled, nonpulsed, and PKH-26-labeled, SIINFEKL-pulsed DC through footpad injection (0.5 × 10^6 cells each/footpad). One day later, cell suspensions from the draining popliteal LN were prepared and the recovery rate of coinjected DCs was determined by FACS. The average proportion of recovered SIINFEKL-pulsed DC (PKH-26+) was presented as a percentage of nonpulsed DC (CFSE+) + SD for three mice from each group.
Ag PRESENTATION BY EXOSOMES IS NOT AFFECTED BY CTL

Ag-loaded DC (33, 34). In this study, we have observed that although the majority of the inoculated DCs are cleared from the draining LN within a few days, a small number of DCs persist until at least day 9. Most interestingly, Ag presentation following DC immunization persists for at least 1 wk following the peak of the CTL response. Further examination revealed that this prolonged Ag presentation was not due to the residual DCs or transfer of Ag to endogenous APC. Thus, we considered alternate explanations for the prolonged Ag presentation despite the presence of activated CTL.

Exosomes are small vesicles secreted by multiple cell types including DCs, B cells, and tumor cells (17, 24, 35). Exosomes derived from DCs carry many of the surface markers found on DCs, such as MHC and costimulatory molecules, and have been shown to possess the ability to activate naive T cells (36–38). Because exosomes are merely vesicles, they should not be affected by the presence of activated CTL. Based on this understanding, we reasoned that exosomes may explain the prolonged Ag presentation observed in our model. Indeed, DC<sub>ex</sub> were able to activate Ag-specific CD8<sup>+</sup> T cells in vitro and in vivo and we have demonstrated that functional exosomes can be recovered in the draining LN following DC immunization. Furthermore, our data demonstrate that exosomes can stimulate naive T cells in the presence of established CTL against another Ag that is simultaneously presented on the same exosome particles, in contrast to Ag-bearing DCs, which are susceptible to CTL-mediated elimination. These data support the hypothesis that exosomes secreted by DCs may mediate Ag presentation after peptide-bearing DCs are cleared from the system.

The mechanisms by which exosomes activate naive T cells remain to be fully elucidated. Some studies show that exosomes or exosome-like membrane vesicles from APC can stimulate T cell clones or naive T cells on their own (24, 38–40), whereas other reports suggest that bystander DCs are required to increase the immunogenicity of exosomes, especially in eliciting CD4<sup>+</sup> T cell responses (28, 38, 41, 42). To minimize the possible contamination of DCs (i.e., CD8<sup>+</sup> DC) in the in vitro proliferation assays, we performed negative selection of OT-I CD8<sup>+</sup> T cells that were stimulated by SIINFEKL-carrying exosomes. No difference was observed between positive and negative selections (data not shown) supporting the idea that exosomes are able to directly stimulate T cells at least under culture conditions. It is likely that both host DC-dependent and -independent pathways coexist in vivo. Several studies have shown that exogenously transferred exosomes can be processed by host DC and presented again in the context of endogenous MHC molecules (35, 43, 44). However, it has also been observed that exosomes at the surface of recipient DC may affect subsequent DC immunization in a nonspecific manner. Furthermore, DC pulsed with two peptides were able to generate antitumor protective immunity in animals without preimmunization confirming the quality of DC vaccine preparation (Fig. 8B). No tumor protection was observed using DCs or DC<sub>ex</sub> alone (without loaded peptide) either in preimmunized or nonimmunized mice (Fig. 8B). These data demonstrate clearly that DCs are sensitive to CTL-mediated elimination but exosomes can effectively present Ag to naive T cells even in the presence of CTL.

Discussion

DCs have been intensively studied as a vaccine platform in preclinical models and clinical trials (5). Although the mechanisms that regulate the ability of DCs to stimulate naive T cells are still being elucidated, a number of studies support the concept of a negative feedback model in which recently activated CTL clear the growth was monitored. Consistent with proliferation data, DC pulsed with both peptides were unable to elicit protective immunity in the presence of SIINFEKL-specific CTL, whereas exosomes were able to elicit protective immunity in 80% of the mice under the same conditions (Fig. 8A). The fact that DC pulsed with KAVYNFATM alone (Fig. 8A) could induce a protective response ruled out the possibility that pre-existing CTL may affect subsequent DC immunization in a nonspecific manner. Furthermore, DC pulsed with two peptides were able to generate antitumor protective immunity in animals without preimmunization confirming the quality of DC vaccine preparation (Fig. 8B). No tumor protection was observed using DCs or DC<sub>ex</sub> alone (without loaded peptide) either in preimmunized or nonimmunized mice (Fig. 8B). These data demonstrate clearly that DCs are sensitive to CTL-mediated elimination but exosomes can effectively present Ag to naive T cells even in the presence of CTL.

FIGURE 8. Exosomes but not DCs can elicit antitumor immunity in the presence of activated CTL. C57BL/6 mice were immunized with 10<sup>6</sup> DC pulsed with SIINFEKL (A) or left untreated (B). Seven days later, mice received a secondary immunization with DCs or exosomes carrying both SIINFEKL and KAVYNFATM peptides. One week after secondary immunization, tumor challenge was performed using F10–gp33 (1 × 10<sup>6</sup> cells) via s.c. injection on the right flank. Results are representative of two experiments with five mice for each group.

Discussion

DCs have been intensively studied as a vaccine platform in preclinical models and clinical trials (5). Although the mechanisms that regulate the ability of DCs to stimulate naive T cells are still being elucidated, a number of studies support the concept of a negative feedback model in which recently activated CTL clear the
the inoculated DC and DC_ex. Particularly, these data offer a mechanism by which Ag presentation can persist in the presence of robust CTL. At present, however, the impact of exosome-mediated Ag presentation on the outcome of T cell responses remains elusive because the requisite duration of antigenic stimulation for optimal CD8 T cell function is unclear and controversial results have been reported in model systems. Using an in vitro culture system with transgenic T cells, van Stipdonk et al. (49) demonstrated that only 20 h of stimulation is required to induce CD8 T cells to acquire full effector function, whereas Stormi et al. (11) showed that Ag had to be presented for several days by activated APCs to trigger protective T cell responses in nontransgenic mice. These observations suggest that the induction of effective T cell immunity under physiological conditions may require Ag presentation for an extended period of time and together with our data, exosomes released by Ag-carrying DCs may play a role in this process. Current studies in our laboratory are focused on the development of a model in which we can control the survival of injected DCs to dissect DC- and DC_ex-mediated Ag presentation in vivo.

In addition to the primary immune response, resistance of exosomes to CTL lysis is likely to have a much greater impact on secondary immune responses. Vaccines designed to elicit cellular immunity typically require repeated vaccination to generate a protective number of memory T cells. Previous studies have demonstrated that the efficacy of readministered DCs is severely reduced by the presence of established CTL (34, 50). Thus, using exosomes as vaccine carrier may provide a novel platform for booster immunizations.

Disclosures

The authors have no financial conflict of interest.

References


