The Journal of Immunology

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J Immunol 2007; 179:2242-2249; ;
doi: 10.4049/jimmunol.179.4.2242
http://www.jimmunol.org/content/179/4/2242

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Effective Treatment of Inflammatory Disease Models with Exosomes Derived from Dendritic Cells Genetically Modified to Express IL-4[1]

Seon Hee Kim,*‡ Nicole R. Bianco,*‡ William J. Shufesky,†§ Adrian E. Morelli,†§ and Paul D. Robbins[2]*‡

In this study, we demonstrate that genetically modified bone marrow-derived dendritic cells (DC) and exosomes derived from the DC, expressing either secreted IL-4 or membrane-bound IL-4, can reduce the severity and the incidence of established collagen-induced arthritis and inhibit inflammation of delayed-type hypersensitivity (DTH) in mice. The ability of the DC and DC-derived exosomes to suppress the DTH response was MHC class II and, in part, Fas ligand/Fas dependent. The DC-derived exosomes were internalized by CD11c$^+$ DC in the dermis at the site of injection and in the draining lymph node as well as by CD11c$^+$ DC and F4/80$^+$ macrophages in the spleen. Moreover, adoptive transfer of CD11c$^+$ or CD3$^+$ splenic cells from mice treated with exosomes showed significant reduction of footpad swelling in the DTH model. These results demonstrate that administration of DC/IL-4 or exosomes derived from DC/IL-4 are able to modulate the activity of APC and T cells in vivo through a MHC class II and partly Fas ligand/Fas-dependent mechanism, resulting in effective treatment of established collagen-induced arthritis and suppression of the DTH inflammatory response. Thus, APC-derived exosomes could be used therapeutically for the treatment of autoimmune disease and inflammatory disorders. *The Journal of Immunology,* **2007, 179: 2242–2249.**

Interleukin-4 is a mediator of Th2 cell commitment and Ig class switching to the Th2-associated isotype IgG1 and IgE. In addition, IL-4 exhibits anti-inflammatory effects such as suppression of IL-1 and TNF-α production by macrophages (1, 2). Using gene transfer or injection of recombinant protein, IL-4 has been shown to be therapeutic in different murine models of autoimmune disease including collagen-induced arthritis (CIA),[3] type I diabetes, and experimental autoimmune encephalomyelitis. In particular, we and others (3–6) have shown that gene transfer of IL-4, either locally or systemically, modulates the severity of CIA.

Dendritic cells (DC) are professional APC that are able to modulate T cell immunity in either a positive or negative manner, depending upon their lineage and state of maturation. There are several subpopulations of DC including myeloid DC, plasmacytoid DC, and Langerhans cells that play different roles in the regulation of the immune responses (7–11). In addition to their ability to stimulate immunity, these different DC populations, under certain conditions, are involved in T cell immunosuppression and/or induction of central and peripheral tolerance. We and others (12–15) have demonstrated that systemic administration of bone marrow (BM)-derived, myeloid DC, genetically modified to express either IL-4 or Fas ligand (FasL), is able to reverse established murine autoimmune arthritis for extended periods of time following a single treatment). Similarly, macrophages modified to express FasL can deplete collagen-reactive T cells in murine CIA (16), down-modulate chronic inflammatory disease (17), and block Ag-specific immune responses (18). Furthermore, BM-DC modified to express FasL are able to induce T cell hyporesponsiveness in allogeneic transplant models (19).

Exosomes are small membrane vesicles, 40–100 nm, released by various cell types through the endocytic pathway. Exosomes from APC carry MHC class I (MHC I) and II (MHC II) and T cell costimulatory molecules on their surface, suggesting that they could play important roles in immune regulation. In murine models, DC-derived exosomes have been shown to be immunostimulatory or suppressive, depending on the type and stage of maturation of the DC (20–26). Tumor cell-derived exosomes carrying tumor Ag have been used effectively to prime mature DC, leading to antitumor immunity in mice and in Phase I human trials (27, 28). Exosomes may also exhibit immunosuppressive ability. Exosome-like vesicles, termed "tolerosomes," produced by rat intestinal epithelial cells in the presence of IFN-γ and the model Ag OVA were able to induce Ag-specific tolerance in untreated mice (29). In addition, allogeneic exosomes from immature BM-DC delayed rejection of heart allografts in rats (30). T lymphocytes and tumor cells release exosome-like vesicles expressing FasL, which are able to induce T cell apoptosis (31, 32). FasL-containing vesicles also have been found in the first trimester syncytiotrophoblast and are involved in conferring maternal tolerance to the placenta (33).

We demonstrated previously that both DC and exosomes derived from immature DC, pretreated with IL-10, produce anti-inflammatory exosomes that suppress the onset of murine CIA and

*Department of Molecular Genetics and Biochemistry, †Department of Surgery and ‡Department of Molecular Medicine and §T. E. Starzl Transplantion Institutes, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

Received for publication July 18, 2006. Accepted for publication June 2, 2007.

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[1] These studies were supported in part by Grants AI56374 (to P.D.R.), HL075512 (to A.E.M.), and HL077545 (to A.E.M.) from the National Institutes of Health and Juvenile Diabetes Research Foundation Grant 7-005-1154 (to P.D.R.).

[2] Address correspondence and reprint requests to Dr. Paul D. Robbins, Department of Molecular Genetics and Biochemistry, W1246 BST, University of Pittsburgh, Pittsburgh, PA 15261. E-mail address: probb@pitt.edu

[3] Abbreviations used in this paper: CIA, collagen-induced arthritis; DC, dendritic cell; BM, bone marrow; FasL, Fas ligand; MHC I/II, MHC class I/II; Ad.FasL, adenovirus encoding FasL; DTH, delayed-type hypersensitivity; mbIL-4, membrane-bound IL-4; sIL-4, secreted IL-4; KLH, keyhole limpet hemocyanin; i.d., intradermal(ly); Ad.IL-4, adenovirus encoding IL-4; Ad.mbIL-4, adenovirus encoding mbIL-4; Ad.sIL-4, adenovirus encoding sIL-4.

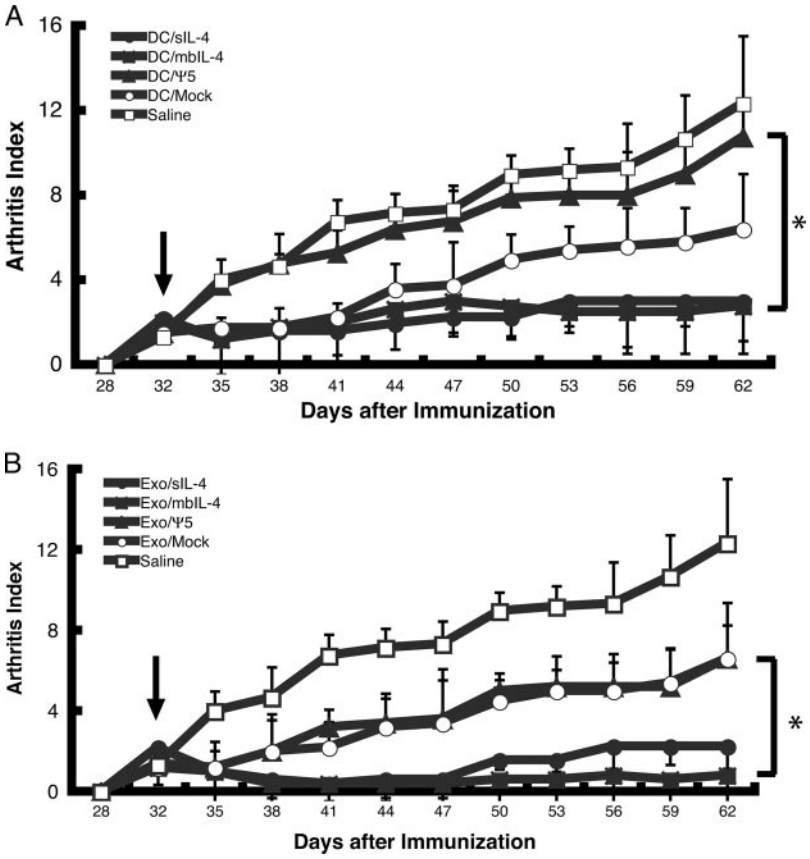

FIGURE 1. Analysis of the therapeutic effect of DC (*A*) and DC-derived exosomes (*B*) in murine CIA. Exosomes were isolated from DBA1 BM-DC that were previously infected with Ad.ψ5, Ad.sIL-4, or mbIL-4. The purified exosomes as well as DC were injected i.v. at day 32 into DBA mice that were immunized with bovine type II collagen and received LPS at day 28 for synchronous disease onset. Mice were monitored periodically by an established macroscopic scoring system on a 0–4. scale: 0, normal; 1, detectable arthritis with erythema; 2, significant swelling and redness; 3, severe swelling and redness from joint to digit; and 4, maximal swelling and deformity with ankylosis. The macroscopic score (mean $\pm$ SD) was expressed as a cumulative value for all paws, with a maximum possible score of 16 ($n = 7$). Arrows, The day of treatment. $*$, Significance at $p = 0.0002463$ (*A*) and $p = 0.0004703$ (*B*).

reduce the severity of established arthritis (25). In fact, exosomes were as effective as the parental DC in suppressing CIA onset. Moreover, DC transduced with recombinant adenovirus encoding FasL (Ad.FasL) produce exosomes able to suppress inflammation in a model of delayed-type hypersensitivity (DTH) and partially reverse established CIA (26) in mice. The ability of the Ad.FasL-transduced DC and DC/FasL-derived exosomes to suppress the DTH response was dependent not only upon FasL in the DC or DC-derived exosomes, but also on the presence of Fas in the host mice. Moreover, the effect was MHC II dependent, but MHC I independent.

It has been demonstrated that systemic administration of BM-DC, genetically modified to express IL-4 by adenoviral-mediated gene transfer, reverse-established CIA in mice (12, 13). However, given that the secreted form of IL-4 was used in these studies, it is possible that the results were due to high local levels of soluble IL-4 with the DC serving only as a vehicle for delivery of IL-4. Thus, in these studies, we also used DC-expressing membrane-bound IL-4 (mbIL-4) to confirm that the immunosuppressive effect is mediated by the DC and not due to elevated levels of secreted IL-4 (sIL-4). In this study, we report that administration of DC expressing either sIL-4 or mbIL-4 resulted in a reversal of established CIA as well as a significant reduction of swelling in a murine DTH model, in both the treated and untreated contralateral paw. Interestingly, exosomes derived from the immunosuppressive DC/IL-4 were as or more suppressive than the parental DC in both CIA and DTH models. In addition, both DC/IL-4 and DC/IL-4-derived exosomes reduced inflammation in the DTH model through a MHC and partly Fas/FasL-dependent mechanism. Moreover, trafficking analysis and adoptive transfer experiments suggest that DC and DC-derived exosomes function through modulating the activity of both APC and T cells in vivo. Because exosomes are more stable and less dynamic than DC, they may be a better therapeutic agent for treatment of arthritis and other autoimmune diseases.

Materials and Methods

DC generation

Murine BM-derived DC were prepared as described previously (13). Briefly, BM was collected from tibias and femurs. Contaminating erythrocytes were lysed and lymphocytes were depleted with a mixture of mAb (RA3-3A1/6.1, anti-B220; 2.43, anti-Lyt2; GK1.5, anti-L3T4; all from American Type Culture Collection). Cells then were cultured for 24 h in complete medium to remove the adherent macrophages. The nonadherent cells then were placed in fresh complete medium containing 1000 U/ml murine GM-CSF and murine IL-4. Cells were cultured for 4 days and harvested for Ad transduction. For Ad infection, 1×10^6 DC were mixed with 5×10^7 PFU of the viruses in a total volume of 1 ml of serum-free medium. After incubation for 24 h, DC were washed intensively three times and incubated for another 48 h. On day 8, culture supernatant was collected for exosome purification and recovery of the Ad-transduced DC.

Exosome isolation

Exosomes were isolated as previously described (25). Collected culture supernatants were centrifuged at $300 \times g$ for 10 min, $1{,}200 \times g$ for 20 min, and $10{,}000 \times g$ for 30 min. The supernatant from the final centrifugation was ultracentrifuged at $100{,}000 \times g$ for 1 h in the ultracentrifuge. The exosome pellet was washed in saline, again centrifuged at $100{,}000 \times g$ for 1 h, and resuspended in saline.

Flow cytometric analysis

For flow cytometry, 100 μg of exosomes was incubated with a fixed number of 4.5-μm beads (Dynabeads; Dynal) precoated with I-A^b or CD11b mAb. Beads coated with exosomes were labeled with the following PE mAb (BD Pharmingen): H-$2K^b$, I-A^b,CD11b, CD11c, CD80, CD86, CD71, and CD178.

For FACS analysis of IL-4 expression on DC-derived exosomes, exosomes were incubated with 5 μl of 4-μm diameter aldehyde/sulfate latex beads in a final volume of 20 μl for 15 min at room temperature. After addition of 10 mg of BSA into the exosome-coated beads, the incubation was continued for 15 min. One milliliter of saline was added in, followed

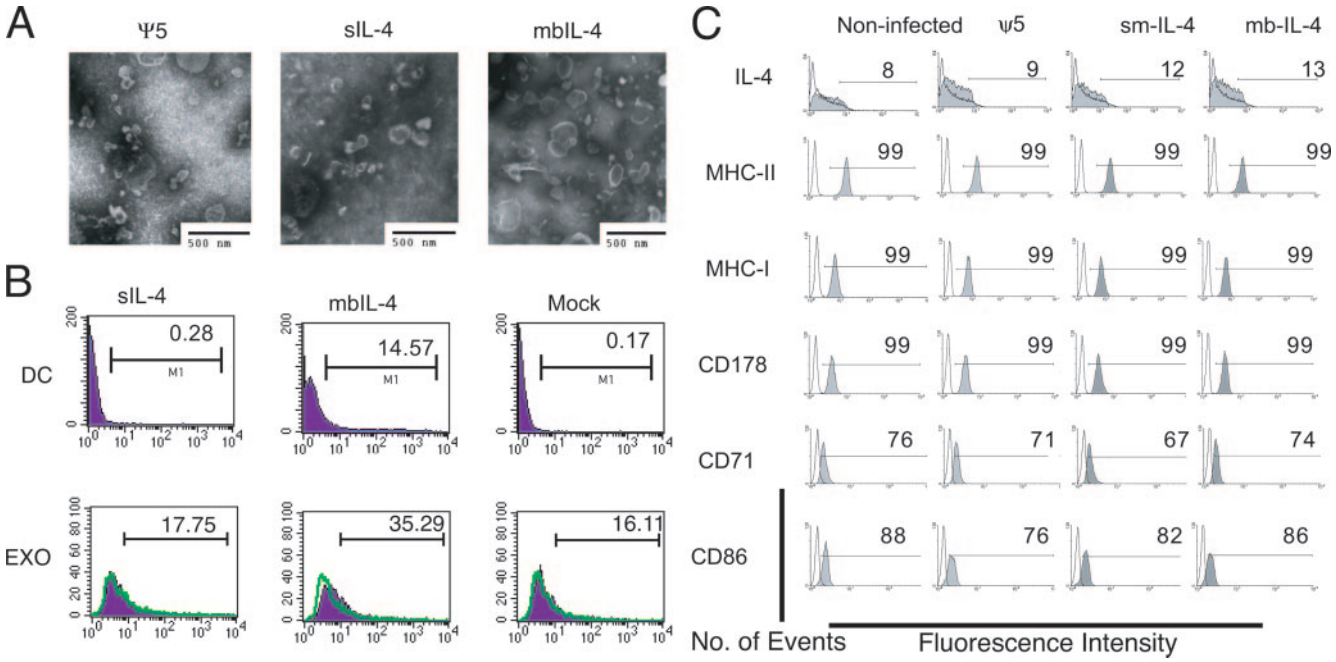

FIGURE 2. *A*, Ultrastructural analysis of DC-derived exosomes. Transmission electron microscopy was performed using a JEOL-1210 computer-controlled high-contrast 120-kV transmission electron microscope. *B*, IL-4 expression on BM-DC and DC-derived exosomes. BM-derived DC were infected with Ad.sIL-4 or Ad.mbIL-4 at day 5. Two days later, DC and DC-derived exosomes were isolated and analyzed by electron microscopy and flow cytometry. Exosomes were isolated from the DC culture supernatants by differential centrifugation and attached to aldehyde/latex beads. DC and exosome-coated beads were stained with IL-4 mAb and analyzed by flow cytometry. *C*, Flow cytometric analysis of DC-derived exosomes. Exosomes were incubated with Dynabeads coated with I-A^b mAb or CD11b mAb. Beads coated with exosomes were labeled with the following PE mAbs: H-$2K^b$, I-A^b, CD11b, CD11c, CD86, CD71, and CD178, or the corresponding isotype controls.

by a 75-min incubation with gentle shaking. Reaction was stopped by incubation for 30 min with 100 mM glycine. Exosome-coated beads were washed twice in FACS buffer (3% FBS and 0.1% NaN_3 in saline) and resuspended in 400 μl of FACS buffer. The DC and exosomes were examined by flow cytometry (FACScan; BD Biosciences).

Electron microscopy

Exosomes were purified by differential ultracentrifugation, 10 μl loaded on a Formvar/carbon-coated grid, negatively stained with 10 μl of neutral 1% aqueous phosphotungstic acid, and viewed using a JEOL-1210 computer-controlled high-contrast 120-kV transmission electron microscope.

Exosome administration into the DTH model

C57BL/6 mice were sensitized by injecting 100 μg of Ag (keyhole limpet hemocyanin (KLH) or OVA) emulsified 1/1 in CFA at a single dorsal site. Ten days later, one hind footpad of the immunized mice was injected with 10^6 DC or 1 μg of DC-derived exosomes, 24 h before challenge with Ag. The contralateral footpad received an equal volume of saline instead of DC or exosomes. Mice were challenged in both footpads by injecting 20 μg of Ag dissolved in 20 μl of saline. Footpad swelling was measured. Results were expressed as the difference in swelling ($\times$0.01 mm) before and after Ag boost injection.

Murine CIA model

Male DBA/1 lacJ (H-2^q) mice, 7–8 wk of age, were purchased from The Jackson Laboratory and maintained in a pathogen-free animal facility at the University of Pittsburgh Biotechnology Center. Bovine type II collagen (Chondrex) in 0.05 M acetic acid at a concentration of 2 mg/ml was emulsified in an equal volume of CFA and injected into the base of the tail.

The mice were monitored by an established macroscopic system ranging from 0 to 4: 0, normal; 1, detectable arthritis with erythema; 2, significant swelling and redness; 3, severe swelling and redness from joint to digit; and 4, maximal swelling and deformity with ankylosis. The average of macroscopic scores was expressed as a cumulative value for all paws, with a maximum possible score of 16 per mouse.

Trafficking analysis

BM-DC-derived exosomes were labeled with PKH67 (green fluorescent cell linker; Sigma-Aldrich) per the manufacturer's protocol. After incubation with the linker, exosomes were washed twice by ultracentrifugation. The labeled exosomes were injected locally (intradermally (i.d.)) into the footpad of one hind paw of the KLH-immunized mice or injected systemically (i.v.) through the tail vein. Tissues were obtained and frozen for histological analysis 48 h after local injection or 24 h after systemic injection.

Immunofluorescence

Cryostat sections (8 μm) were fixed in 4% paraformaldehyde, blocked with 10% goat serum, and incubated with the biotin anti-CD11c or biotin anti-F4/80 (BD Pharmingen) mAbs followed by 1/3000 cyanin 3-streptavidin (Jackson ImmunoResearch Laboratories). Nuclei were stained with 4′,6′-diamidino-2-phenylindole (Molecular Probes).

Adoptive transfer

Exosomes were injected either systemically (i.v.) via the tail vein or i.d. into a hind paw of the KLH-immunized mice. Three days after injection, peritoneal macrophages, lymph nodes, and spleen were obtained and subjected for isolation of $CD3^+$, or $CD11c^+$ cells. Isolated subpopulations were injected into a hind paw of KLH-immunized mice for analysis in the DTH model.

Statistical analysis

Results were compared using Student's t test and by ANOVA. Values of $p < 0.05$ were considered to be statistically significant.

Results

Administration of DC/IL-4 or Exo/IL-4 reduces severity of murine CIA

Previously, others and we demonstrated that systemic administration of DC transduced with Ad.IL-4 suppresses established CIA (13). However, in these studies, a secreted form of IL-4 was used, allowing for the possibility that the observed effects were due to elevated local levels of a secreted form of IL-4. To demonstrate that the effect of DC/IL-4 was due to the immunosuppressive function of DC, BM-DC were infected with a recombinant Ad vector

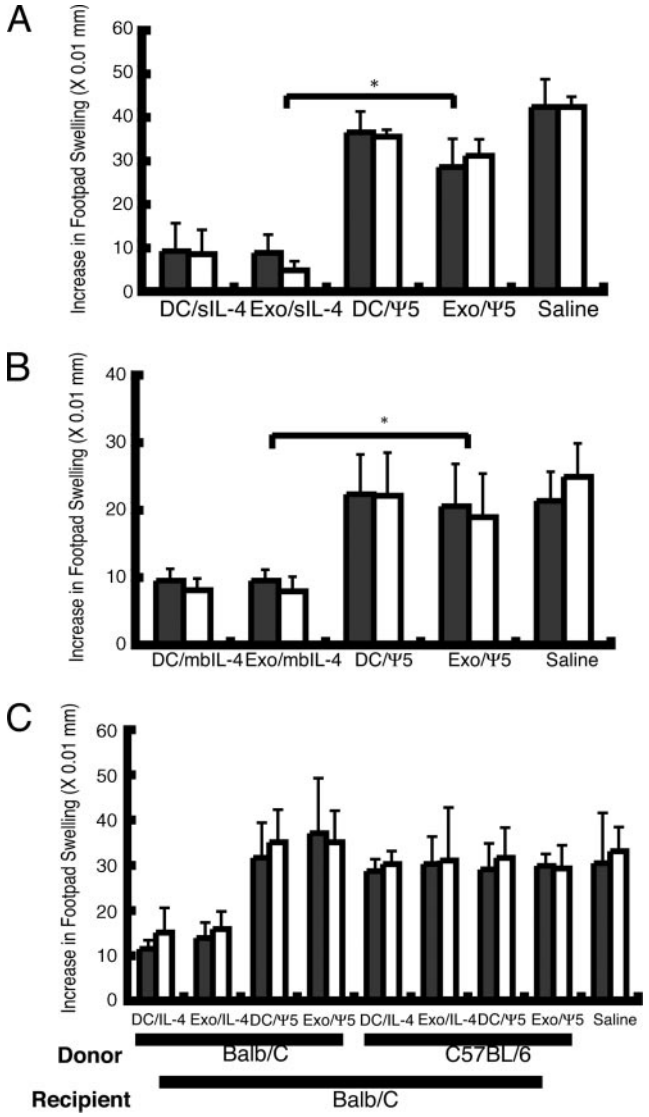

FIGURE 3. Suppression of DTH by DC and DC-derived exosomes. Exosomes were isolated from BM-DC that were either infected with the Ad.ψ*5* (control) or Ad.sIL-4 (*A*) or Ad.mbIL-4 (*B*). *C*, DTH suppression of exosomes is MHC dependent. Exosomes were separately isolated from allogeneic BALB/c BM-DC that were infected with Ad.ψ*5* or Ad.mbIL-4. The purified exosomes were injected into the right footpad of KLH-immunized BALB/c and C57BL/6 mice. Twenty-four hours postinjection of DC or DC-derived exosomes, KLH was injected into both hind footpads and the extent of swelling was measured at 48 h. Difference of footpad thickness in the treated paws (■) and in the nontreated contralateral paws (□). ∗, Significance of $p = 0.002095$ (treated), $p = 0.000316$ (contralateral) in *A* and $p = 0.005651$ (treated), $p = 0.007306$ (contralateral) in *B*. $p = 0.001776$ (treated), $p = 0.041229$ (contralateral) in *C*.

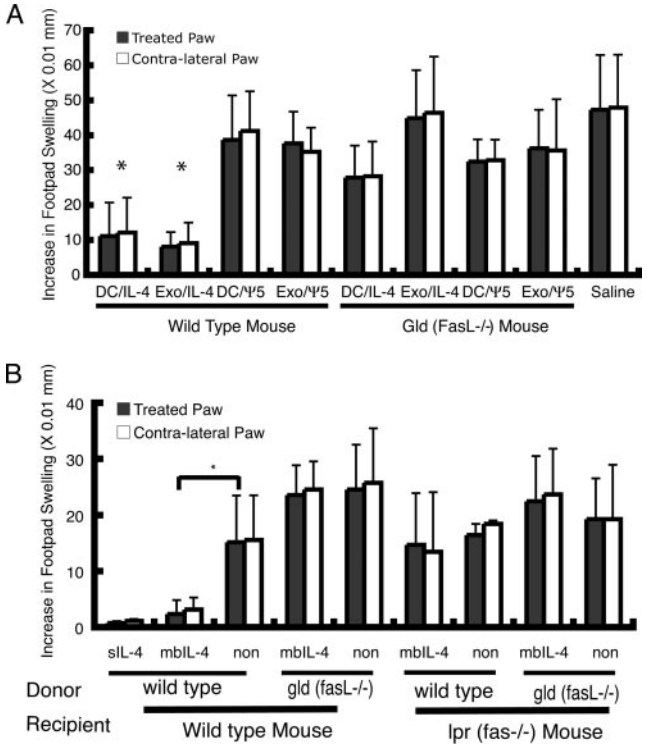

FIGURE 4. DTH suppression by exosomes is partially dependent on Fas/FasL. Exosomes were isolated from BM-DC from wild-type C57BL/6 mice or *gld* mice (deficient in functional FasL) previously transduced with Ad.ψ*5* (control) or Ad.IL-4. The DC and exosomes from the genetically modified DC were tested in wild-type recipient mice (*A*) or wild-type and Fas-deficient *lpr* mice (*B*). The purified exosomes as well as DC were injected into the right footpad of KLH-immunized mice. Twenty-four hours postinjection of DC or DC-derived exosomes, KLH Ag was injected into both hind footpads and the extent of swelling was measured at 48 h. Difference of footpad thickness in the treated paws (■) and in the nontreated contralateral paws (□). ∗, Significance of $p = 0.000936$ (treated), $p = 0.002347$ (contralateral) in *A* and $p = 0.001189$ (treated), $p = 0.004438$ (contralateral) in *B*.

expressing a membrane-bound form of IL-4 (Ad.mbIL-4) containing the CD80 transmembrane domain fused to IL-4, a secreted form of IL-4 (Ad.sIL-4) or a control Ad, Ad.ψ*5*. The DC were injected i.v. on day 32 after immunization into DBA1 mice with established arthritis. Treatment with DC/sIL-4 or DC/mbIL-4 (Fig. 1*A*) was able to suppress the progression of arthritis, consistent with our previous study with DC/sIL-4. This result suggests that the effect of DC/IL-4 is not mediated only by soluble, sIL-4, but also by the genetically modified DC-carrying mbIL-4. However, because the levels of sIL-4 and mbIL-4 expressed in the genetically modified DC may not be identical, we can not conclude whether the efficacy of the DC/sIL-4 and DC/mbIL-4 are indeed identical, only that DC/mbIL-4 are immunosuppressive.

Because we have demonstrated previously that exosomes from IL-10-treated DC were immunosuppressive, we also examined the ability of exosomes derived from the IL-4-expressing DC to reverse established CIA (Fig. 1*B*). Systemic injection of 1 μg of DC-derived exosomes, equivalent to the amount of exosomes isolated from 10^6 DC per 24 h, from either DC/sIL-4 or DC/mbIL-4 ameliorated disease severity in established CIA, whereas the Exo/ψ*5* control group showed an intermediate effect on disease progression when compared with mock control and the saline control (Fig. 1*B*). Similarly, injection of Exo/sIL-4 or Exo/mbIL-4 also delayed disease onset in the mice, which were not given LPS on day 28 (data not shown). These results suggest that a single i.v. injection of exosomes derived from DC expressing either sIL-4 or mbIL-4 was able to suppress established CIA with efficacy similar to injection of 10^6 parental DC.

Characterization of exosomes derived from DC/sIL-4 and DC/mbIL-4

To examine the composition of the exosomes isolated from the DC modified to express IL-4, exosomes were isolated from DC transduced with Exo/sIL-4, Exo/mbIL-4, and Exo/ψ*5* 48 h after infection and analyzed by electron microscopy and flow cytometry.

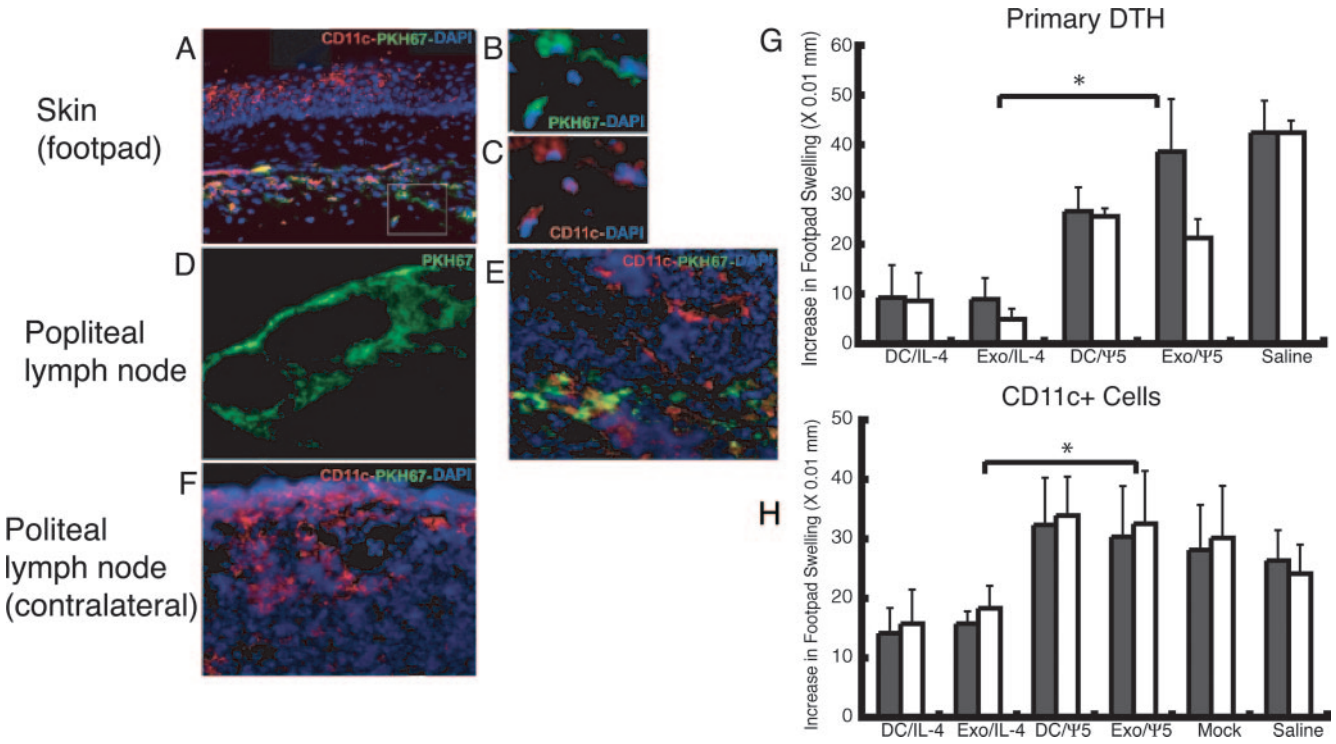

FIGURE 5. Analysis of trafficking of exosomes following local i.d. footpad injection. PKH67-labeled (green) exosomes obtained from DC were locally injected into a footpad 2 days before analysis. *A–C*, Examples of dermal $CD11c^+$ DC with internalized PKH67 exosomes are indicated with arrows and are shown in detail in the *inset*s. *D* and *E*, $CD11c^+$ DC with $PKH67^+$ inclusions (arrows) were detected in the draining (popliteal) lymph node in treated side, but were absent in the contralateral lymph node (*F*). $CD11c^+$ cells from mice locally treated with DC and DC-derived exosome-treated mice were adoptively transferred to the DTH model (*G* and *H*). The exosomes from the Ad-transduced DC were prepared and the DC and exosomes were injected locally into a hind paw of KLH-immunized mice. The results of the therapeutic effect in the primary DTH model are shown in *G*. Three days postinjection, spleens were isolated and $CD11c^+$ cells were isolated using a MACS column. Isolated $CD11c^+$ cells were injected into a hind paw of KLH-immunized mice followed by boosting with KLH 24 h after injection of $CD11c^+$ cells (*H*). Footpad swelling was measured 48 h after injection. Difference of footpad thickness in the treated paws (■) and in the nontreated contralateral paws (□). ∗, Significance of $p = 0.002095$ (treated), $p = 0.000316$ (contralateral) in *G* and $p = 0.011632$ (treated), $p = 0.019239$ (contra-lateral) in *H*.

Analysis of exosomes by electron microscopy showed similar saucer-shaped vesicles in the preparations from Exo/sIL-4, Exo/mbIL-4, and Exo/ψ5 (Fig. 2*A*). Flow cytometric analysis of the Ad-transduced DC and the exosomes released by the DC showed the presence of IL-4 on the surface of the DC with a low, but detectable level of membrane-associated IL-4 in the exosomes (Fig. 2*B*). Exosomes derived from the Ad.sIL-4-, Ad.mbIL-4-, and Ad.ψ5-transduced DC as well as the control-transduced DC were positive for MHC I and II molecules, CD11c, and the costimulatory molecule CD86 (Fig. 2*C*). Interestingly, exosomes from the different Ad-transduced DC express CD178 (FasL), confirmed by Western blot analysis (data not shown).

Local administration of Exo/sIL-4 or Exo/mbIL-4 inhibits the DTH response

To determine whether the DC genetically modified to express sIL-4 or mbIL-4, as well as exosomes derived from the modified DC, were therapeutic in an Ag-specific model of inflammation more amenable to analysis of mechanism, a DTH mouse model was used. In this model, mice were immunized to a specific Ag, KLH, and then a Th1-mediated inflammatory response was induced 2 wk postimmunization by i.d. injection of the specific Ag into the hind footpads. We have used this model previously to demonstrate that Ad.vIL-10 and Ad.FasL-transduced DC and DC-derived exosomes were anti-inflammatory. The KLH-immunized mice received either 10^6 DC or 1 μg of exosomes injected into one hind paw 12 h before a KLH boost injection into both hind paws. Local injection of DC/sIL-4, DC/mbIL-4, DC/sIL-4-derived exosomes, and DC/mbIL-4 exosomes (Fig. 3, *A* and *B*) significantly suppressed paw swelling not only in the treated paw, but also in the untreated contralateral paw at 24, 48, and 72 h postinjection of Ag (the results are only shown for the 48-h time point). In contrast, injection of DC/ψ5 or exosomes derived from the control DC was unable to inhibit the DTH response. These results demonstrate that a single, local footpad injection of genetically modified DC expressing either sIL-4 or mbIL-4 as well as exosomes derived from the DC/sIL-4 or DC/mbIL-4 are able to suppress the DTH response in not only the treated paw, but also in the contralateral, untreated paw.

Suppression of DTH by syngeneic, but not allogeneic exosomes

To determine whether the ability of the exosomes to inhibit the DTH response is MHC dependent, we examined whether allogeneic exosomes were able to suppress the DTH response in vivo. BM-DC derived from C57BL/6 ($H\text{-}2^b$, $I\text{-}A^b$) mice were used as a source of syngenic exosomes, whereas DC from BALB/c ($H\text{-}2^d$, $I\text{-}A^d$) mice were used as a source for allogeneic exosomes. Syngeneic DC/mbIL-4 and exosomes from them were able to suppress the DTH response (Fig. 3*B*). In contrast, paw swelling was not inhibited following injection of either Ad.mbIL-4-transduced DC or DC/mbIL-4-derived exosomes from allogeneic mice (Fig. 3*C*). However, the BALB/c DC and DC-derived exosomes were able to suppress the DTH response in BALB/c syngeneic recipients (Fig. 3*C*). The in vivo results in the DTH model demonstrate that the

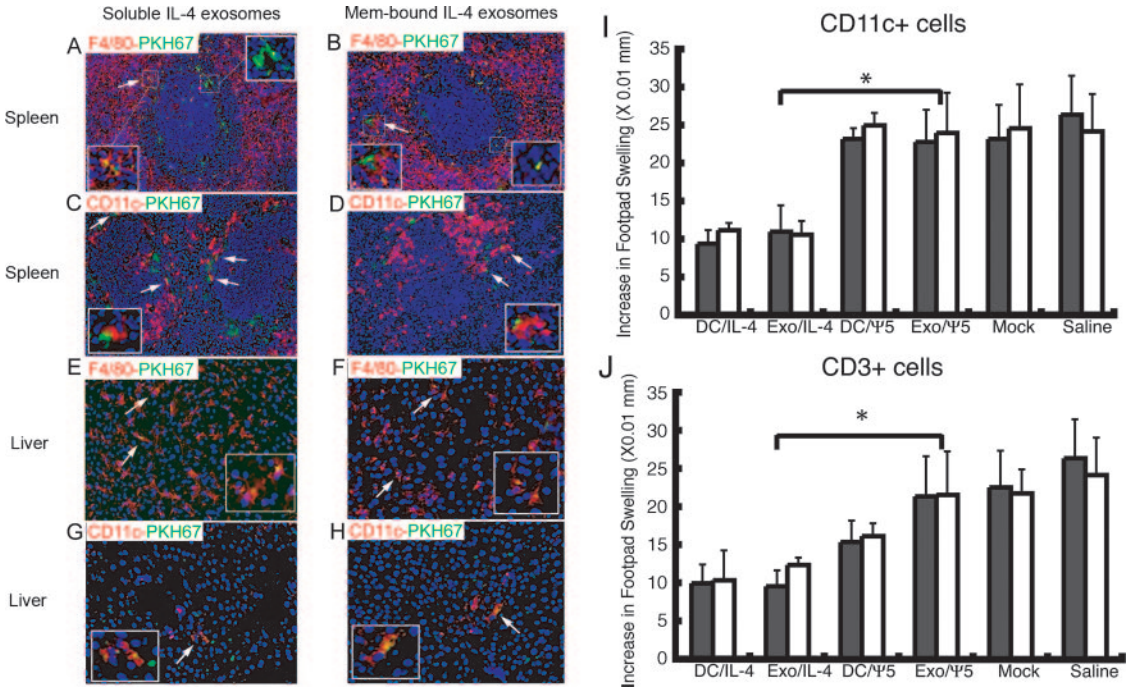

FIGURE 6. Analysis of trafficking of exosomes following systemic injection. Exosomes were labeled with PKH67 and injected i.v. in C57BL/6 mice (50 μg/mouse). Twenty-four hours after i.v. injection, sIL-4 and mbIL-4 exosomes were captured by splenic F4/80$^+$ macrophages (*A* and *B*, arrows and *insets*) and splenic CD11c$^+$ DC (*C* and *D*, arrows and *insets*). *A* and *B*, Following labeling for detection of the macrophage marker F4/80, it was clear that two populations of splenic cells phagocytes had internalized PKH67$^+$ exosomes: 1) F4/80$^+$ splenic macrophages and 2) F4/80$^-$ cells (mostly DC). Examples of both cellular subsets are indicated by the squares and magnified in the *insets*. Blood-borne sIL-4 and mbIL-4 exosomes were also entrapped by hepatic F4/80$^+$ Kupffer cells (*E* and *F*, arrows and *insets*) and a few CD11c$^+$ DC (*G* and *H*, arrows and *insets*) in the liver. Nuclei were stained with 4′,6′-diamidino-2-phenylindole. Immunofluorescence, ×200; *insets*, ×1000. Cells from mice injected i.v. with DC and DC-derived exosome were adoptively transferred (*I* and *J*). BM-DC were infected with either Ad.IL-4 or Ad.ψ5. The exosomes from the Ad-transduced DC were prepared and the DC and DC-derived exosomes were injected systemically via the tail vein. Three days after injection, spleens were isolated and subjected for separation of CD11c$^+$ (*I*) or CD3$^+$ (*J*) cells using a MACS column. The isolated CD11c$^+$ and CD3$^+$ cells were injected into a hind paw of KLH-immunized mice followed by boosting with KLH 24 h after injection. Footpad swelling was measured 48 h after injection. Difference of footpad thickness in the treated paws (■) and in the nontreated contralateral paws (□). ∗, Significance of $p = 0.002832$ (treated), $p = 0.001446$ (contralateral) in *I* and $p = 0.003364$ (treated), $p = 0.013560$ (contralateral) in *J*.

effect of Exo/IL-4 is MHC dependent, consistent with our previous results demonstrating the anti-DTH effect of Ad.FasL-transduced DC-derived exosomes was MHC II dependent, but MHC I independent (26).

Effect of exosomes is Fas/FasL dependent

As outlined above, flow cytometric and Western blot analysis of the exosomes demonstrated that they contain FasL on their surface membrane. Because we observed previously that Ad-mediated gene transfer of FasL to DC results in immunosuppressive DC and DC-derived exosomes, we tested whether the FasL in the DC-derived exosomes was important for the observed anti-inflammatory effect in the DTH model. DC were prepared from either wild-type or FasL-deficient (*gld*) mice and infected with Ad.sIL-4 or Ad.ψ5. Subsequently, exosomes were isolated from DC/IL-4 or DC/ψ5 and injected into the hind paws of KLH-immunized mice. Injection of the genetically modified DC and DC-derived exosomes from wild-type mice significantly suppressed the DTH response, whereas the IL-4-expressing DC and DC/IL-4-derived exosomes from FasL-deficient *gld* mice were significantly reduced in their ability to suppress the DTH response (Fig. 4*A*). Similarly, DC-derived exosomes from both wild-type DC/IL-4 and *gld* DC/IL-4 had reduced suppressive activity in Fas-deficient (*lpr*) recipient mice (Fig. 4*B*). These results suggest that the inhibitory effect of DC/IL-4 and exosomes from DC/IL-4 in the DTH model is, in part, Fas/FasL dependent. However, there was no significant difference in the level of FasL between the suppressive and nonsuppressive DC and DC-derived exosomes, suggesting that although FasL is important, additional factors are required to render the DC/IL-4 and DC/IL-4-derived exosomes immunosuppressive.

Trafficking of DC-derived exosomes

To examine the mechanism of action of DC-derived exosomes in vivo, the trafficking of injected exosomes was examined in mice. DC-derived exosomes were cultured and labeled with the lipophilic dye PKH67. The PKH67-labeled exosomes were injected i.d. into a hind paw or injected i.v. 48 h or 24 h, respectively before tissue isolation. The PKH67-labeled exosomes (green) produced by the Ad-transduced DC were found associated with CD11c$^+$ cells (red) at the site of injection in the dermis (Fig. 5, *A–C*). Similarly, CD11c$^+$ cells with PKH67$^+$ inclusions were detected in the draining (popliteal) lymph node from the treated paw side (Fig. 5, *D* and *E*), but were absent in the contralateral lymph node (Fig. 5*F*). The footpad-injected exosomes were not detected at significant levels in liver or spleen. It is important to note that the results with exosomes from DC/IL-4 were identical to the results observed with exosomes from unmodified DC.

The systemically injected DC/sIL-4- and DCmbIL-4- derived exosomes were mostly captured by the spleen and liver (Fig. 6). The results demonstrate that two populations of splenic phagocytes internalized the PKH67$^+$ exosomes: 1) F4/80$^+$ splenic

macrophages (Fig. 6, *A* and *B)* and 2) F4/80$^-$ cells mostly CD11c$^+$ DC (Fig. 6, *C* and *D*). Blood-borne sIL-4 and mbIL-4 exosomes were also entrapped by hepatic F4/80$^+$ Kupffer cells (Fig. 6, *E* and *F*) and a few CD11c$^+$ DC (Fig. 6, *G* and *H*) in the liver. These results showed that exosomes migrate to and interact with parenchymal CD11c$^+$ DC and F4/80$^+$ macrophages in vivo.

Adoptive transfer of CD11c$^+$ and CD3$^+$ cells from exosome-treated mice suppresses the DTH response

The trafficking results suggest that the exosomes are able to interact with CD11c$^+$ and F4/80$^+$ cells. To determine the functional targets for action of the exosomes, adoptive transfer experiments were performed where CD11c$^+$ and CD3$^+$ cells were isolated from exosome-treated mice and injected into naive, KLH-immunized mice. BM-DC were infected with Ad.IL-4 or Ad.ψ*5* and the resulting exosomes were injected either locally i.d. into the hind paw or systemically i.v. into KLH-immunized mice. Three days after injection, splenic CD11c$^+$ DC and CD3$^+$ T lymphocytes (2×10^5) were isolated and injected into one footpad of KLH-immunized mice before injection of Ag. The local injection into the footpad of the splenic CD11c$^+$ cells (Fig. 5*H*) isolated from DTH mice treated locally with DC/IL-4 and DC/IL-4-derived exosomes (Fig. 5*G*) partially suppressed the DTH response compared with controls. Similarly, injection of CD11c$^+$ (Fig. 6*I*) and CD3$^+$ (Fig. 6*J*) cells from DTH mice treated systemically with DC/IL-4 and Exo/IL-4, but not control DC and exosomes, inhibited the DTH response. Although transfer of CD3$^+$ cells from the DTH mice locally treated with Exo/IL-4 showed reduction in paw swelling, the results did not reach significance (data not shown). Taken together with the trafficking studies, these results suggest that the suppressive exosomes are able to interact with and are internalized by APC in the spleen and lymph nodes, modifying their function, rendering them suppressive. In addition, the vesicles can directly or indirectly modify the function of T cells, possibly by inducing a regulatory subset and/or depleting Ag-reactive Th1 cells.

Discussion

We have demonstrated previously that i.v. injection of DC, genetically modified to express IL-4, reverses established CIA (13). However, the previous studies did not address whether the therapeutic effect was simply due to elevated levels of soluble IL-4 instead of a direct contribution of the Ad.IL-4-modified DC. In this report, we have shown that DC, modified to express either a secreted or membrane-bound form of IL-4, were both therapeutic in murine models of established CIA and DTH. The observation that DC modified to express mbIL-4 are suppressive suggest that the observed therapeutic effects are not mediated solely by high concentrations of sL-4, but are instead mediated by the genetically modified DC.

Interestingly, we also have demonstrated that exosomes produced by DC expressing either sIL-4 or mbIL-4 were able to suppress established CIA and reduce inflammation in the DTH model. The suppression conferred by the exosomes was observed following injection of the amount of exosomes produced by 10^6 DC per 24 h, suggesting that the DC-derived vesicles are highly suppressive. It is important to note that the therapeutic effect was observed with a highly enriched exosome fraction, not with pure exosomes. However, we have demonstrated that the therapeutic efficacy of the enriched exosome fraction was sensitive to freeze-thaw and sonication (data not shown) and could be abrogated by immunodepletion with anti-MHC II Abs. Moreover, the therapeutic effect appears to be MHC dependent as well as Fas/FasL dependent. Taken together, these results strongly suggest that the therapeutic agent is indeed an intact vesicle carrying MHC II and FasL. We also would like to note that there is a slight therapeutic effect in the CIA model when treated with mock DC or mock exosomes, which has been observed in other studies as well (26). This may be due in part to the fact that there was a significant number of still immature (and therefore anti-inflammatory, tolerogenic) BM-DC in the preparation of mock DC.

We also have demonstrated that treatment of BM-DC with higher doses of rIL-4, similar to results with rIL-10 and rTGF-β1, results in exosomes that are able to suppress the DTH response (data not shown). This result further demonstrates that the effect of IL-4 is on the phenotype of the BM-DC and the DC-derived exosomes.

To determine the composition of the immunosuppressive DC/IL-4-derived exosomes, in contrast to exosomes from control DC, we performed flow cytometric analysis on the different exosome fractions. However, we did not detect a difference in the levels of MHC I and II as well as CD86 between the different groups, at least at the sensitivity of the assay. We currently are performing proteomic analysis of the different exosome populations to identify the nature of the component(s) that renders the DC/IL-4-derived exosomes immunosuppressive.

Interestingly, we observed that FasL was present in all of the exosome populations examined. Our results using *gld*, FasL-deficient donor mice and *lpr*, Fas-deficient recipient mice clearly demonstrate the importance of FasL in the DC/IL-4-derived exosomes as well as Fas in the recipient for the therapeutic effect (26). Moreover, FasL also was important for the suppression of the DTH response by parental DC/IL-4. These results suggest that FasL plays a prominent role in conferring the effects of immunosuppressive DC. How the effect of the DC and DC-derived exosomes is conferred through Fas/FasL is unclear because we have not observed extensive T cell apoptosis in draining lymph nodes and spleen by the immunosuppressive DC and DC-derived exosomes (data not shown). Preliminary analysis of the composition of DC-derived exosomes has shown that they also carry other costimulatory molecules, which have been implicated in blocking Ag-specific responses. Thus, although the observed effects of DC-derived exosomes are dependent upon MHC and FasL, it is likely that other factors also play important roles, explaining why DC/IL-4 and DC/IL-4 derived exosomes retained a slight therapeutic effect in the Fas-deficient mouse. In preliminary experiments, using DC and DC-derived exosomes from different knockout strains, we have shown that the suppressive effects of both the DC and exosomes requires CD80 and CD86, but not PD-L1 and PD-L2.

The therapeutic effects of DC/IL-4 and DC/IL-4-derived exosomes is MHC dependent in that allogenic DC and DC-derived exosomes are ineffective. This result is consistent with our previous result demonstrating that the ability of Ad.FasL-transduced DC and the DC/FasL exosomes to suppress the DTH response is through an MHC II-dependent, but MHC I-independent mechanism. The fact that the therapeutic effects of DC and DC-derived exosomes are MHC and FasL dependent suggests that both DC and exosomes can regulate T cell responses, either directly or indirectly. Furthermore, the fact that adoptive transfer of CD3$^+$ splenic T lymphocytes from DC/IL-4 and DC/IL-4-derived exosomes can partially suppress the DTH response suggests that T cell activity is being regulated, possibly through the induction of a regulatory T cell population.

Trafficking analysis of PKH67-labeled exosomes demonstrated that the vesicles are able to interact with and be internalized into CD11c$^+$ and F4/80$^+$ APC at the site of injection in the draining lymph node and in the spleen. These results are consistent with our

previous study demonstrating that allogeneic DC-derived exosomes are internalized by a subset of APC in the spleen following systemic delivery, resulting in modification of function of the target APC (22, 26). Our adoptive transfer experiments of $CD11c^+$ cells from mice treated with DC/IL-4-derived exosomes strongly suggest that exosomes are able to modulate the activity of endogenous APC, rendering the APC immunosuppressive. Whether the effect of the exosomes on endogenous APC is MHC and/or Fas/FasL dependent is currently unclear, but is under investigation. Interestingly, treatment of mice with DC/IL-4 also results in generation of suppressive, endogenous APC similar to the DC/IL-4-derived exosomes. This result suggests that exogenous immunosuppressive DC might modulate the activity of endogenous APC, possibly through a mechanism involving release of exosomes in vivo.

In this report, we have demonstrated the ability of DC-derived exosomes to reverse established arthritis as efficiently as the parental DC. However, unlike DC, which can undergo phenotypic changes following injection making them more stimulatory, exosomes derived from the DC presumably reflect the phenotype of the DC at the time of isolation. Thus, the use of exosomes derived from immunosuppressive DC for treatment of autoimmune diseases such as rheumatoid arthritis may be safer as well as more effective than using modified DC.

Acknowledgments

We thank Joan Nash, Ana Lopez, and Sue Schoonover for technical help.

Disclosures

The authors have no financial conflict of interest.

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