CD169 mediates the capture of exosomes in spleen and lymph node

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Exosomes are lipid nanovesicles released following fusion of the endosome limiting membrane with the plasma membrane; however, their fate in lymphoid organs after their release remains controversial. We determined that sialoadhesin (CD169; Siglec-1) is required for the capture of B cell-derived exosomes via their surface-expressed α2,3-linked sialic acids. Exosome-capturing macrophages were present in the marginal zone of the spleen and in the subcapsular sinus of the lymph node. In vitro assays performed on spleen and lymph node sections confirmed that exosome binding to CD169 was not solely due to preferential fluid flow to these areas. Although the circulation half-life of exosomes in blood of wild-type and CD169−/− mice was similar, exosomes displayed altered distribution in CD169−/− mice, with exosomes freely accessing the outer marginal zone rim of SIGN-R1+ macrophages and F4/80+ red pulp macrophages. In the lymph node, exosomes were not retained in the subcapsular sinus of CD169−/− mice but penetrated deeper into the paracortex. Interestingly, CD169+/− mice demonstrated an enhanced response to antigen-pulsed exosomes. This is the first report of a role for CD169 in the capture of exosomes and its potential to mediate the immune response to exosomal antigen. (Blood. 2014;123(2):208-216)

Key Points

- This study has identified a novel capture mechanism for host-derived vesicles within the spleen and lymph node.
- This pathway modulates the immune response to circulating particulate antigens.

Introduction

Exosomes are vesicles released from multivesicular endosomes following fusion with the plasma membrane. Exosomes are a potential source of self-antigen for modulating the immune response against self-tissues, including tumors.1,2 Several cellular and molecular interactions direct the binding of exosomes to populations of leukocytes or stromal cells.3-10 In lymphoid organs, antigen presenting cells (APCs) in the marginal zone (MZ) of the spleen11 and follicular dendritic cells (DCs) in the B-cell areas of lymph node (LN)12 have been suggested to interact with exosomes, although the presence of a specific exosome receptor has yet to be demonstrated.

Sialic acid binding immunoglobulin lectins (Siglec) are sialic acid binding molecules expressed on a variety of leukocytes and stromal cells. CD169 (Sialoadhesin), the first Siglec family member identified, contains 17 immunoglobulin-like domains with the sialic acid binding site within the V-set terminal immunoglobulin domain. The short cytoplasmic tail of CD169 lacks signal transduction and endocytosis motifs, although recent data have implicated CD169 in endocytosis.13 Sialic acids decorate the surface of all cells and most secreted proteins14; however, due to the low (millimolar) affinity of CD169 for sialic acid, only heavily sialylated, multimeric structures bind strongly to CD169+ macrophages.13 CD169−/− mice do not display overt immune response defects but have depressed immunoglobulin M (IgM) levels and subtle alterations in the proportions of T- and B-cell subsets.15 Interestingly, CD169−/− mice show lower levels of autoreactive T-cell activation in mouse models of multiple sclerosis and uveoretinitis autoimmune mice, likely due to altered regulatory T-cell activity.13,16

CD169 is strongly expressed on the subcapsular sinus (SCS) and medullary macrophages in LN and on marginal metallophilic macrophages in the MZ of the spleen.13,17 CD169+ macrophages sample a wide variety of antigens and participate in generation of immunity to tumors and viruses but may also down-regulate immune responses to self-tissue.13 CD169+ macrophages directly present captured antigen to T cells or natural killer (NK) T cells17 and are adept at transferring antigen to CD8+ DC and B cells.17 LN CD169+ macrophages transfer their own membrane material to closely associated T cells and NK cells.18

Exosomes express carbohydrate modifications, such as complex N-linked glycans, high mannose, poly lactosamine, and sialic acids.19-21 We report that the preferred ligand of CD169, α2,3-linked sialic acid,22 is enriched on B cell–derived exosomes, allowing their capture by CD169+ macrophages in both spleen and LN. In the absence of this pathway, exosome access to the lymphoid system is dysregulated, resulting in aberrant trafficking of exosomes into the splenic red pulp or LN cortex. In addition, CD169−/− mice demonstrate enhanced cytotoxic T-cell responses to exosomal antigen. This suggests that CD169 controls the access of exosomes to lymphoid organs, possibly to minimize immune responses to self-antigen.
Materials and methods

Mice

C57BL/6 (wild-type) mice and ovalbumin peptide-specific OT-I and OT-II transgenic T-cell ovalbumin were obtained from Jackson Laboratories. OT-I and OT-II mice were crossed with CD45.1<sup>+</sup> B6.SJL-Ptprc<sup>−/−</sup>Pepck<sup>−/−</sup>BoyJArc (Animal Resources Centre) to generate F<sub>1</sub>, CD45.1<sup>+</sup> OT-I or OT-II progeny. CD169<sup>−/−</sup> mice<sup>15</sup> were from the University of Dundee. All mice were bred in specific pathogen-free conditions at the University of Otago Hercus Taieri Resource Unit as described.<sup>26</sup> All intravenous injections (100 μL phosphated-buffered saline [PBS]) were in the lateral tail vein. Subcutaneous injections were in the forelimb with 50 μL PBS. Animal studies were approved by the regional Animal Ethics Committee.

Exosome purification and labeling

Exosomes were isolated from anti-CD40 (FGK-45; 5 μg/mL) and interleukin-4 (50 ng/mL; R&D Systems, Auckland, NZ) stimulated C57BL/6 DCs generated from C57BL/6 bone marrow cells as previously described for biotin. Peptide and protein loading was performed using the Bradford assay, and exosome quality was routinely controlled by flow cytometry analysis (BD LSRRoFtessa; FlowJo).

Modified Stamper-Woodruff assay

Frozen naïve C57BL/6 and CD169<sup>−/−</sup> mouse spleen or LN (axillary, brachial, inguinal, and mesenteric) tissue was cryosectioned as previously described.<sup>26</sup> Sections were blocked with 1% BSA/PBS for 10 minutes, and 50 μg/mL Exo-bio (±sialidase treatment) diluted in 0.1% BSA/Isocrow’s modified Dulbecco’s media (Gibco #12440-053) was added for 2 hours at 37°C in a humid box. Sections were rinsed with PBS, fixed with 1% paraformaldehyde/PBS, quenched with 100 mM glycine/PBS, and blocked with 1% goat serum (GS)/PBS each for 10 minutes. Sections were incubated for 1 hour with primary antibodies from cell supernatant prepared from MOMA-1 (anti-CD169), F4/80, and ER-TR9 (anti-SIGN-R1) hybridomas obtained from Professor Georg Kraal (VU Medical Centre, Amsterdam, Netherlands). Primary antibodies were detected with 10 μg/mL anti-rat IgG-Alexa-488 or -Alexa-594 (Invitrogen #A21208 and Invitrogen #A21209, respectively) in 1% GS/PBS. Biotin was detected with 5 μg/mL streptavidin-Alexa-488 or -Alexa-594 (Invitrogen #S11223 and Invitrogen #S11227, respectively), and nuclei were counterstained with 25 ng/mL 4',6-diamidino-2-phenylindole (DAPI; Invitrogen #D3571). Sections were mounted in Prolong Gold anti-fade (Invitrogen #P36930). For blocking experiments, sections were first incubated with 10 μg/mL neutralizing antibody anti-CD169 (clone SER-4) or negative control antibody anti-interleukin-4 (clone 11B11; purified in house) diluted in 1% BSA/PBS for 1 hour. Biotinylated exosomes (50 μg/mL) were then added directly to sections (without removal of blocking antibody) for 2 hours at 37°C. Sections were viewed with an Olympus BX-51 upright fluorescence microscope with UPLAN FL lenses (FN26.5) with identical exposure conditions and images analyzed using DP Manager software.

Fluorescence microscopy of in vivo captured exosomes and beads

C57BL/6 or CD169<sup>−/−</sup> mice were injected intravenously or subcutaneously with 100 or 50 μg of Exo-bio, respectively, and 2 × 10<sup>10</sup> or 1 × 10<sup>11</sup> 100 nm fluorescence microspheres (ex-488 nm; Polysciences #24061), respectively. For intravenous and subcutaneous routes, respectively, mice were killed at 5, 60, or 120 minutes and spleen and liver were harvested or at 30 minutes and draining LN (axillary and brachial) was harvested. Organs were cryosectioned, fixed, and quenched before being blocked with 1% GS/PBS and labeled for CD169, F4/80, or SIGN-R1. Exosomes were detected as detailed above.

Colocalization analysis

Colocalization between exosome signal (red, Alexa-594) or 100 nm microspheres (green, Alexa-488) and macrophage marker signal green (Alexa-488; red, Alexa-594) was determined from intravenously injected C57BL/6 or CD169<sup>−/−</sup> mice killed at 5 minutes. Colocalization was calculated from 10 individual photos (×20 objective lens) per mouse, using the Manders’ coefficient (fraction of exosome or bead signal overlapping with macrophage signal) with the software ImageJ and JACoP plugin using autostretching.<sup>27</sup>

In vivo T-cell proliferation

CD45.1<sup>+</sup> splenocytes and LN cells (5 × 10<sup>7</sup> cells/mL) from OT-I or OT-II mice were labeled with differing dyes; 2.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen #C34554) or 2.5 μM cell proliferation dye (CPD) V450 (BD #562158) for 7 minutes at 37°C and quenched with 5 mM FCS. Cells were washed once in 10% FCS/PBS and twice in PBS. Labeled cells were intravenously injected into recipient mice (Molecular Probes #A8244A); BSA-conjugated microspheres were used as a negative control. Exosomes were analyzed for α<sub>2</sub>,3- and α<sub>2</sub>,6-linked sialic acid expression with biotinylated lectins 5 μg/mL MAL-II (Vector #B-1265) or 0.6 μg/mL SNA-I (Vector #B-1305), or for CD9, CD19, CD21, CD24, MHC, and immunoglobulin expression (see supplemental Methods) before flow cytometric analysis (BD LSRFortessa; FlowJo).
immunization with 10^7 OT-I splenocytes. Seven days after immunization, targets were stated, mice were adoptively transferred intravenously 1 day prior to and mice were immunized intravenously with equivalent volumes. Where mice were unpulsed (R10) or pulsed with 100 ng of biotinylated B cell–derived exosomes (Exo-bio) purified by ultracentrifugation, respectively. As a control, the pellet (OT-I and OT-II cells; 10^7 each were pooled and cotransferred). For LN or splenic responses, C57BL/6 or CD169^+^ mice were intravenously or intravenously with PBS, 50 or 100 µg sucrose cushion purified Exo257/323, respectively, 50 µg sucrose cushion purified Exo-pro, or 10^5 peptide- or protein-pulsed B cells or DCs. As a control, the pellet from Exo257/323, respectively, 50 µg sucrose cushion purified Exo-pro, or 10^5 peptide- or protein-pulsed B cells or DCs. As a control, the pellet from Exo-pro sucrose cushion purification was washed and resuspended identically to Exo257/323 fractions, and mice were immunized subcutaneously with equivalent volumes. Five days later, draining LN or spleen was harvested, and T cells were labeled for CD4, CD8, and CD45.1 and analyzed by flow cytometry (supplemental Methods).

### In vivo cytotoxicity assays

C57BL/6 or CD169^+^ mice were anesthetized with ketamine and medetomidine as previously described and maintained on a warming tray at 37°C. Biotinylated B cell–derived exosomes (100 µg exosomal protein/mouse) were intravenously injected. Total mouse blood volume was estimated to be 5.5% of body weight. Blood was removed from the opposite lateral tail vein to that of exosome administration. MHC-II^+^ exosomes were detected by enzyme-linked immunosorbent assay. In brief, Nunc Maxisorp plates were coated with 1 µg/mL streptavidin (Jackson #016-000-084) in PBS overnight at 4°C. Plates were washed with 0.02% Tween-20/PBS and blocked for 10 minutes at 20°C with 0.1% caseinate (Arotech)/PBS. Exosome concentration in plasma was determined using a standard of biotinylated exosomes of known protein concentration. EDTA plasma samples were diluted 1/20 with PBS and added overnight at 4°C. After washing, 1 µg/mL fluorescence isothiocyanate (FITC)-MS5/114 (anti-mouse I-Ab,a/I-Ed,k; purified and FITC-conjugated in-house) was added for 1 hour at 37°C to detect bound MHC-II^+^ exosomes. Plates were analyzed for colocalization of green (Alexa-488) and red (Alexa-594) signal using the Manders’ coefficient with ImageJ. Each point represents an individual photograph; line indicates mean. Circles, exosomes; squares, beads; closed symbols, C57BL/6 mice; open symbols, CD169^+^ mice.

Figure 1. Aberrant distribution of exosomes in lymphoid tissues of CD169^+^ in vivo. C57BL/6 or CD169^+^ mice were (A [spleen],B,C,F) intravenously (IV) or (A [LN],E) subcutaneously (SC) injected in the forelimb with 100 or 50 µg of biotinylated B cell–derived exosomes (Exo-bio; purified by ultracentrifugation), respectively. (D,F,G) Alternatively, C57BL/6 or CD169^+^ mice were IV or SC injected with 2 × 10^11 or 1 × 10^10 100 nm fluorescent microspheres, respectively (green). For IV or SC routes, mice were killed at 5 minutes with spleens and livers harvested or at 30 min and draining LN harvested, respectively. (A-C,E) Exo-bio was detected with streptavidin-Alexa-594 (red) and nuclei were counterstained with DAPI (blue). Original magnification, (A [LN],D [spleen],E) 250, and (C) 400. Bar represents (A [spleen],B,D [LN],G) 200 nm. All results are representative of ≤4 mice per group. (F) Percent colocalization was calculated from fluorescent microscopy photos of (A [spleen],B,G) spleen sections. Ten individual photographs per mouse (original magnification, ×200) were analyzed for colocalization of green (Alexa-488) and red (Alexa-594) signal using the Manders’ coefficient with ImageJ. Each point represents an individual photograph; line indicates mean. Circles, exosomes; squares, beads; closed symbols, C57BL/6 mice; open symbols, CD169^+^ mice. One-way ANOVA with Bonferroni postcorrection test was performed: ns, not significant; *P < .05; **P < .01; ****P < .0001.
washed and then incubated with anti-FITC-horseradish peroxidase (1/5000; Roche #1426346) for 1 hour at 37°C. After washing, the plate was developed in tetramethyl benzidine (Zymed #00-2023), and the reaction was developed in tetramethyl benzidine (Zymed #00-2023), and the reaction was stopped with 2 N H₂SO₄. Optical density at 450 nm was determined using a Tecan Infinite M200 microplate reader.

Statistical analysis
All statistical analyses were performed with GraphPad Prism 6 by 1-way (or 2-way in Figure 3) ANOVA with a Bonferroni postcorrection test.

Results
Identification of spleen, liver, and LN as the main exosome targets
To determine the in vivo target of exosomes, primary B cell–derived exosomes were isolated. Quality controls demonstrated effective enrichment of MHC-II⁺ exosomes using ultracentrifugation alone (supplemental Figure 1). Vesicles were then biotinylated and injected intravenously or subcutaneously into mice. Results showed distinct MZ or SCS distribution of exosomes in the spleen or LN, respectively (Figure 1). Further analysis showed a distinct colocalization of exosome and CD169 labeling (Figure 1A), suggesting a role for CD169⁺ macrophages in exosome capture. To investigate the potential for CD169⁺ macrophage-mediated binding of exosomes, we used CD169⁻/⁻ mice. Compared with B6 mice, CD169⁻/⁻ mice exhibited altered distribution of intravenously transferred exosomes, with exosomes penetrating the splenic red pulp and outer MZ sinus, the signal overlapping with SIGN-R1⁺ and F4/80⁺ macrophages (Figure 1B). To a lesser extent, liver macrophages (Kupffer cells) also bound intravenously transferred exosomes (Figure 1C). Kupffer cells also express CD169, but at levels severalfold below that of splenic or LN macrophages. Interestingly, binding of exosomes to Kupffer cells was not altered in CD169⁻/⁻ mice (Figure 1C).

Although CD169⁻/⁻ mice retain populations of marginal metallophilic macrophages in the MZ of the spleen and SCS macrophages in LN, it is possible that these macrophages display altered barrier properties to particulate antigens. However, intravenous or subcutaneous administration of inert 100 nm microspheres into wild-type and CD169⁻/⁻ mice demonstrated no strain difference in distribution of microspheres into the splenic MZ or SCS of the LN, respectively (Figure 1D), suggesting no loss of MZ or SCS barrier function in CD169⁻/⁻ mice.

Subcutaneous administration of exosomes resulted in localization of exosomes to CD169⁺ macrophages within the SCS (Figure 1E). However, in CD169⁻/⁻ mice, exosome binding to SCS macrophages was reduced, and exosomes penetrated deeper areas of the LN cortex (Figure 1E). Exosome binding to splenic CD169⁺ macrophages in wild-type mice was significantly greater than to
other macrophage subsets (Figure 1F). In contrast, inert 100 nm beads preferentially colocalized with SIGN-R1 + macrophages (Figure 1F; supplemental Figure 1). In the absence of CD169, exosomes showed greater access to SIGN-R1 + and F4/80 + macrophages (Figure 1B,F).

**Exosome binding to splenic and lymph node macrophages in vitro**

It could be argued that colocalization of exosomes to CD169 + macrophages was simply due to anatomical constraints, because the primary site of fluid entry into the spleen and LN is the MZ and SCS, respectively. We developed an in vitro assay (modified Stamper-Woodruff assay25; Figure 2A-B) using Exo-bio (Figure 2C) applied to tissue sections to determine if specific exosome receptors were present in the spleen and LN. Interestingly, the distribution pattern of exosome binding matched that of exosome capture in vivo (cf. Figure 1A with Figure 2A-B). These results clearly demonstrated that exosomes bound CD169 + macrophages in both wild-type spleen and LN but not CD169 −/− mice (Figure 2B,D). BSA-biotin failed to bind to tissue sections, ruling out nonspecific binding effects due to amine-linked biotinylation (supplemental Figure 3). In addition, labeling of exosomes with an alternate fluorochrome confirmed these results (supplemental Figure 3), and cold (nonbiotinylated) exosomes effectively inhibited exosome binding to tissue sections (supplemental Figure 3). Blocking experiments with an anti-CD169 neutralizing antibody (SER-4)31 further confirmed that binding of both B cell–derived (Figure 2D) and DC-derived (supplemental Figure 4) exosomes was CD169 dependent.

Exosomes were shown to display high levels of α2,3-linked sialic acids—the preferred ligand of CD169 (Figure 2E)29,32. Sialidase-treated exosomes (Figure 2C) failed to bind the SCS or splenic MZ (Figure 2D), further confirming that exosome binding was CD169 dependent. Sialidase treatment did not alter exosome buoyant density on the sucrose cushion (supplemental Figure 1), exosome morphology (Figure 2C), α2,6-linked sialic acid levels (Figure 2E), or the detection of surface marker expression. Note, sialidase treatment caused a modest increase in antibody binding to the CD19 and CD24 glycoproteins (Figure 2E).

**Plasma clearance rates of exosomes in wild-type and CD169 −/− mice**

Surprisingly, despite our observation of aberrant exosome trafficking in lymphoid organs of CD169 −/− mice, MHC-II + exosomes were cleared from the blood of wild-type and CD169 −/− mice at similar rates, with a half-life of ~2 minutes (Figure 3A). Interestingly, after 120 minutes, exosomes were still detectable in the spleen, indicating that longer-lived reservoirs of exosomes may persist after the majority of exosomes have been cleared from circulation (Figure 3B).

**Enhanced responses of CD169 −/− mice to protein and peptide-loaded exosomes**

Because B cell–derived exosomes express both MHC-I and MHC-II,24 we next compared their ability to induce an immune response in wild-type and CD169 −/− mice. Interestingly, although protein-pulsed exosomes induced both CD4 + and CD8 + T-cell proliferation, peptide-pulsed exosomes did not reproducibly induce CD4 + T-cell proliferation (Figures 4 and 5; supplemental Figures 5 and 6). No differences in OT-I or OT-II proliferation were noted between wild-type and CD169 −/− mice by either the intravenous or subcutaneous route for both peptide and protein (Figures 4 and 5; supplemental Figures 5 and 6). Surprisingly, peptide- or protein-pulsed parental B cells did not, or only weakly induced, CD4 + or CD8 + T-cell proliferation, or cytotoxicity, by both subcutaneous and intravenous routes (Figures 4-7).

Consistent with a recent report on DC-derived exosomes,10 peptide-pulsed exosomes induced relatively weak cytotoxic T-lymphocyte (CTL) responses. This response was not enhanced by inclusion of the T-cell helper epitope peptide OVA323-339 (Figure 6). However, supplementation of naïve OT-I T cells resulted in a significantly enhanced CTL response to Exo257, demonstrating that increasing CTL precursor levels significantly enhanced the cytotoxic response induced by peptide-pulsed exosomes. Interestingly, the level of cytotoxicity was dependent on antigen type, with significantly higher levels of cytotoxicity noted in mice immunized with protein-pulsed compared with peptide-pulsed exosomes (Figures 6 and 7; P < .0001 for both B6 or CD169 −/− mouse strains). Control experiments demonstrated that particulate antigenic material (either protein or peptide) was not responsible for the observed immune responses (Figure 7; supplemental Figure 5). Strikingly, compared with wild-type mice, CD169 −/− mice demonstrated significantly enhanced CTL responses to peptide-pulsed (intravenous only; Figure 6) and protein-pulsed exosomes (intravenous and subcutaneous; Figure 7). This difference was maintained using differing amounts (50 and 100 μg) of protein-pulsed exosomes via intravenous immunization (Figure 7). A small but significant increase in the CTL response of CD169 −/− mice to subcutaneously injected peptide-pulsed, but not protein pulsed, DCs was also noted (Figure 6B).
Discussion

We identified CD169 as a specific binding-partner for exosomes in lymphoid tissue. Its restriction to subpopulations of macrophages dedicated to antigen capture and direct presentation and/or transfer of antigen to other APCs \(^\text{17}\) suggests an important role of CD169-mediated capture in the immune response to exosomal antigen.

CD169\(^+\) macrophages are strategically situated at antigen entry points into the spleen and LN and are involved in capture of sialylated pathogens \(^\text{13,30}\); however, a role for CD169 in capturing exosomes has not previously been demonstrated.

Despite the low affinity (\(K_d \sim 1.4 \text{ mM}\)) of CD169 for \(\alpha^2 \text{3-linked sialic acid}\), \(^\text{33}\) we observed abundant binding of exosomes to this receptor. This may be a result of high level expression of \(\alpha^2 \text{3-linked sialic acid}\) expression on exosomes, as linear increases in CD169 ligand availability results in logarithmic increases in avidity. \(^\text{13}\) Moreover, due to their size, exosomes may suffer less shear stress, therefore gaining greater access to receptors, compared with larger cells or particles.

In the spleen, McGaha et al noted a similar role for MZ macrophages in the exclusion of particulate antigen from the red pulp following depletion of MZ macrophages by clodronate. \(^\text{34}\) Thus, the MZ macrophage barrier acts together with the MZ MADCAM-1\(^+\) sinus-lining cells to limit antigen entry into the red pulp. \(^\text{35}\) Such partitioning of antigen may optimize antigen availability for the initiation of appropriate immune responses at the MZ and white pulp. \(^\text{35}\) Interestingly, McGaha et al observed enhanced immune responses to antigen associated with apoptotic cells in mice depleted of MZ macrophages. \(^\text{34}\) It has previously been demonstrated that exosomes entering the venous circulation localize to the splenic MZ. \(^\text{11}\) In addition, there are several reports of MZ macrophages being involved in immune tolerance to apoptotic cells, either through modulation of cytokine secretion or by the induction of the immunosuppressive enzyme indoleamine 2,3-dioxygenase. \(^\text{34,36,37}\)

DC-derived exosomes abundantly express the ligand milk-fat globule lactadherin, a potential ligand for the \(\alpha_\beta_3/\beta_5\) integrins. \(^\text{38}\) Others have proposed that a number of molecules, including CD9, CD11a, CD81, CD91, intercellular adhesion molecule-1, and phosphatidyl serine, are involved in exosome capture by DCs. \(^\text{3,6,8,11}\) Stromal interactions of exosomes to collagen or fibronectin may be facilitated in humans by the \(\beta_1\) and \(\beta_2\) interactions. \(^\text{4,7}\) Additionally, C3b deposition on exosomes enhances antigen presentation and splenic uptake. \(^\text{10,39}\) There is 1 description of the rat-restricted Galectin 5 mediating the binding of erythrocyte exosomes to macrophages, \(^\text{40}\) but to our knowledge, ours is the first report of a macrophage-specific exosome receptor expressed in lymphoid tissue.

Similar exosome clearance rates were noted in the circulation of wild-type and CD169\(^{-/-}\) mice, suggesting that CD169-independent exosome clearance mechanisms are active in CD169\(^{-/-}\) mice. Removal of exosomes by complement-mediated destruction and uptake by phagocytes, \(^\text{10,39}\) or uptake by fenestrated endothelium in the liver sieve, \(^\text{41}\) may mask the contribution of a selectively expressed receptor present only in a subpopulation of LN and splenic macrophages. In contrast to our findings, liposomes displaying high-affinity glycan ligands for CD169 exhibited delayed clearance from circulation in CD169\(^{-/-}\) mice. \(^\text{42}\) However, the combination of multivalent, high-affinity glycan ligands expressed on these liposomes together with enhanced stealth properties due to polyethylene glycolylation may mask the contribution of a selectively expressed receptor present only in a subpopulation of LN and splenic macrophages. In contrast to our findings, liposomes displaying high-affinity glycan ligands for CD169 exhibited delayed clearance from circulation in CD169\(^{-/-}\) mice. \(^\text{42}\) However, the combination of multivalent, high-affinity glycan ligands expressed on these liposomes together with enhanced stealth properties due to polyethylene glycolylation may mask the contribution of a selectively expressed receptor present only in a subpopulation of LN and splenic macrophages.

Figure 4. In vivo T-cell proliferation in response to exosomal-peptide antigen. C57BL/6 or CD169\(^{-/-}\) mice were immunized IV or SC in the forelimb with (A) PBS, 100 \(\mu\)g sucrose cushion purified Exo\(_{257/323}\), and \(10^7\) DC\(_{257/323}\) or (B) \(10^5\) parental B cell 257/323. Exosomes and cells were all pulsed simultaneously with 1 \(\mu\)M ovalbumin peptides OVA257-264 and OVA323-339. T-cell proliferation of adoptively cotransferred OT-I (CD8) and OT-II (CD4) cells (CFSE or CPD V450) were analyzed 5 days after immunization by flow cytometry. Black line, test group; shaded peak, PBS-immunized mice. Results representative of \(\approx 6\) mice per group.
glycol modification may explain this difference. A key distinction between exosomes and glycan-expressing liposomes is that the latter does not present any ligands for host receptors, apart from sialic acid. In contrast, exosomes possess many potential host ligands including adhesion molecules and complement receptors. As this is the first report on exosome circulation clearance rates, we are unable to compare our findings. However, similar rapid rates of reticulocyte microvesicle uptake and destruction were reported by Willekens et al.

Interestingly, protein- but not peptide-pulsed exosomes induced strong endogenous CTL responses in agreement with another study. This enhancement was reportedly due to cooperation of helper T cells with antigen-specific B cells. This is an important finding as it distinguishes exosomes from cellular APCs namely, DCs, which have been reported to be 50-fold more efficient at presenting OVA peptides compared with equimolar concentrations of whole OVA. Given the inherent inefficiency of processing proteins for cross-priming, it is surprising that protein immunization resulted in high-level CTL activity by exosomes.

Our results are the first report of exosome capture by CD169 and of enhanced cytotoxicity to protein-pulsed exosomes in CD169−/− mice. Similarly, it was shown that CD169 macrophages promote an inhibitory effect in response to apoptotic cells, although CD169 may alternatively promote autoimmunity and inflammatory responses to sialylated pathogens. It is possible that the outcome of antigen targeted to CD169 macrophages is

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**Figure 5. T-cell proliferation in response to exosomal-protein antigen.** C57BL/6 or CD169−/− mice were immunized IV or SC in the forelimb with (A) PBS, 50 μg sucrose cushion purified exosomes derived from B cells cultured with 200 μg/mL ovalbumin protein for 2 days (Exo-pro), and 10⁶ DC-pro or (B) 10⁵ parental B cell-pro. DC and B cells were cultured with 200 μg/mL ovalbumin protein for 2 days. T-cell proliferation of adoptively cotransferred OT-I (CD8) and OT-II (CD4) cells (CFSE or CPD V450) were analyzed 5 days after immunization by flow cytometry. Black line, test group; shaded peak, PBS-immunized mice. Results representative of n=6 mice per group.

**Figure 6. Enhanced cytotoxic responses to intravenous exosomal-peptide in CD169−/− mice.** C57BL/6 or CD169−/− mice were immunized (A) IV or (B) SC with PBS and 100 or 50 μg Exo257, respectively, 100 μg Exo257/323 (IV), 10⁵ DC257, or 10⁵ parental B cell257. Where stated, mice were supplemented IV with 10⁷ OT-I splenocytes prior to immunization. Seven days after immunization, mice were adoptively transferred with unpulsed (CFSE low) or OVA257-264-pulsed (CFSE high) target cells. In vivo killing was analyzed 18 hours later by flow cytometry. Results representative of n=6 mice per group using exosomes purified by ultracentrifugation. Line, mean. One-way ANOVA with Bonferroni postcorrection was performed: ns, not significant; **P < .01; ****P < .0001.
greatly influenced by the antigenic context, such as the presence of microbial products or inflammation.\(^1\)\(^3\)\(^1\)\(^7\) Our current focus is the role of APC subsets mediating the enhanced immune response observed in CD169\(^{\sim}\) mice.

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**References**


**Authorship**

Contribution: S.C.S. performed the research presented, analyzed the data, prepared the figures, and aided in design of the study and manuscript preparation; A.C.D. provided technical assistance and aided in manuscript preparation; P.R.C. provided antibodies and the CD169\(^{\sim}\) mice; and A.D.M. designed the study, assisted with laboratory work, and wrote the manuscript.

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CD169 mediates the capture of exosomes in spleen and lymph node

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