CD169 mediates the capture of exosomes in spleen and lymph node

Running title: Exosome capture by CD169

Sarah C. Saunderson1,3, Amy C. Dunn1, Paul R. Crocker2 and Alexander D. McLellan1,3

1Department of Microbiology & Immunology, Otago School of Medical Sciences, University of Otago, P.O. Box 56, Dunedin, Otago, New Zealand. 3Corresponding author, TEL +64 3 34797728, FAX +64 3 34798540, E-mail: alex.mclellan@otago.ac.nz or sarah.saunderson@otago.ac.nz.

2College of Life Sciences, University of Dundee, Dundee, Scotland.
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 antigen

Abstract

Exosomes are lipid nanovesicles released following fusion of the endosomal limiting membrane with the plasma membrane, however their fate in lymphoid organs following their release remains controversial. We have 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<sup>−/−</sup> mice was similar, exosomes displayed altered distribution in CD169<sup>−/−</sup> mice, with exosomes freely accessing the outer marginal zone rim of SIGN-R1<sup>+</sup> macrophages and F4/80<sup>+</sup> red pulp macrophages. In the lymph node, exosomes were not retained in the subcapsular sinus of CD169<sup>−/−</sup> mice, but penetrated deeper into the paracortex. Interestingly, CD169<sup>−/−</sup> 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.
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.\textsuperscript{1,2} Several cellular and molecular interactions direct the binding of exosomes to populations of leukocytes or stromal cells.\textsuperscript{3-10} In lymphoid organs, antigen presenting cells (APC) in the marginal zone (MZ) of the spleen\textsuperscript{11} and follicular dendritic cells (DC) in the B cell areas of lymph node (LN)\textsuperscript{12} have been suggested to interact with exosomes, though the presence of a specific exosome receptor has yet to be demonstrated.

Sialic acid binding immunoglobulin (Ig) 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 Ig-like domains with the sialic acid binding site within the V-set terminal Ig-domain. The short cytoplasmic tail of CD169 lacks signal transduction and endocytosis motifs, although recent data have implicated CD169 in endocytosis.\textsuperscript{13} Sialic acids decorate the surface of all cells and most secreted proteins,\textsuperscript{14} however due to the low (millimolar) affinity of CD169 for sialic acid, only heavily sialylated, multimeric structures bind strongly to CD169\textsuperscript{+} macrophages.\textsuperscript{13} CD169\textsuperscript{+/-} mice do not display overt immune response defects, but have depressed IgM levels and subtle alterations in the proportions of T and B cell subsets.\textsuperscript{15} Interestingly, CD169\textsuperscript{--} 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.\textsuperscript{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.\textsuperscript{13,17} CD169\textsuperscript{+} macrophages sample a wide variety of antigens and participate in generation of immunity to tumors and viruses, but may also downregulate immune responses to self-tissue.\textsuperscript{13} CD169\textsuperscript{+} macrophages directly present captured antigen to T cells or natural killer (NK) T cells\textsuperscript{17} and are adept at transferring antigen to
CD8α+ DC and B cells. LN CD169+ macrophages transfer their own membrane material to closely associated T cells and NK cells.

Exosomes express carbohydrate modifications, such as complex N-linked glycans, high mannose, polylactosamine, and sialic acids. We report that the preferred ligand of CD169, α2,3-linked sialic acid, 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 (USA). OT-I and OT-II mice were crossed with CD45.1+ B6.SJL-Ptprc<sup>Pep3b</sup>/BoyJArc (obtained from Animal Resources Centre, Australia) to generate F1 CD45.1+ OT-I or OT-II progeny. CD169− mice were from the University of Dundee (UK). All mice were bred in SPF conditions at the University of Otago Hercus Taieri Resource Unit as described. All intravenous injections (100 µl 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 IL-4 (50 ng/mL; R&D Systems, Auckland, NZ) stimulated C57BL/6 splenocytes cultured at 2 × 10<sup>6</sup> / mL for three days in R10
(RPMI-1640; Gibco #31800-022) supplemented with 10% vesicle-depleted fetal calf serum (FCS; PAA Laboratories, Austria), 100 U/mL penicillin (Gibco #15140-122), 100 µg/mL streptomycin (Gibco #15140-122), 55 µM β-mercaptoethanol (Gibco #21985-023) and 2 µg/mL NaHCO₃.²⁴ In brief, culture supernatant was centrifuged at 450 ×g for five minutes, then 2000 ×g for twenty minutes (4°C) to deplete cells and debris respectively. The supernatant was 0.2 µm filtered and exosomes pelleted by ultracentrifugation at 120000 ×g for one hour at 4°C. Pellets were washed twice in PBS. Where stated, exosomes were resuspended in 12 mL of PBS, overlaid onto 4 mL of 30% sucrose/200 mM Tris/D₂O cushion and ultracentrifuged at 100000 ×g for 75 minutes at 4°C. Exosomes were located 1 mL above to 2 mL below the interface; these fractions were pooled (see supplemental Figure 1). Pellets were resuspended in the final 0.5 mL. Sucrose was removed by washing twice in PBS by ultracentrifugation. Protein content of exosome preparations was performed using the Bradford assay and exosome quality routinely controlled by flow cytometry and electron microscopy.²⁴ For in vivo capture experiments and modified Stamper-Woodruff²⁵ assays, exosomes were biotinylated (‘Exo-bio’) for ten minutes at 4°C using 1 mg/mL sulfo-NHS-LC-biotin (Pierce #21335) in Phosphate Buffered Saline (PBS; Gibco #21600-010), quenched with 100 mM glycine/PBS (pH 7.4) and washed twice with 30 mL PBS by ultracentrifugation. Alternatively, where stated exosomes were labeled with 1 mg/mL sulfo-NHS-LC-Fluorescein (Pierce #46410) following the same procedure as described for biotin.

**Peptide and protein loading**

Dendritic cells were generated from C57BL/6 bone marrow cells as previously described,²⁶ matured overnight at day six with 200 ng/mL lipopolysaccharide (Salmonella Typhimurium; Sigma #L6511) and harvested on day seven. For peptide experiments, pelleted exosomes, parental B cells or DC were pulsed for four hours at 37°C, with 1 µM ovalbumin (OVA) peptides OVA257-264 and/or OVA323-339 (Genscript, USA; Exo257/323, B cell257/323 and DC257/323 respectively). Alternatively, B cells or DC were incubated for two days with 200 µg/mL ovalbumin protein (Sigma #A5503).
cell derived exosomes (Exo-pro) were then isolated from ovalbumin-pulsed B cell supernatants. Exosomes were washed twice in 30 mL PBS by ultracentrifugation. Exo257/323 and Exo-pro were subsequently sucrose-cushion purified. Peptide- and protein-pulsed B cells and DC were washed twice in PBS.

**Sialidase treatment of exosomes**

Biotinylated exosomes were treated with 0.1 U/mL *Vibrio cholerae*-derived sialidase (SIAL-V; Roche #11-080-725-001) in digestion buffer (14.7 mg CaCl2-dihydrate, 580 mg NaCl to 100 mL 0.1 M Na-acetate, pH 5.5) for 30 min at 37°C. Exosomes were washed twice in 0.1% bovine serum albumin (BSA; Gibco #30063-572)/PBS by ultracentrifugation. Where stated, exosomes were sucrose-cushion purified. Untreated or SIAL-V treated exosomes were bound to 4 µm aldehyde-sulfate microspheres24 (Molecular Probes #A8244A); BSA-conjugated microspheres were used as a negative control. Exosomes were analyzed for α2,3- and α2,6-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-II and immunoglobulin expression (see Supplemental Methods) before flow cytometric analysis (BD LSRFortessa; FlowJo).

**Modified Stamper-Woodruff Assay**

Frozen naïve C57BL/6 and CD169− mouse spleen or LN (axillary, brachial, inguinal and mesenteric) tissue was cryosectioned as previously described.26 Sections were blocked with 1% BSA/PBS for ten minutes and 50 µg/mL Exo-bio (± sialidase treatment) diluted in 0.1% BSA/IMDM (Iscove’s Modified Dulbecco’s Media; Gibco #12440-053) added for two 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 ten minutes. Sections were incubated for one hour with primary antibodies from cell supernatant prepared from MOMA-1 (anti-CD169), F4/80 and ER-TR9 (anti-SIGN-R1) hybridomas obtained
from Prof. 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; Invitrogen #A21209) in 1% GS/PBS. Biotin was detected with 5 µg/mL streptavidin-Alexa-488 or -Alexa-594 (Invitrogen #S11223; Invitrogen #S11227) and nuclei counterstained with 25 ng/mL DAPI (4’,6-diamidino-2-phenylindole; 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-IL-4 (clone 11B11; purified in house) diluted in 1% BSA/PBS for one hour. Biotinylated exosomes (50 µg/mL) were then added directly to sections (without removal of blocking antibody) for two hours at 37°C. Sections were viewed with an Olympus BX-51 upright fluorescent 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>11</sup> or 1 × 10<sup>11</sup> 100 nm fluorescent microspheres (ex-488 nm; Polysciences #24061) respectively. For intravenous and subcutaneous routes respectively, mice were sacrificed at 5, 60 or 120 min and spleen and liver harvested, or at 30 min and draining LN (axillary and brachial) harvested. Organs were cryosectioned, fixed and quenched, before being blocked with 1% GS/PBS, labeled for CD169, F4/80 or SIGN-R1. Exosomes were detected as detailed above.

**Co-localization analysis**

Co-localization between exosome signal (red; Alexa-594) or 100 nm microspheres (green; ex-488 nm) and macrophage marker signal (green; Alexa-488 or red; Alexa-594 respectively) was determined from intravenously injected C57BL/6 or CD169<sup>-/-</sup> mice sacrificed at five minutes. Co-
localization was calculated from ten individual photos (×20 objective lens) per mouse, using the Manders’ co-efficient (fraction of exosome or bead signal overlapping with macrophage signal) with the software ImageJ and JaCoP plugin using auto-thresholding.27

**In vivo T cell proliferation**

CD45.1+ splenocytes and LN cells (5 × 10⁷ cells/mL) from OT-I or OT-II mice were labeled with differing dyes; 2.5 µM CFSE (carboxyfluorescein diacetate succinimidy ester; Invitrogen #C34554) or 2.5 µM cell proliferation dye (CPD) V450 (BD #562158) for seven minutes at 20°C and quenched with 5 mL FCS. Cells were washed once in 10% FCS/PBS and twice in PBS. Labeled cells were intravenously injected into recipient mice (OT-I and OT-II cells; 10⁷ each, were pooled and co-transferred). For LN or splenic responses C57BL/6 or CD169⁻ mice were immunized subcutaneously or intravenously with PBS, 50 or 100 µg sucrose-cushion purified Exo257/323 respectively, 50 µg sucrose-cushion purified Exo-pro, or 10⁵ peptide- or protein-pulsed B cells or DC. As a control, the pellet from Exo257/323 sucrose-cushion purification was washed and resuspended identically to Exo257/323 fractions and mice immunized subcutaneously with equivalent volumes. Five days later, draining LN or spleen were removed and T cells labeled for CD4, CD8, and CD45.1 and analyzed by flow cytometry (see supplemental Methods).

**In vivo cytotoxicity assays**

C57BL/6 or CD169⁻ mice were immunized intravenously or subcutaneously with PBS, Exo257 or Exo-pro (50 µg sucrose-cushion purified, or 100 µg by ultracentrifugation only), 10⁵ peptide or protein pulsed B cells or DC. As a control, the pellet from Exo-pro sucrose-cushion purification was washed and resuspended identically to Exo-pro fractions and mice immunized intravenously with equivalent volumes. Where stated, mice were adoptively transferred intravenously one day prior to immunization with 10⁷ OT-I splenocytes. Seven days post-immunization, targets were prepared as follows; naïve C57BL/6 splenocytes (2 × 10⁷ cells/mL) were unpulsed (R10), or pulsed with 1 µM
OVA\textsubscript{257} in R10 for one hour at 37°C, then washed in 0.1% BSA/PBS/2 mM EDTA. Unpulsed or pulsed cells were stained with 0.2 or 2 µM CFSE respectively, for seven minutes at 20°C, quenched and washed as described above. Equal numbers of unpulsed and pulsed targets were pooled (1.5 × 10\textsuperscript{7} total) for intravenous injection. Mice were sacrificed 18 hours later and spleens analyzed by flow cytometry.

**Kinetics of exosome clearance from blood**

C57BL/6 and CD169\textsuperscript{-/-} mice were anesthetized with ketamine and medetomidine as previously described\textsuperscript{23} 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\textsuperscript{+} exosomes were detected by ELISA, in brief Nunc Maxisorp plates were coated with 1 µg/mL purified streptavidin (Jackson #016-000-084) in PBS overnight at 4°C. Plates were washed with 0.02% Tween-20/PBS and blocked for ten 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: PBS. EDTA-plasma samples were diluted 1/20 with PBS and added overnight at 4°C. After washing, 1 µg/mL fluorescein isothiocyanate (FITC)-M5/114 (anti-mouse I-A\textsuperscript{b,d,q}/I-E\textsuperscript{d,k}; purified and FITC-conjugated in house) was added for one hour at 37°C to detect bound MHC-II\textsuperscript{+} exosomes.\textsuperscript{28} Plates were washed then incubated with anti-FITC-horse radish peroxidase (1/5000; Roche #1426346) for one hour at 37°C. After washing, the plate was developed in tetramethyl benzidine (TMB; Zymed #00-2023) and the reaction stopped with 2N H\textsubscript{2}SO\textsubscript{4}. 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 one-way (or two-way in Figure 3) ANOVA with Bonferroni post-correction test.

Results

Identification of spleen, liver and lymph node 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 co-localization 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 utilized CD169$^{-/-}$ mice. Compared to 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 several fold 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 co-localized with SIGN-R1⁺ macrophages (Figure 1F-G and supplemental Figure 2). 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 co-localization of exosomes to CD169⁺ macrophages was simply due to anatomical constraints, as 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 non-specific 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’ (non-biotinylated) 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).\textsuperscript{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 bouyant density on sucrose-cushion (supplementary 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\textsuperscript{-/-} mice**

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

**Enhanced responses of CD169\textsuperscript{-/-} mice to protein and peptide-loaded exosomes**

Since B cell-derived exosomes express both MHC-I and MHC-II,\textsuperscript{24} we next compared their ability to induce an immune response in wild-type and CD169\textsuperscript{-/-} mice. Interestingly, although protein-pulsed exosomes induced both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell proliferation, peptide-pulsed exosomes did not reproducibly induce CD4\textsuperscript{+} T cell proliferation (Figure 4-5 and supplemental Figure 5-6). No differences in OT-I or OT-II proliferation were noted between wild-type and CD169\textsuperscript{-/-} mice, by either intravenous or subcutaneous route for both peptide and protein (Figure 4-5, supplemental Figure 5-6). Surprisingly, peptide- or protein-pulsed parental B cells did not, or only weakly induced, CD4\textsuperscript{+} or CD8\textsuperscript{+} T cell proliferation, or cytotoxicity, by both subcutaneous and intravenous routes (Figures 4-7).
Consistent with a recent report on DC-derived exosomes, 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 OVA\textsubscript{323-339} (Figure 6). However, supplementation of naïve OT-I T cells, resulted in a significantly enhanced CTL response to Exo\textsubscript{257}, 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 to peptide-pulsed exosomes (cf. Figure 6 and Figure 7; p<0.0001 for both B6 or CD169\textsuperscript{-/-} 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 to wild-type mice, CD169\textsuperscript{-/-} 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 (Figures 7). A small but significant increase in the CTL response of CD169\textsuperscript{-/-} mice to subcutaneously injected peptide-pulsed, but not protein pulsed, DC was also noted (Figure 6B).
**Discussion**

We identified CD169 as a specific binding-partner for exosomes in lymphoid tissue. Its restriction to sub-populations of macrophages dedicated to antigen capture and direct presentation and/or transfer of antigen to other APC, 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, however a role for CD169 in capturing exosomes has not previously been demonstrated.

Despite the low affinity (K_d ~1.4 mM) of CD169 for α2,3-linked sialic acid, we observed abundant binding of exosomes to this receptor. This may be a result of high level expression of α2,3-linked sialic acid expression on exosomes, as linear increases in CD169 ligand availability results in logarithmic increases in avidity. Moreover, due to their size exosomes may suffer less shear stress, therefore gaining greater access to receptors, compared to 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. Thus the MZ macrophage barrier acts together with the MZ MADCAM-1+ sinus lining cells to limit antigen entry into the red pulp. Such partitioning of antigen may optimize antigen availability for the initiation of appropriate immune responses at the MZ and white pulp. Interestingly, McGaha et al. observed enhanced immune responses to antigen associated with apoptotic cells in mice depleted of MZ macrophages. It has previously been demonstrated that exosomes entering the venous circulation localize to the splenic MZ. 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.
DC-derived exosomes abundantly express the ligand milk-fat globule (MFG)-lactadherin, a potential ligand for the $\alpha_\text{v}\beta_3/\beta_5$ integrins. Others have proposed that a number of molecules, including, CD9, CD11a, CD81, CD91, ICAM-1 and phosphatidyl serine are involved in exosome capture by DC. Stromal interactions of exosomes to collagen or fibronectin may be facilitated in humans by the $\beta_1$ and $\beta_2$ interactions. Additionally, C3b deposition on exosomes enhances antigen presentation and splenic uptake. There is one description of the rat-restricted Galectin 5 mediating the binding of erythrocyte exosomes to macrophages, 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, or uptake by fenestrated endothelium in the ‘liver sieve’, may mask the contribution of a selectively expressed receptor present only in a sub-population 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. However, the combination of multi-valent, high affinity glycan ligands expressed on these liposomes together with enhanced ‘stealth’ properties due to polyethylene glycol modification, may explain this difference. A key distinction between exosomes and glycan-expressing liposomes is that the latter do 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 co-operation of helper
T cells with antigen-specific B cells.\textsuperscript{10,44} This is an important finding as it distinguishes exosomes from cellular APC namely, DC, which have been reported to be 50-fold more efficient at presenting OVA peptides, compared to equimolar concentrations of whole ovalbumin.\textsuperscript{45} Given the inherent inefficiency of processing proteins for crosspriming, 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\textsuperscript{+} mice. Similarly, it was shown that CD169\textsuperscript{+} macrophages promote an inhibitory effect in response to apoptotic cells,\textsuperscript{36} although CD169 may alternatively promote autoimmunity and inflammatory responses to sialylated pathogens.\textsuperscript{13,30} It is possible that the outcome of antigen targeted to CD169\textsuperscript{+} macrophages is greatly influenced by the antigenic context, such as the presence of microbial products or inflammation.\textsuperscript{13,17} Our current focus is the role of other APC subsets in mediating the enhanced immune response observed in CD169\textsuperscript{+} mice.
Acknowledgements

The authors would like to thank Tri Phan and Robert Brink (Garvan Institute) for critical input into this study, Vernon Ward for support and Lane Black for performing initial bead localization experiments. The authors acknowledge the scientific assistance from the Otago Centre for Electron Microscopy (OCEM; University of Otago). This work was supported by the Marsden Fund (08-U00-106), a University of Otago Research grant, a bequest from the Otago School of Medical Sciences and a PhD scholarship from New Zealand Lottery Health Research. PRC was funded by a Wellcome Trust Senior Research Fellowship, WT081882.

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-/- mice. A.D.M. designed the study, assisted with laboratory work and wrote the manuscript.

Conflict-of-interest disclosure: The authors have no financial conflicts of interest.

Correspondence: Dr. Alexander McLellan or Sarah Saunderson, Department of Microbiology & Immunology, Otago School of Medical Sciences, University of Otago, P.O. Box 56, Dunedin, Otago, New Zealand; e-mail: alex.mclellan@otago.ac.nz or sarah.saunderson@otago.ac.nz.
References


19. Colino J, Snapper CM. Dendritic cell-derived exosomes express a Streptococcus pneumoniae capsular polysaccharide type 14 cross-reactive antigen that induces protective immunoglobulin


Figure Legends

**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. Alternatively C57BL/6 or CD169−/− mice were IV or SC injected with 2 × 10^{11} or 1 × 10^{11} 100 nm fluorescent microspheres respectively (green; D,F-G). For IV or SC routes, mice were sacrificed, at five minutes with spleens and livers harvested, or at 30 min and draining LN harvested respectively. Exo-bio were detected with streptavidin-Alexa-594 (red; A-C,E). Sections were co-labeled for (A,G) marginal metallophilic or subcapsular sinus macrophages with anti-CD169 (MOMA-1), (B) MZ or red pulp macrophages with anti-SIGN-R1 (ER-TR9) or anti-F4/80 respectively, or (C) Kupffer cells with anti-F4/80. Primary antibodies were detected with anti-rat IgG-Alexa-488 (green; A-C), or anti-rat IgG-Alexa-594 (red; G) and nuclei counterstained with DAPI (blue). Original magnification ×100 (A:LN,D:Spleen,E), ×200 (A:Spleen,B,D:LN,G) and ×400 (C). Bar represents 200 µm (A:Spleen,B,D:LN,G), 250 µm (A:LN,D:Spleen,E) and 50 µm (C). All results representative of at least four mice per group. Percent co-localization (F) was calculated from fluorescent microscopy photos of spleen sections (A:Spleen,B,G). Ten individual photos per mouse (original magnification ×200) were analyzed for co-localization of green (Alexa-488) and red (Alexa-594) signal using the Manders’ co-efficient with ImageJ. Each point represents an individual photo; line indicates mean. Circles = Exosomes; Squares = Beads; Closed symbols = C57BL/6 mice; Open symbols = CD169−/− mice. One-way ANOVA with Bonferroni post-correction test performed: ns = not significant; * P <.05; ** P <.01; **** P <.0001

**Figure 2:** Exosomes are bound by CD169+ macrophages in the spleen and lymph node in the absence of blood or lymph flow. Exo-bio were applied to naïve C57BL/6 or CD169−/− spleen and LN sections using a modified Stamper-Woodruff assay. Biotin was detected with streptavidin-
Alexa-488 (green; A), or -Alexa-594 (red; B) and marginal metallophilic or subcapsular sinus macrophages stained with anti-CD169 (MOMA-1). Primary antibody was detected with anti-rat IgG-Alexa-488 (green; B) and nuclei counterstained with DAPI (blue). (C) Exo-bio ± treatment with *Vibrio cholerae*-derived sialidase (α2,3-linked sialic acid preferential cleavage; SIAL-V) were purified using a sucrose-cushion, negatively stained and visualized under TEM. No apparent differences in morphology were observed between samples; diameter range 70-120 nm. Photos are representative of the preparations as a whole. (D) Exo-bio were bound to naïve C57BL/6 sections in the presence of negative control antibody or CD169 neutralizing antibody (SER-4). Alternatively, Exo-bio or SIAL-V treated Exo-bio were applied to naïve C57BL/6 or CD169<sup>−/−</sup> sections respectively. Exosomes and nuclei were detected as described for panel A. Exosomes ± SIAL-V treatment were bound to aldehyde-sulfate microspheres and analyzed by flow cytometry for α2,3- and α2,6-linked sialic acid expression using biotinylated MAL-II and SNA lectins respectively. In addition, CD9, CD24, MHC-II, CD19, Ig and CD21 expression was measured. Shaded peak indicates negative control (BSA conjugated aldehyde-sulfate microspheres); Black line indicates untreated exosomes; Dashed line indicates SIAL-V treated exosomes. Results representative of at least three separate experiments and/or exosome preparations, with (D:LN) LN sections from at least four anatomically distinct locations per experiment. Original magnification ×40 (A:Spleen), ×100 (A:LN), ×200 (B,D), ×24500 (C:Exo-bio) and ×17500 (C:SIAL-V treated Exo-bio). Bar represents 500 µm (A:Spleen), 250 µm (A:LN), 200 µm (B,D), 500 nm (C) and 100 nm (C:inset).

**Figure 3: Exosome clearance and distribution in vivo.** (A) C57BL/6 or CD169<sup>−/−</sup> mice were anesthetized and then IV injected with 100 µg Exo-bio (purified by ultracentrifugation). Mice were tail bled at the indicated time points. MHC-II<sup>+</sup> exosome concentration was analyzed by ELISA from plasma samples; Exo-bio spiked plasma was used as a standard. Closed circles = C57BL/6 mice; Open circles = CD169<sup>−/−</sup> mice. (B) C57BL/6 or CD169<sup>−/−</sup> mice were IV injected with 100 µg Exo-bio. Mice were sacrificed at the indicated time points and spleens harvested. Exo-bio were
detected with streptavidin-Alexa-594 and nuclei counterstained with DAPI. Original magnification ×100 for panel B. Bar represents 500 µm. Results representative of six mice (A,B:120 min) 3-6 mice (B:5 min and 60 min).

**Figure 4: In vivo T cell proliferation in response to exosomal-peptide antigen.** C57BL/6 or CD169⁻/⁻ were immunized IV or SC in the forelimb with (A) PBS, 100 µg sucrose-cushion purified Exo257/323, 10⁵ DC-257/323, or (B) 10⁵ parental B cell257/323; Exosomes and cells were all pulsed simultaneously with 1 µM ovalbumin peptides OVA257-264 and OVA323-339. T cell proliferation of adoptively co-transferred OT-I (CD8) and OT-II (CD4) cells (CFSE or CPD V450) were analyzed five days post-immunization by flow cytometry. Black line indicates test group; Shaded peak indicates PBS immunized mice. Results representative of at least six mice per group.

**Figure 5: T cell proliferation in response to exosomal-protein antigen.** C57BL/6 or CD169⁻/⁻ 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 two days (Exo-pro), 10⁵ DC-pro or (B) 10⁵ parental B cell-pro. DC and B cells cultured with 200 µg/mL ovalbumin protein for two days. T cell proliferation of adoptively co-transferred OT-I (CD8) and OT-II (CD4) cells (CFSE or CPD V450) were analyzed five days post-immunization by flow cytometry. Black line indicates test group; Shaded peak indicates PBS immunized mice. Results representative of at least six 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, 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 post-immunization, mice were adoptively transferred with unpulsed (CFSE low), or OVA257-264 pulsed (CFSE high).
target cells. *In vivo* killing was analyzed 18 h later by flow cytometry. Results representative of at least six mice per group using exosomes purified by ultracentrifugation. Line indicates mean. One-way ANOVA with Bonferroni post-correction was performed: ns = not significant; ** P<.01; **** P<.0001

**Figure 7: Enhanced cytotoxic responses to exosomal-protein in CD169−/− mice.** C57BL/6 or CD169−/− mice were immunized (A) IV or (B) SC with PBS, pellet from exosome sucrose-cushion purification (IV: B6 Sucr. Pellet), 50 µg sucrose-cushion purified Exo-pro or 100 µg Exo-pro (purified by ultracentrifugation), 10^5 DC-pro, or 10^5 parental B cell-pro. Seven days post-immunization, mice were adoptively transferred with unpulsed (CFSE low), or OVA 257-264 pulsed (CFSE high) target cells. *In vivo* killing was analyzed 18 h later by flow cytometry. Results representative of at least six mice per group. One-way ANOVA with Bonferroni post-correction was performed: * P<.05; **** P<.0001
Figure 2

A

Exo-bio

Spleen

LN

B

Exo-bio

CD169

Spleen

LN

C

Exo-bio

SIAL-V treated

Exo-bio

D

Spleen

LN

Control

α-CD169 (SER-4)

SIAL-V treated

CD169−

E

α2,3

α2,6

CD9

CD24

MHC-II

CD19

Ig

CD21

Shaded = Control

Black = Untreated

Dashed = SIAL-V treated
Figure 3

A

Exosomes (μg/ml)

Time (min)

C57BL/6

CD169−/−

B

5 min  60 min  120 min

B6

CD169−/−
Figure 4

A

Exo\textsubscript{257/323}  DC\textsubscript{257/323}  Exo\textsubscript{257/323}  DC\textsubscript{257/323}

B

Parental B cells\textsubscript{257/323}

CD8

CD4

B6

CD169\textsuperscript{−/−}

CFSE / CPD V450

CFSE / CPD V450

IV

SC
Figure 5

A

Exo-pro  DC-pro  Exo-pro  DC-pro

B

Parental B cell-pro

CD8

CD4

B6

CD169⁻/⁻

CD8

CD4

B6

CD169⁻/⁻

CFSE / CPD V450
Figure 6

A  IV CTL

B  SC CTL

% Killing

B6 PBS  B6 Exo257  B6 Exo257/CD257  B6 Exo257 + OT-I  B6 DC257  B6 B cell257  CD169−  B cell257

% Killing

B6 PBS  B6 Exo257  B6 Exo257/CD257  B6 Exo257 + OT-I  B6 DC257  B6 B cell257  CD169−  B cell257
CD169 mediates the capture of exosomes in spleen and lymph node

Sarah C. Saunderson, Amy C. Dunn, Paul R. Crocker and Alexander D. McLellan