Exosomal regulation of lymphocyte homing to the gut

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Key Points

- Exosomes secreted by gut-imprinted T cells contribute to altering the expression of gut-homing molecules in the microenvironmental niche.
- This exosomal function may represent a novel mechanism for downsizing excessive lymphocyte homing to the intestinal tissue.

Exosomes secreted from T cells have been shown to affect dendritic cells, cancer cells, and other T cells. However, little is known about how T-cell exosomes (T exosomes) modulate endothelial cell functions in the context of tissue-specific homing. Here, we study the roles of T exosomes in the regulation of gut-specific T-cell homing. The gut-tropic T cells induced by retinoic acid secrete the exosomes that upregulate integrin $\alpha 4\beta 7$ binding to the MAdCAM-1 expressed on high endothelial venules in the gut. T exosomes were preferentially distributed to the villi of the small intestine in an $\alpha 4\beta 7$ -dependent manner. Exosomes from gut-tropic T cells suppressed the expression of MAdCAM-1 in the small intestine, thereby inhibiting T-cell homing to the gut. Moreover, microRNA (miRNA) profiling analysis has shown that exosomes from gut-tropic T cells were enriched with miRNAs targeting NKX2.3, a transcription factor critical to MAdCAM-1 expression. Taken together, our study proposes that $\alpha 4\beta 7$ -expressing T exosomes distribute themselves to the small intestine and modify the expression of microenvironmental tissues such that any subsequent lymphocyte homing is precluded. This may represent a novel mechanism by which excessive lymphocyte homing to the intestinal tissues is downsized.

Introduction

The trafficking patterns and destinations of lymphocytes circulating in tissues throughout the body are highly regulated to enhance the ability of antigen-specific effector/memory T cells to encounter pathogens containing cognate antigens.^{1–3} A subset of naïve lymphocytes activated by cognate-antigen-bearing dendritic cells (DCs), which are transformed into effector/memory T cells in gut-associated lymphoid tissues, are destined to return to gut compartments such as the lamina propria regions.^{4,5} The underlying molecular mechanism by which effector/memory T cells home back to the gut tissue lamina propria regions adjacent to those lymphoid tissues where T cells are activated by cognate antigen-expressing gut DCs involves the upregulation of the cell-adhesion molecule integrin $\alpha 4\beta 7$ and the chemokine receptor CCR9.^{6,7}

The upregulation of integrin $\alpha 4\beta 7$ on T cells is induced by the unique ability of gut DCs to imprint upon T cells a propensity to home to the intestine.^{8–11} This singular ability of gut DCs derives from the gut cell type-specific expression of the cytosolic retinal dehydrogenase (RALDH) enzyme, which catalyzes the formation of all-trans retinoic acid (RA) specifically at the gut microenvironment.^{6,12} RA upregulates the cell-surface expression of integrin $\alpha 4\beta 7$ and CCR9 and/or CCR10¹³ on T cells by binding to the nuclear receptor RAR α . Upregulated integrin $\alpha 4\beta 7$ enables flowing T cells to roll along and subsequently stop on the immunoglobulin superfamily ligand MAdCAM-1 preferentially expressed on high endothelial venules (HEVs) of the gut tissue, thereby allowing them to play the central role in enabling gut-specific lymphocyte homing.^{14,15} The interaction of $\alpha 4\beta 7$ with MAdCAM-1 is implicated not only by the induction of mucosal immunity elicited by vaccines such

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as that stemming from rotavirus infections,¹⁶⁻¹⁸ but also by the pathogenesis of inflammatory bowel diseases.¹⁹⁻²¹

Exosomes, the nano-sized lipid bilayer bioparticles secreted from cells, encapsulate bioactive materials such as microRNAs (miRNAs) and protein and lipid mediators, by which they play important roles in intercellular communication between neighboring cells and among distant cells.^{22,23} T-cell-derived exosomes have been shown to be critically involved in several important aspects of immune responses, including the immunosuppressive activities of regulatory T cells as well as cytotoxic and immune stimulatory activities of effector T cells.²⁴⁻²⁶ However, it remains to be elucidated whether exosomes might affect the gut-specific homing of T cells, and if so, how they do it.

Integrins present on the surface of cancer exosomes have been shown to regulate the tissue specificities of tumor metastases. Integrin $\alpha 6 \beta 4$ -displaying exosomes secreted from certain types of primary tumors enter the circulation and subsequently reach the lung tissue where they then precondition those resident cells that are permissive for metastasis.²⁷ By contrast, $\alpha v \beta 5$ -displaying exosomes secreted from another kind of primary tumor reach and precondition liver tissue permissive for metastasis.²⁷ In this way, integrins present on cancer exosomes contribute to the establishment of premetastatic niches, thereby determining the tissue tropism of metastatic cancers.

Here, we sought to elucidate the potential roles of the exosomal integrin $\alpha 4 \beta 7$ in the regulation of gut-specific T-cell homing. We have shown that RA-treated $\alpha 4 \beta 7^{\text{high}}$ gut-tropic T cells secrete exosomes that display high levels of integrin $\alpha 4 \beta 7$. Exosomal $\alpha 4 \beta 7$ retains the ability to bind to MAdCAM-1 and supports the preferential distribution of T exosomes to the villi of the small intestine. Interestingly, $\alpha 4 \beta 7$ -displaying T exosomes suppressed the expression of MAdCAM-1 as well as ICAM-1, VCAM-1, and CCL28 in the small intestine in vivo and in an endothelial cell line in vitro. miRNA profiling has revealed that miRNAs targeting NKX2.3 (the transcription factor critical for MAdCAM-1 expression) along with ICAM-1, VCAM-1, and/or CCL28 were enriched in $\alpha 4 \beta 7$ -displaying T exosomes. Furthermore, the pretreatment of recipient mice with $\alpha 4 \beta 7$ -displaying T exosomes inhibited the homing of donor lymphocytes to the gut. These results support the idea that exosomes secreted from RA-treated $\alpha 4 \beta 7^{\text{high}}$ gut-tropic T cells might negatively regulate gut-specific homing, potentially playing an important role in balancing trafficking to the gut.

Materials and methods

Mice

The C57BL/6J mice age 10 to 12 weeks were obtained from CLEA Japan (Tokyo, Japan) and maintained at the Experimental Animal Facility of Mie University. Experimental animal protocols were approved by the Ethics Review Committee for Animal Experimentation of Mie University.

Cell culture

Splenocytes and lymph node cells were isolated from C57BL/6J mice. After lysis of red blood cells using ammonium-chloride-potassium (ACK) lysing buffer (Thermo Fisher Scientific, Waltham, MA), lymphocytes were suspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin and

cultured with or without 200 nM RA (Sigma) on culture dishes coated with anti-CD3 (3 $\mu\text{g/mL}$) and anti-CD28 (3 $\mu\text{g/mL}$) antibodies (BD Biosciences, San Jose, CA) in a 37°C incubator supplied with 5% CO₂. After 48 hours, the activated T cells were transferred to antibody-uncoated dishes and cultured for 72 hours with 1 ng/mL interleukin-2 (R&D Systems, Minneapolis, MN). Mouse lymphoma TK-1 cells (ATCC, Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10% FBS (Equitech-Bio, Kerrville, TX), penicillin (100 U/mL)/streptomycin (100 $\mu\text{g/mL}$) (Nakalai, Kyoto, Japan), 2 mM L-glutamine (Sigma, St. Louis, MO), and phorbol-12-myristate-13-acetate (100 nM) (Wako, Osaka, Japan) in an incubator maintained at 37°C with 5% CO₂ for 48 hours. Mouse endothelial bEnd.3 cells (ATCC) were cultured in Dulbecco's modified Eagle medium (Nakalai) containing 10% FBS with or without tumor necrosis factor α (TNF- α ; 5 ng/mL) (R&D Systems) for 48 hours. FBS was used after being depleted of extracellular vesicles by ultracentrifugation (110 000g for 18 hours) followed by filtration through a 0.22- μm filter.²⁸

Exosome isolation and size characterization

Exosome isolation was performed as previously described with minor modifications.^{29,30} In brief, the culture medium was spun at 1000g for 10 minutes at 4°C to remove pelleted cells. The supernatant was centrifuged at 2000g for 20 minutes at 4°C to remove apoptotic pelleted bodies. The supernatant was then transferred to ultracentrifuge tubes (Beckman Coulter, Brea, CA) and centrifuged with an L60 Ultracentrifuge (Beckman Coulter) at 26 000g for 20 minutes at 4°C. The supernatant was transferred to new ultracentrifuge tubes and spun at 140 000g for 2 hours at 4°C to pellet the exosomes, which were then resuspended in phosphate-buffered saline. The resuspended solution was filtered through a 0.22- μm filter, transferred to new tubes, and centrifuged at 140 000g for 2 hours at 4°C. The pellet was resuspended in phosphate-buffered saline. Protein concentrations of the isolated exosomes were measured by using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Exosome size was characterized by a nanoparticle-tracking analysis system that included a NanoSight LM10 microscope and nanoparticle-tracking analysis software (version 2.3).³¹

Flow cytometry analysis to detect integrin expression

The exosomes (conjugated with microbeads) were stained with fluorescently labeled antibodies to integrins including $\alpha 4$ (PS/2) (Southern Biotech, Birmingham, AL), $\beta 7$ (M293) (BD Biosciences), $\alpha 4 \beta 7$ (DATK32) (BD Biosciences), or αL (2D7) (BioLegend). Approximately 1 μg of exosomes was stained and then washed twice. The expression was determined by flow cytometry using a BD Accuri C6 cytometer (BD Biosciences).

MAdCAM-1-binding assay using flow cytometry

MAdCAM-1-Fc (5 μg) was conjugated to 10 μL of 4- μm latex beads (Thermo Fisher Scientific) as described above. Exosomes (25 μg) were stained with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Dojindo, Kumamoto, Japan) at 5 μM . The bead-conjugated MAdCAM-1-Fc (1 μg) was mixed with BCECF-AM-labeled exosomes (5 μg) along with 1 mM EDTA, 1 mM Ca²⁺/Mg²⁺, or 1 mM Mn²⁺. In some experiments, the same amount of bead-conjugated Fc was used as a negative control. Binding was determined by flow cytometry using BD Accuri C6 (BD Biosciences).

Knockdown of $\beta 7$ integrin in TK-1 cells by electroporation with $\beta 7$ -short hairpin RNA

TK-1 cells were transfected with scrambled (scr) or $\beta 7$ short hairpin RNAs (shRNAs) (Santa Cruz, Dallas, TX) using Amaxa Nucleofector II (Lonza, Basel, Switzerland) according to the manufacturer's instructions. The stably transfected cells were stained with anti- $\beta 7$ antibody, and the reduction in $\beta 7$ integrin expression was determined by flow cytometry as described above.

In vivo exosomal and lymphocyte homing assay

The exosomes isolated from $\beta 7$ -scr (control) and $\beta 7$ -knockdown ($\beta 7$ -KD) TK-1 cells were stained with an Exo-Glow kit (System Biosciences, Palo Alto, CA) according to the manufacturer's instructions. Briefly, scr or $\beta 7$ -KD exosomes (30 μ g each) were labeled with EXO-Red or EXO-Green dyes, respectively, and vice versa, mixed, and injected intravenously into C57BL/6J mice. After 16 hours of injections, the recipient mice were euthanized so that frozen sections of the tissues (5 μ m thick) could be prepared using O.C.T. Compound (Sakura, Torrance, CA) and a Cryostat CM1850 (Leica Biosystems, Wetzlar, Germany) and stained using 4',6-diamidino-2-phenylindole (DAPI) with NucBlue (Thermo Fisher Scientific). The fluorescent signals were counted by using a BZ-X700 Fluorescent Microscope (Keyence, Itasca, IL) to acquire the numbers of scr (control) and $\beta 7$ -KD exosomes distributed in different tissues. The measurements were performed in randomly selected fields by 3 independent examiners in a blinded fashion, and results were expressed as the mean $\pm$ standard error of the mean.

To study the exosomal effect on lymphocyte homing, the mice were pretreated with intravenous injection of either RA⁺ or RA⁻ T exosomes (50 μ g per mouse) or vehicle. Three hours after the injection, mice were intravenously administered carboxyfluorescein diacetate succinimidyl ester (CFSE; Thermo Fisher Scientific)-labeled donor lymphocytes (2×10^7 cells per mouse). Donor cells were isolated from spleen and lymph nodes of syngeneic C57BL/6J mice and were labeled with CFSE (at 2.5 μ M final concentration) according to the manufacturer's instructions. After 16 hours of donor-cell administration, the recipient mice were euthanized, tissues (small intestine, large intestine, and mesenteric lymph nodes) were harvested, and mononuclear cells were isolated as previously described.³² The distribution of CFSE-labeled donor cells was analyzed by flow cytometry using a BD Accuri C6 analyzer. In some experiments, portions of the harvested tissues were set aside for preparation of the frozen sections and total RNA extraction for reverse transcription quantitative polymerase chain reaction (RT-qPCR). The sections were stained with DAPI and were used to enumerate the CFSE-labeled donor cells under microscopic analysis as described in the previous section. RT-qPCR was performed to measure the relative expressions of the genes of interest as described in the next section.

miRNA analysis via deep sequencing of a small RNA library of T exosomes and T cells

After the T exosomes were isolated, as described above, total RNA was isolated by using Trizol reagent. In parallel, total RNA was isolated from the T cells. A small RNA library was constructed by The Center for Molecular Biology and Genetics (Mie University, Japan) by using Ion Total RNA-Seq Kit v2 (Thermo Fisher

Scientific) according to the manufacturer's instructions. The small RNA libraries were sequenced on the Ion PGM system (Thermo Fisher Scientific). Data collection was performed using Torrent Suite v4.0.1 software.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean for each group. Student *t* tests were used for statistical analyses unless otherwise indicated. *P* < .05 was considered significant. Additional information about materials and methods is supplied in supplemental Data.

Results

RA-induced gut-tropic T cells secrete exosomes that display integrin $\alpha 4\beta 7$

Activation of naïve T cells by mucosal DCs upregulates the expression of integrin $\alpha 4\beta 7$ and CCR9 in an RA-dependent manner, thereby giving rise to gut tropism.⁶ RA treatment of T cells stimulated by CD3/CD28 crosslinking has been shown to restore the ability of mucosal DCs to induce gut-tropic T cells. Capitalizing on the advantage of RA treatment, we generated activated T cells that upregulated $\alpha 4\beta 7$ (Figure 1A). By using differential centrifugation as previously described,²⁹ we isolated exosomes from the culture supernatant of RA-induced gut-tropic T cells. Both RA⁺ and RA⁻ T exosomes had similar diameters (supplemental Figure 1A) and expressed comparable levels of the exosome markers CD9 and Alix (supplemental Figure 1B). Consistent with the upregulated cell surface expression of integrin $\alpha 4\beta 7$, RA-positive T exosomes displayed an increased surface expression of $\alpha 4\beta 7$ compared with RA⁻ T exosomes (Figure 1B; supplemental Figure 2A). Furthermore, $\alpha 4\beta 7$ on RA⁺ T exosomes was functionally active because it bound to MAdCAM-1, whereas the expression of integrin $\alpha 4\beta 7$ on RA⁻ T exosomes was too low to support such binding (Figure 1E). Antibody inhibition confirmed that the binding of RA⁺ T exosomes to MAdCAM-1 was specifically mediated by integrin $\alpha 4\beta 7$ (Figure 1F). As is the case with integrins expressed on T cells, the binding of exosomal integrin $\alpha 4\beta 7$ to MAdCAM-1 in vitro required stimulation by Mn²⁺, a reagent known to mimic inside-out activation signals (Figure 1E-F).

The integrin $\alpha 4\beta 7$ displayed on exosomes supports their distribution to the gut

To further investigate the roles of exosomal integrin $\alpha 4\beta 7$, we sought to knock down its expression. Because of the technical ease involved in generating stable transfectants, we used the murine TK-1 T-cell line as a model, and thereby established stable T-cell line transfectants expressing shRNAs against integrin $\beta 7$. TK-1 cells expressed high levels of integrin $\alpha 4\beta 7$ without RA treatment (Figure 1C). We obtained TK-1 cell clones stably transfected to express the shRNAs targeting $\beta 7$ integrin. These exhibited reduced cell surface expression of integrin $\alpha 4\beta 7$ compared with those TK-1 cells stably transfected to express scr shRNAs (Figure 1C). By using ultracentrifugation, we isolated exosomes secreted from TK-1 cells stably transfected with either $\beta 7$ or scr shRNAs. Both types of TK-1 exosomes exhibited similar diameters and expressed comparable levels of the exosome markers CD9 and Alix (supplemental Figure 1C-D). TK-1 exosomes from $\beta 7$ integrin-knockdown cells (TK-1 KD exosomes) showed reduced surface levels of integrin $\alpha 4\beta 7$ and a reduced ability to bind to MAdCAM-1 compared with

Figure 1. T exosomes bind MAdCAM-1 via RA-increased integrin $\alpha 4 \beta 7$. (A-D) Expression levels of the indicated integrins on T and TK1 cells and their exosomes immobilized on beads are shown in representative flow cytometry histograms. (A-B) RA^+ indicates the activation condition of T cells with CD3/CD28/interleukin-2/RA, whereas RA^- are those with CD3/CD28/interleukin-2. RA effect on upregulating the expression of integrins $\alpha 4$ and $\beta 7$ is shown by red (RA^+) or blue (RA^-) lines; gray and black lines indicate the background staining with isotype controls for RA^+ and RA^- samples, respectively. (C-D) Flow cytometry histograms showing the effect of $\beta 7$ silencing (by treating shRNAs in TK-1 cells) on altering integrin expression. Integrin expression for the $\beta 7$ -KD (by $\beta 7$ shRNA [purple lines]) and scr (by scr shRNA [green lines]) TK-1 cells is shown. Background staining with isotype controls for $\beta 7$ -KD (gray lines) and scr (black lines) is also shown. Analysis of binding of T exosomes (E-F) or TK-1 exosomes (G) to MAdCAM-1 coated on beads. (E) Binding of RA^+ (red bars) and RA^- (blue bars) T exosomes to MAdCAM-1 is shown in the presence of 1 mM $\text{Ca}^{2+}/\text{Mg}^{2+}$, 1 mM Mn^{2+} , or 1 mM EDTA. (F) Binding specificity was tested by adding $\alpha 4 \beta 7$ -blocking antibody (Ab) or isotype (rat immunoglobulin G [IgG]) in the presence of 1 mM Mn^{2+} . (G) Binding of $\beta 7$ -KD (purple bars) and scr (green bars) TK-1 exosomes to MAdCAM-1. (A-D) The flow cytometry histograms are representative of 3 independent experiments that yielded similar results. (E-G) The bar graphs represent the mean $\pm$ standard error of the mean (SEM) for mean fluorescent intensity (MFI) values obtained from 3 to 5 independent experiments. Iso, isotype control IgG; mAb, monoclonal antibody. * $P < .05$ between indicated groups.

TK-1 exosomes from control scr shRNA-expressing cells (TK-1 control exosome) (Figure 1D,G; supplemental Figure 2B).

To study the roles played by the exosomal integrin $\alpha 4 \beta 7$ in regulating the tissue distribution of exosomes, we performed a competitive exosomal homing assay. TK-1 $\beta 7$ -KD and scr (control) exosomes were fluorescently labeled green or red, respectively, and equal amounts of both were mixed and intravenously injected into recipient mice. The organs were harvested 16 hours after injection, and frozen sections were made. We then examined the $\beta 7$ -KD and scr exosomes by using a fluorescence microscope. To minimize any biases during examination resulting from artifacts (eg, background signals in the intestinal lumen), the microscopic fields were randomly selected and the fluorescent dots in tissues were quantitated in a double-blind fashion by 3 independent examiners

to ensure an objective validation. We have found that the $\beta 7$ -KD exosomes did not distribute as well to the mucosa of the small intestine as the scr exosomes did. However, both exosomes were distributed to spleen, Peyer's patch, mesenteric lymph node (MLN), and liver in equal fashion (Figure 2). Similar results were obtained by another experiment in which $\beta 7$ -KD and scr exosomes were alternately labeled, thereby excluding any effects of the different fluorescent dyes. Thus, these results imply that exosomal $\alpha 4 \beta 7$ promotes the distribution of exosomes to the small intestine.

Integrin $\alpha 4 \beta 7$ -displayed RA^+ T exosomes suppress lymphocyte homing to the small intestine

After demonstrating the proof-of-principle that exosomal integrin $\alpha 4 \beta 7$ supports the preferential distribution of exosomes to the small

Figure 2. TK-1 exosomes home to the small intestine in an $\alpha 4\beta 7$ -dependent manner. TK-1 exosomes (30 μ g each) were fluorescently labeled with either Exo-Green or Exo-Red, mixed ($\beta 7$ -scr-Green/ $\beta 7$ -KD-Red or $\beta 7$ -KD-Green/ $\beta 7$ -scr-Red), and injected intravenously into mice. Sixteen hours after injection, recipient mice were euthanized, and organs were harvested for preparation of frozen tissue sections. Microscopic analysis was performed to count exosomes distributed to the tissues. The absolute number of fluorescently labeled exosomes was counted in randomly selected microscopic image fields of tissue samples using identical magnification. Fluorescent spots inside each villus were counted in the small intestines to exclude those exosomes in the regions of external tissues such as the lumen. Fluorescent microscopic analyses were performed by 3 independent examiners in a double-blinded fashion, and the results are expressed as the mean $\pm$ SEM. *** $P < .001$ (vs $\beta 7$ -scr).

intestine using TK-1 exosomes, we further investigated the effects of T exosomes on the regulation of lymphocytes homing to the gut. We pretreated recipient mice with RA⁺ or RA⁻ T exosomes, allowing the exosomes 3 hours to reach the organs before donor T cells were injected. Fluorescently labeled donor lymphocytes were then intravenously injected into recipient mice pretreated with either RA⁺ or RA⁻ T exosomes. Adoptively transferred fluorescently labeled donor lymphocytes were allowed to home to organs for 16 hours. Subsequently, mononuclear cells were isolated from each organ and subjected to flow cytometry analysis to measure the frequency of donor cells. Lymphocyte homing to the small intestine was suppressed by RA⁺, but not RA⁻, T-exosome treatment, whereas homing to the large intestine or spleen remained unaffected (Figure 3A-B; data not shown). Donor cell frequency in each tissue was alternatively studied by fluorescent microscopic examination of tissue sections, confirming all results except that adoptively transferred lymphocytes homed to MLNs increased with both RA⁺ and RA⁻ T-exosome treatment (supplemental Figure 3).

To study the potential underlying molecular mechanism by which RA⁺ T exosomes suppressed lymphocyte homing to the small intestine, we analyzed the gene expression of integrin ligands and chemokines in the gut. We found that the expressions of MAdCAM-1, ICAM-1, and VCAM-1 along with the chemokine CCL28 were reduced in the small intestine of RA⁺, but not RA⁻, T-exosome-treated mice (Figure 4). In contrast, in the large intestine, RA⁺ and RA⁻ T-exosome treatments suppressed CCL28 expression, while enhancing MAdCAM-1 expression. RA⁻, but not RA⁺, T-exosome treatment also enhanced ICAM-1 and VCAM-1 expression in the large intestine. RA⁺ T-exosome treatment was shown to markedly upregulate messenger RNA expressions of ICAM-1, VCAM-1, MAdCAM-1, CCL25, and CCL28 in MLNs.

To substantiate (at the cellular level) the in vivo findings obtained in the tissues that RA⁺ T exosomes suppressed MAdCAM-1 expression, we used a murine endothelial cell model using a bEnd.3, which enabled us to treat the cells with RA⁺ or RA⁻ T exosomes. We found that RA⁺, but not RA⁻, T exosomes suppressed the TNF-induced expression of MAdCAM-1, ICAM-1, and VCAM-1 (Figure 5). In TNF-untreated bEnd.3 cells, both RA⁺ and RA⁻ T

exosomes suppressed the expression of CCL28 and NKX2.3, the latter being an important transcription regulator for MAdCAM-1.^{33,34} CCL25 expression was suppressed only by RA⁺ T exosomes in TNF-untreated bEnd.3 cells (Figure 5). These in vitro results may be only partially consistent with in vivo results observed in the small intestine, because bEnd.3 cells were originally derived from brain tissue.

Profiling the miRNAs enriched in the RA⁺ T exosomes reveals the potential candidates that target endothelial homing molecules

To further investigate the mechanisms by which RA⁺ T exosomes suppressed the expression of the endothelial molecules that support lymphocyte homing to the small intestine, we examined the possible roles they played by performing miRNA expression profiling. Expression profiling of miRNA by small RNA deep sequencing detected 332 miRNAs that showed a value of more than 10 reads per kilobase of transcript per million mapped reads (RPKM).³⁵ The 39 and 45 miRNAs were higher (for the RPKM values of twofold or more) in RA⁺ and RA⁻ T exosomes, respectively (Figure 6A; supplemental Table 1). A bioinformatics analysis using a TargetScan database predicted that 133 miRNAs would target gut-homing-related integrin-ligand or chemokine genes (supplemental Table 1; supplemental Figure 4). Although no miRNA candidates that targeted MAdCAM-1 were detected, several miRNAs predicted to target NKX2.3, a transcription factor critical in regulating the expression of MAdCAM-1,^{33,34} were found. By using qPCR, we confirmed the upregulation of several miRNAs at increased levels in the RA⁺ T exosomes (Figure 6B). Remarkably, some NKX2.3-targeting miRNAs were also predicted to target other endothelial molecules that supported lymphocyte homing: miR-132 and miR-212 targeted VCAM-1 and NKX2.3, and miR-431 targeted ICAM-1 and NKX2.3 (supplemental Table 1; supplemental Figure 4).

We have also performed miRNA profiling analysis of T cells in the presence or absence of RA treatment (supplemental Table 2; supplemental Figure 5). Comparative analysis of the miRNA profiles of T exosomes (supplemental Table 1) and T cells (supplemental Table 2) have shown that some miRNAs are enriched upon RA

Figure 3. Pretreatment with RA⁺ T exosomes suppresses lymphocyte homing to the small intestine. (A) We investigated the effect of exosomal pretreatment of recipient mice on the homing of adoptively transferred T cells. Recipient mice were pretreated with an intravenous injection of either vehicle (mock), RA⁻ T exosomes, or RA⁺ T exosomes. Three hours after injection, recipient mice were intravenously administered an identical number of CFSE-labeled lymphocytes (2×10^7). Sixteen hours after lymphocytes were administered, organs were harvested, mononuclear cells were isolated, and the percentages of donor cells were examined by flow cytometry. Representative flow cytometry dot plots of the small intestine lamina propria lymphocytes (LPLs), large intestine LPLs, and MLN cells are shown. CFSE-labeled donor cells are circled and the percentages are shown. (B) Bar graphs represent the percentages of homed donor cells in different tissues. Results are expressed as the mean $\pm$ SEM of 3 independent experiments. * $P < .05$ (vs mock).

treatment in either T exosomes or T cells. However, key miRNAs in this study, such as miR-132 and miR-212, predicted to target T cell homing-regulating molecules VCAM-1 and NKX2.3 were enriched in both T exosomes and T cells upon RA treatment.

Discussion

Similarities and differences in cancer exosome-mediated formations of premetastatic niches

Exosomes act not only as local mediators that support the intercellular communication between neighboring cells but also as systemic mediators that deliver biological signals to distant cells by flowing throughout the circulatory system.³⁶ In cancers,

the destination of this systemic delivery of biological signals by exosomes has been shown to be regulated by integrins present on the surface of exosomes.²⁷ As Hoshino et al²⁷ reported, cancer exosomes that display integrin $\alpha 6 \beta 4$ were preferentially taken up by the lung, whereas those displaying $\alpha V \beta 5$ were taken up by the liver. In this way, different types of exosomal integrins determine the tissue-specific biodistribution of exosomes secreted from cancer cells. Furthermore, cancer exosomes deliver biological signals to the specific organ, thereby modifying the gene expression of the tissue-resident cells to precondition the premetastatic niches that promote metastasis. It has been shown that exosomal integrins can remodel microenvironments, thereby promoting cancer metastasis.²⁷ These findings regarding cancer exosomes led us to investigate the potential involvement of T exosomes in remodeling the microenvironment, specifically their role in regulating the gut-specific homing of T cells, which is a central mechanism for maintaining effective mucosal immunity.

We have shown that gut-tropic RA-activated T cells secrete exosomes that display high levels of integrin $\alpha 4 \beta 7$ compared with non-gut-tropic activated T cells that secrete exosomes that display low levels of integrin $\alpha 4 \beta 7$. Enhanced expression of integrin $\alpha 4 \beta 7$ facilitates the biodistribution of exosomes to the gut. Furthermore, $\alpha 4 \beta 7^{\text{high}}$ T exosomes secreted by gut-tropic T cells suppressed T-cell homing to the small intestine, possibly by altering the gene expression of gut tissues via miRNA-mediated mechanisms. These results suggest a potential negative regulatory role played by the T exosomes secreted by gut-tropic T cells as compared with their subsequent gut-specific homing. This is in contrast to the reported roles played by cancer exosomes in promoting the formation of premetastatic niches.²⁷ Because HEVs in the gut constitutively express MAdCAM-1 thereby forming a microenvironment that supports the homing of gut-tropic T cells, our results show that RA⁺ T exosomes suppress the gut homing that supports the microenvironment, thus negatively regulating gut-specific T-cell homing.

Novel features underlying the dual functionalities of RA-mediated gut imprinting

The results of this study have highlighted the novel dual roles of RA locally produced on mucosal DCs that express the RALDH enzyme. RA has been implicated in positively regulating the gut-specific imprinting of T-cell homing.³⁷ T-cell activation in the presence of RA, which recapitulates the T-cell activation by DCs in the gut mucosa, promotes the ability of T cells to home to the gut by enhancing the cell surface expression of integrin $\alpha 4 \beta 7$ and the chemokine receptors CCR9 and/or CCR10. Conversely, our results have shown that RA induces T cells to secrete $\alpha 4 \beta 7^{\text{high}}$ T exosomes, which preferentially distribute to the gut where the gut-homing supporting phenotypes of the small intestinal microenvironment are suppressed by downregulating the expression of MAdCAM-1 and chemokines. The RA-driven induction of these 2 apparently contrasting effects on gut homing might suggest the presence of a balancing mechanism related to gut-specific imprinting. Intriguingly, the positive effect is exerted on integrin $\alpha 4 \beta 7$ on T cells at the MLNs and Peyer's patches (ie, inductive sites), whereas the negative effect mediated by exosomes is exerted on MAdCAM-1 on the endothelial cells of gut-lamina propria (ie, effector sites).³⁸ This phenomenon supports the notion that RA differentially regulates gut-specific imprinting at 2 distinct locations (at inductive and effector sites)

Figure 4. Reduced lymphocyte homing may be partly due to exosome-mediated suppression of the tissue expression of integrin ligands related to gut homing. The gene expression of *icam-1*, *vcam-1*, *madcam-1*, *ccl25*, and *ccl28* was analyzed by RT-qPCR using RNA extracted from the tissues of mice pretreated with mock or T exosomes (either RA⁻ or RA⁺). Data from qPCR analysis were normalized to the controls that represent samples from the mock-treated mice after being normalized to a reference β -actin gene (2^{-ddCT}). Bar graphs represent the mean $\pm$ SEM obtained from 8 to 10 reactions using the tissues from at least 2 independent homing experiments. * $P = .01$ to $<.05$; ** $P = .001$ to $<.01$; *** $P < .001$ (vs mock). # $P = .01$ to $<.05$; ### $P = .001$ to $<.01$; #### $P < .001$ (vs RA⁻).

thereby constituting a novel mode of T-exosome-mediated regulation of gut-specific homing.

Mechanisms that support the $\alpha 4\beta 7$ integrin-mediated biodistribution of exosomes to the gut

The integrin $\alpha 4\beta 7$ -mediated preferential distribution of exosomes to the small intestine demonstrated in this study is consistent with the recent finding by Guzzo et al³⁹ that a certain type of HIV virion particles (~150-nm diameter) that displayed integrin $\alpha 4\beta 7$ was efficiently taken up by MAdCAM-1-expressing mucosal gut endothelial cells. Interestingly, unlike integrins on the cell surface, the ability of $\alpha 4\beta 7$ integrins on exosomes and HIV virions to bind MAdCAM-1 is unlikely to be dynamically upregulated by inside-out signaling. Integrin $\alpha 4\beta 7$ on T exosomes and HIV virions might maintain their basal activity to bind MAdCAM-1. Because shear stress has been shown to enhance the ability of integrins to bind ligand, independently of the activation by inside-out signaling,⁴⁰ exosomally and virally displayed $\alpha 4\beta 7$ integrins might upregulate binding to MAdCAM-1 under shear stress in circulation. This could explain the mechanisms by which $\alpha 4\beta 7^{\text{high}}$ T exosomes are preferentially taken up by HEVs in the small intestine.

Mechanisms used by exosomes to suppress lymphocyte homing to the gut

We have shown that RA⁺ T exosomes potently suppress the homing of adoptively transferred lymphocytes to the small intestine.

Different mechanisms can be postulated for the exosome-mediated suppression of small intestine-specific T-cell homing. Because recipient mice were administered with exosomes 3 hours before the infusion of donor cells, it is believed that the inhibitory effects of exosomes are mediated predominantly through exosomal miRNAs acting on recipient tissues, but not on donor cells. However, we are aware of the possibility that parts of the injected exosomes that remain in circulation for more than 3 hours could be taken up not only by endothelial cells but also by donor cells, thereby modifying T-cell functions. Alternatively, miRNA-independent mechanisms could be involved. For example, exosomal $\alpha 4\beta 7$ might compete with cellular $\alpha 4\beta 7$ for endothelial MAdCAM-1, thereby inhibiting cell adhesion to the HEVs of the small intestine. This possibility is partly supported by a previous study that showed how a soluble integrin domain competitively inhibited integrin-mediated T-cell adhesion to endothelial cells in vivo.⁴¹

Consistent with our hypothesis that the inhibitory effects of exosomes were mediated predominantly on recipient tissues, treatment of recipient mice with RA⁺ T exosomes resulted in the reduced gene expression of endothelial molecules promoting lymphocyte homing to gut tissues. Remarkably, we have observed a reduction in MAdCAM-1 expression in the small intestine, which may constitute the principal mechanism by which RA⁺ T exosomes mediate the suppression of lymphocyte homing to the small intestine. The potent ability of RA⁺ T exosomes to suppress MAdCAM-1 expression in tandem with the suppression of ICAM-1 and/or VCAM-1 was recapitulated in an in vitro model using a monolayer of a mouse

Figure 5. Endothelial expression of integrin ligands is altered by RA⁺ T exosomes. To support the hypothesis that exosomes mediate the alteration of the tissue expression of integrin ligands and chemokines, the effect of T exosomes on gene expression was tested with a bEnd.3 endothelial cell line. Expression of *icam-1*, *vcam-1*, *madcam-1*, *nx2.3*, *cc25*, and *cc28* was investigated on bEnd.3 cells (left) without or (right) with TNF. Data from qPCR analysis were normalized to those controls that represented samples from the mock-treated cells after being normalized to a reference β -actin gene (2^{-ddCT}). Bar graphs represent the mean $\pm$ SEM obtained from at least 4 separate experiments. * $P = .01$ to $<.05$; *** $P < .001$ (vs mock).

endothelial cell line.^{32,42} Whereas suppression of MAdCAM-1 expression is thought to play a major role in the dampening of gut homing, the concomitant suppression of ICAM-1 and VCAM-1 expression on endothelial cells at the mucosa would further weaken adhesive interactions with T cells, thereby boosting the suppressive effects of lymphocyte homing to the gut. Although treatment with RA⁺ T exosomes did not change the expression of CCL25, RA⁺ and RA⁻ T exosomes both decreased that of CCL28. Because CCL28 and CCL25 have been implicated in attracting

CCR10-positive and CCR9-positive effector/memory T cells, respectively, to the small intestine,⁴³ and because both activate integrin $\alpha 4 \beta 7$ to bind to MAdCAM-1,⁴⁴ suppression of CCL28 by RA⁺ T exosomes might be biologically relevant. However, in our simplified homing assay system, because the majority of donor cells were naïve lymphocytes that possibly express little CCR9 or CCR10, the inhibitory effect of RA⁻ T exosomes was likely to have been mediated by the suppression of MAdCAM-1 along with the concomitant suppression of ICAM-1 and VCAM-1.

Although RA⁺ T exosomes play a predominant role in suppressing the expression of the adhesion molecules and chemokines in the small intestine that regulate T-cell homing, both RA⁺ and RA⁻ T exosomes exhibited mixed effects compared with the expression of these molecules in the large intestine. RA⁺ and RA⁻ T exosomes both suppressed CCL28 expression but enhanced MAdCAM-1 expression in the large intestine, which suggests the presence of certain biological effects independent of RA treatment. In addition, RA⁻, but not RA⁺, T exosomes enhanced the expression of ICAM-1 and VCAM-1 in the large intestine. Moreover, RA⁺, but not RA⁻, T exosomes increased the expression of ICAM-1, VCAM-1, and MAdCAM-1 in MLNs. Interestingly, despite such alterations in the expression of cell adhesion molecules and chemokines, the number of adoptively transferred lymphocytes that homed to the large intestine and the MLNs was hardly changed in T-exosome-treated recipient mice. We observed an increased homing to the MLNs in both RA⁺ and RA⁻ T-exosome-treated recipient mice in some of our results (supplemental Figure 3), which is inconsistent with the RA⁺ T-exosome-specific effects that increase ICAM-1, VCAM-1, and MAdCAM-1 messenger RNA expression in MLNs. These discrepancies are still unexplained; however, it is possible that efficient T-cell homing to the large intestine might require additional homing receptors such as GPR15 and/or CCR10.^{45,46} Alternatively, the enhanced expression of MAdCAM-1 induced by T exosomes might result in aberrant glycosylation of these adhesion molecules, which would fail to fully support T-cell homing to the large intestine and MLNs, because effective glycosylation is required for MAdCAM-1 to support T-cell homing.⁴⁷

Interestingly, contrary to its effect on gut homing, RA⁺ T-exosome treatment resulted in increased ligand expression and homing frequency to the MLNs (Figures 3 and 4). Although its implication remains to be elucidated, the net effect of RA⁺ T exosomes seems to be a redirection of lymphocyte homing to the MLNs from the small intestine.

Exosomal miRNA profiling reveals the possible presence of molecular mechanisms that modify preexisting niches for lymphocyte homing

Exosomal miRNAs are the major bioactive molecules that modify the functionality of target cells.⁴⁸⁻⁵⁰ Of the miRNAs shown by our miRNA profiling analysis to be upregulated in RA⁺ T exosomes, we have identified several miRNA candidates that predict the targeting of NKX2.3, a transcription factor critical for the expression of MAdCAM-1 along with ICAM-1, VCAM-1, and/or CCL28. The NKX2.3-targeting miRNA candidates upregulated in RA⁺ T exosomes include miR-132 and miR-212 (predicted to target NKX2.3 and VCAM-1), miR-431 (NKX2.3 and ICAM-1), and miR-670 (NKX2.3). As previously shown by the NKX2.3 knockout mice that lacked the expression of MAdCAM-1, NKX2.3 is required to activate

Figure 6. Expression of several miRNA candidates associated with the downregulation of endothelial-ligand expression. (A) Data represent the numbers of miRNAs detected in RA⁻ and RA⁺ T exosomes. (B) Several representative miRNAs were validated for their upregulated expression in RA⁺ T exosomes by using a qPCR assay. U6 was used as an endogenous reference gene for normalizing miRNA levels. Data from qPCR analysis were normalized to the controls that represented samples from the RA⁻ T exosomes after being normalized to a reference U6 gene (2^{-ddCT}). Data are presented as the mean $\pm$ SEM obtained from 3 or 4 independent experiments. *P = .01 to <.05; **P = .001 to <.01 (vs RA⁻).

MAdCAM-1 transcription, by which the expression of MAdCAM-1 is induced.³³ We believe that suppression of MAdCAM-1 expression might be mediated by the additive effects of multiple NKX2.3-targeting miRNAs contained in RA⁺ T exosomes, which would inhibit T-cell homing to the small intestine. Notably, miR-132 and/or miR-212 have been implicated in the regulation of vascular integrity and proliferation by targeting Sirt1⁵¹ and p120RasGAP.⁵² miR-431 has been shown to protect neurons from ischemia-reperfusion injury⁵³ by targeting the ρ/ρ -kinase signaling pathway, which might also be involved in the regulation of endothelial cell integrity.

In summary, on the basis of the results discussed in this study, we believe that the exosomes secreted from gut-tropic memory/effector T cells act as a negative regulator that can adjust the levels of gut-specific lymphocyte homing by suppressing MAdCAM-1 expression in the small intestine. Future investigations will be required to substantiate and validate this concept under physiologic and pathophysiologic conditions.

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Authorship

Contribution: E.J.P. and O.P. performed all experiments and analyzed the results; Z.Y.S., S.D., M.G.A., and E.K. assisted in the isolation and analysis of exosomes; F.M. and H.S. helped characterize the exosomes; E.J.P. and M.S. designed all experiments and wrote the paper; and all authors approved the final manuscript.

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