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Intestinal epithelial cell-derived integrin αβ6 plays an important role in the induction of regulatory T cells and inhibits an antigen-specific Th2 response

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ABSTRACT

Toleroge nic DCs and Tregs are believed to play a critical role in oral tolerance. However, the mechanisms of the generation of tolerogenic DCs and activation of Tregs in the gut remain poorly understood. This study aims to dissect the molecular mechanisms by which IECs and protein antigen induce functional tolerogenic DCs and Tregs. Expression of αvβ6 by gut epithelial cell-derived exosomes, its coupling with food antigen, and their relationship with the development of functional tolerogenic DCs and Tregs were examined by using in vitro and in vivo approaches. The results show that IECs up-regulated the integrin αvβ6 upon uptake of antigens. The epithelial cell-derived exosomes entrapped and transported αvβ6 and antigens to the extracellular environment. The uptake of antigens alone induced DCs to produce LTGFβ, whereas exosomes carrying αvβ6/antigen resulted in the production of abundant, active TGF-β in DCs that conferred to DCs the tolerogenic properties. Furthermore, αvβ6/OVA-carrying, exosome-primed DCs were found to promote the production of active TGF-β in Tregs. Thus, in vivo administration of αvβ6/OVA-laden exosomes induced the generation of Tregs and suppressed skewed Th2 responses toward food antigen in the intestine. Our study provides important molecular insights into the molecular mechanisms of Treg development by demonstrating an important role of IEC-derived exosomes carrying αvβ6 and food antigen in the induction of tolerogenic DCs and antigen-specific Tregs. *J. Leukoc. Biol.* 90: 751–759; 2011.

Introduction

Oral tolerance plays an important role in the maintenance of intestinal homeostasis. The tolerogenic DCs are considered critical to this process by inducing the development of Tregs [1, 2]. Although the mechanisms still remain to be fully understood, it is believed that one of the tolerogenic DC-derived molecules involved in Treg induction is the TGF-β and/or IL-10 [3, 4]. However, very much still remains to be unraveled about the cellular and molecular mechanisms of tolerogenic DC development and that required for Treg induction/activation in the gut.

TGF-β is produced first in LTGFβ in tolerogenic DCs and Tregs. The generation of the bioactive form of TGF-β requires LTGFβ to be dissociated from its LAP [5]. Thus, the activation of LTGFβ represents an important regulatory step in the acquisition of tolerogenic or immunosuppressive properties of tolerogenic DCs and Tregs. Although several molecules, including integrin αvβ6 [5], αvβ8 [6], plasmin [7], thrombin [8], and matrix metalloproteinases [9], are involved in the activation of LTGFβ, the molecular mechanisms for the activation of LTGFβ in intestinal tolerogenic DCs and Tregs are still unclear.

It is believed that IECs play an important role in the generation of tolerogenic DCs and Tregs [10]. Like immune cells, such as DCs and mast cells, epithelial cells can generate and secrete small vesicles—exosomes [11]; published data [12] indicate that exosomes can serve as a carrier to bring antigens to remote tissue or organs to modulate the local immune responses. Conceivably, the IEC-derived exosomes would be able to carry not only the captured food antigens but also the surface integrin molecules such as αvβ6. However, the level of αvβ6 is generally low or unde-

Abbreviations: BMDC=bone marrow-derived DC, CIHR=Canadian Institutes of Health Research, Foxp3=forkhead box p3, IEC=intestinal epithelial cell, LAP=latency-associated peptide, LTGFβ=latent TGF-β, SEB=staphylococcal enterotoxin B, siRNA=small interfering RNA, Treg=regulatory T cell

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tectable in naïve epithelial cells [5], and the crypt area of intestinal epithelia is an exception [13]. Whether food antigen uptake can induce αvβ6 expression on IECs and what the relationship is between IEC-derived exosomes and oral tolerance, i.e., induction of tolerogenic DCs, Tregs, and LTGFβ activation, have remained unclear.

Thus, the present study aims to investigate whether IECs produce αvβ6 upon uptake of protein antigens; whether IEC-derived exosomes carry αvβ6 and antigens and whether such exosomes are able to facilitate the generation of tolerogenic DCs and antigen-specific Tregs; and what the role is of αvβ6 and antigen-carrying exosomes in the activation of LTGFβ. Our study for the first time has provided the important evidence that IEC-derived αvβ6 and food antigen-carrying exosomes play an important role in the development of tolerogenic DCs and antigen-specific Tregs. Furthermore, we show that administration of αvβ6 and antigen-carrying exosomes inhibits the antigen-specific Th2 immune responses in vivo. Thus, our study provides important molecular insights into the molecular mechanisms of oral tolerance.

MATERIALS AND METHODS

Mice

Balb/c mice, 6–8 weeks old, were purchased from Charles River Canada (St. Constant, QC, Canada). OVA TCR-transgenic DO11.10 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were maintained in a pathogen-free environment. The procedures of experiments in this study were approved by the Animal Care Committee at McMaster University (Hamilton, ON, Canada).

Cell culture

Mouse IEC line IEC4.1 cells were cultured at 37°C with 5% CO_2 in a 1:1 mixture of DMEM and Ham's F-12 medium, supplemented with 2% penicillin/streptomycin, 0.6% L-glutamine, 1.92% $NaHCO_3$, and 10% FCS.

Exosome preparation

IECs were incubated with antigens for 24 h; the supernatants were collected and centrifuged at 300 *g* (10 min), 1200 *g* (20 min), and 10,000 *g* (30 min) to remove cell debris. Exosomes were pelleted at 100,000 *g* for 1 h and resuspended in PBS for further experiments. The protein in exosomes was quantified using a Bradford assay.

Electron microscopy

Jejunal tissue was processed for ultrathin sections. Exosomes were prepared as aforementioned, except fixatives were added to the culture supernatant for 2 h before ultracentrifugation at a final concentration of 0.75% (glutaraldehyde) and 2% (paraformaldehyde). Gelatin solution (10%) 0.5 ml was added to the centrifuge tubes to collect the precipitated exosomes. A gelatin block was formed in a few minutes and cut to small blocks of 2 × 2 × 5 mm in size. The samples were processed with established protocol and embedded with LR White resin. Ultrathin sections were stained with antibodies against OVA and αvβ6 and then with gold particle-labeled second antibody (6 nm for OVA; 12 nm for αvβ6). Sections were counterstained with 2% aqueous uranyl acetate, followed by 0.2% lead citrate. Samples were observed with a JEOL JEM-1200 EX TEM.

Flow cytometry

Cells were collected and incubated with primary antibodies on ice for 30 min (for the intracellular staining, cells were fixed with 1% paraformaldehyde on ice for 30 min and incubated with permealization reagents for 30 min on ice). The stained cells were analyzed using a FACSArray (BD Biosciences, San Jose, CA, USA). Data were analyzed with FlowJo software.

RNA interference

siRNA transfection was performed following our established procedures [14] and detailed in Supplemental Materials.

Statistics

All values were expressed as the means ± SD of at least three separate experiments. The values were analyzed using the two-tailed unpaired Student's *t* test when data consisted of two groups or by ANOVA when three or more groups were compared. $P < 0.05$ was accepted as statistically significant.

In addition, reagent information, BMDC generation, Western blotting, and immune staining were presented in Supplemental Materials.

RESULTS

IECs up-regulate αvβ6 upon uptake of protein antigen

Integrin αvβ6 has been found to play a role in converting LTGFβ to bioactive TGF-β, which contributes to the maintenance of immune homeostasis in the body [15]. Although the expression of αvβ6 is restricted in epithelial cells, its level is, in general, low in naïve status but is increased rapidly upon activation, as seen in wounds or under inflammatory conditions [5]. Considering that the absorption of certain proteins also induces a series of activities in epithelial cells, we hypothesize that uptake of protein antigen could increase the expression of αvβ6 in the IECs. By quantitative PCR, confocal microscopy, and Western blotting assays, weak expression of integrin αvβ6 was detected in murine jejunal epithelial cells and the IEC4.1 cell line (IEC, derived from Balb/c mouse small IECs) at a naive state. After addition of a protein antigen, OVA, peanut protein, or BSA to the culture, expression of αvβ6 was similarly induced in IEC in a dose-dependent manner at mRNA and protein levels (**Fig. 1A** and **B**). In addition, an increase in αvβ6 expression was detected in the Caco-2 cell line, HT-29 cell line, and IEC-6 cell line in response to OVA uptake (data not shown). To see if microbial products were involved in an OVA-induced increase in αvβ6 expression, we exposed IEC to OVA, together with LPS, SEB, or flagellin in culture. However, the exposure to those microbial products suppressed the expression in IEC (Fig. 1C). Similar to cell-culture results, feeding with a regular protein-containing diet induced in vivo expression of αvβ6 in jejunal epithelial cells of the mice (Fig. 1D, 1). A switch to feeding with a no-protein diet resulted in the loss of αvβ6 expression within 5 days (Fig. 1D, 2–4), which could be restored by gavage feeding with OVA (Fig. 1D, 5; also see Supplemental Fig. 1). These results suggest that the uptake of protein antigens up-regulates αvβ6 expression in IECs.

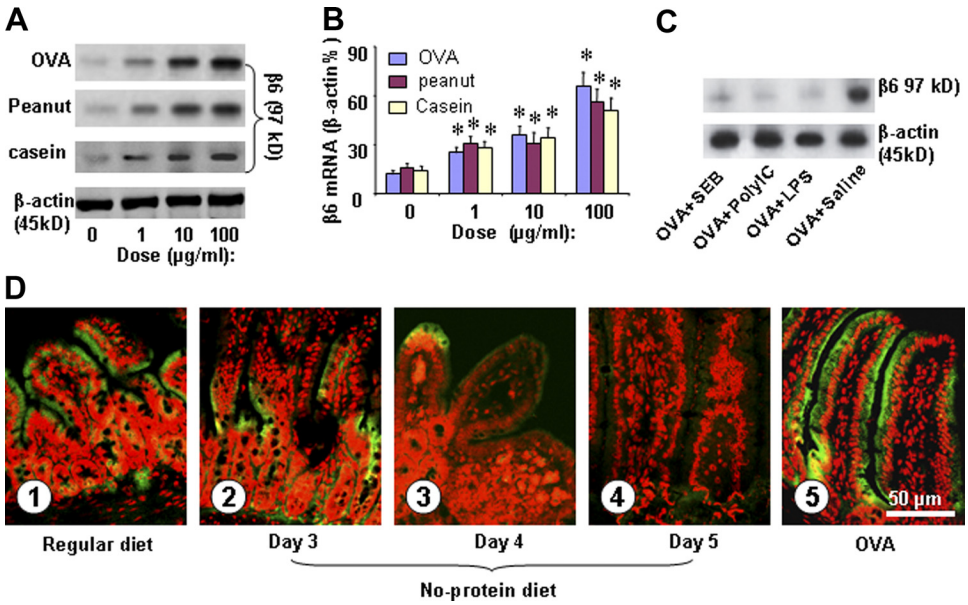

Figure 1. IECs express *αvβ6*. (A and B) Mouse IEC line IEC4.1 cells were cultured with serum-free media overnight and then exposed to OVA, peanut protein, or casein for 6 h. The cells were then collected and processed for measurement of expression of β6. The immunoblots in A and bars in B indicate the level of β6 in cellular extract (data in B are presented as mean±SD). Data represent three separate experiments. *Compared with group 0. (C) IECs were cultured in the presence of OVA + SEB (50 ng/ml), OVA + polyinosinic:polycytidylic acid (PolyIC; 100 ng/ml), OVA + LPS (200 ng/ml), or OVA + saline for 24 h. The immune blots show β6 protein in IEC extracts. (D) Expression of β6 (stained in green) in mouse jejunal epithelial cells (six mice/group). Regular diet: naïve mice that were fed with normal food. No-protein diet: mice were fed with no-protein food. OVA: Five hours after mice were gavage-fed with OVA (10 mg/mouse). Randomly chosen 20 fields were observed for each mouse. The nuclei were stained in red for morphological view. No stain was observed in sections stained with isotype IgG or the sections omitted the primary antibody (data not shown).

Epithelial cell-derived exosomes carry αvβ6 and foreign antigen

As αvβ6 is not a secreted protein and is expressed on the plasma membrane, a shuttle carrier is required for transporting αvβ6, if indeed this molecule is involved in the induction of tolerogenic DCs. The putative carrier can be exosomes, nanovesicles originating from multivesicular bodies and found to be secreted by a variety of cell types including IECs [11, 12]. To investigate if this may be the case, we treated IECs with OVA in culture for 24 h and then purified the exosomes from culture media with our established procedures [16]. Indeed, using electron microscopy and immunogold-labeled antibodies, the αvβ6 and absorbed OVA were colocalized inside the purified exosomes from OVA-loaded IECs but not in those from the IEC without OVA treatment (**Fig. 2A–C**). On the other hand, αvβ6/OVA-containing exosomes were also found in the jejunal subepithelial region of mice fed with OVA (Supplemental Fig. 2). The proteins of αvβ6 and OVA were identified further in purified exosomes by Western blotting (Fig. 2D).

To investigate if DCs are able to capture the epithelial exosomes, immature BMDCs were prepared and exposed to αvβ6/OVA-containing exosomes in culture for 30 min. As shown by flow cytometry, >90% BMDCs captured the αvβ6/OVA-containing exosomes (Fig. 2E and F; also see Supplemental Fig. 3). These findings together suggest that IEC-derived exosomes can serve as a carrier to shuttle the protein antigen-induced αvβ6 and protein antigen from epithelial cells to the periepithelial cell microenvironment, where it is to be picked up by antigen-presenting DCs.

αvβ6 and antigen-carrying exosomes modulate the expression of TGF-β in DCs

Tolerogenic DCs are important in the maintenance of immune homeostasis in the body [2, 17]. However, the mechanisms regulating the development of TGF-β-producing tolerogenic DCs are not fully understood, particularly in the intestine rich in such tolerogenic DCs [18]. As one of the main functions of the intestine is to absorb nutrients and transport some of these nutrients to the subepithelial region, whereby nutrients interact with immune cells, and we have shown above that IEC-derived exosomes containing food antigen and αvβ6 molecules can be picked up by DCs, it is likely that such exosomes may promote the expression of TGF-β and/or IL-10 in immature DCs. To examine whether this might be true, we first examined the expression of LTGFβ in BMDCs without exposing it to intestinal exosomes. As expected, after it was exposed to OVA in culture for 24 h, the expression of LTGFβ, shown as the level of the LAP but not active TGF-β in BMDCs, was increased in an OVA dose-dependent manner (**Fig. 3A**). The expression of IL-10 in these BMDCs was under a detectable level (data not shown).

To examine whether the epithelial cell-derived exosomes carrying OVA antigen and αvβ6 could not only induce LTGFβ expression in immature DCs but also convert LTGFβ to TGF-β in these cells, immature BMDCs were exposed to OVA, αvβ6, both OVA/αvβ6, or OVA/αvβ6-carrying exosomes for 48 h. As shown by the Western blotting assay, the amounts of TGF-β were increased significantly in BMDCs exposed to OVA/αvβ6 or OVA/αvβ6-carrying exosomes but not in those exposed to OVA alone, αvβ6 alone, or medium alone (Fig. 3B). Furthermore, TGF-β was de-

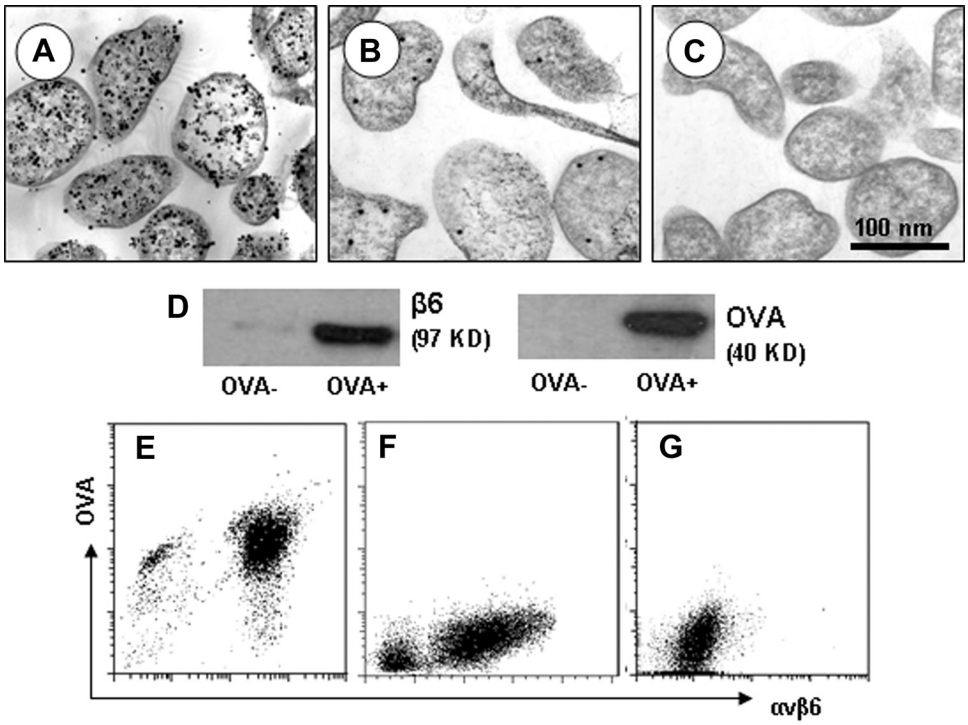

Figure 2. IEC-derived exosomes carry αvβ6 and antigen. OVA (20 μg/ml) were added to subconfluent IEC culture overnight. Exosomes were purified from the supernatants, embedded with gelatin/resin, and processed for ultrathin sections, which were subjected to immunogold electron microscopy. (A–C) Immunogold electron microphotograph shows that exosomes contain OVA (labeled by the small gold particles; 6 nm) and αvβ6 (labeled by the large gold particles; 12 nm); exosomes were purified from OVA-treated IEC (A) or naïve IEC (B); (C) a negative-staining control. (D) Western blotting gels show immune blots of αvβ6 and OVA in extracts of lysed exosomes from naïve (OVA–; IECs were not exposed to OVA) or OVA-treated (OVA+) IECs. (E–G) Flow cytometry plots show that BMDCs captured the αvβ6 and OVA-containing (E) or no-OVA (F) exosomes. (G) Naïve control. Each experiment was repeated three times.

tected on the surface of DCs treated with OVA/αvβ6 or OVA/αvβ6-carrying exosomes, as demonstrated by flow cytometry. Treatment with αvβ6-deficient but OVA-laden exosomes, with OVA alone or with αvβ6 alone, did not result in TGF-β expression in DCs (Fig. 3C). These results indicate that IEC-derived antigen/αvβ6-carrying exosomes have the capacity to induce the development of the DCs of the tolerogenic phenotype.

As expressing only moderate levels of costimulatory molecules is another major feature of tolerogenic DC [19], we also examined the expression of CD80 and CD86 in BMDCs treated with OVA/αvβ6-carrying exosomes. As shown by flow cytometry, levels of CD80 and CD86 were significantly lower in BMDCs treated by exosomes than those treated with LPS but still higher than those treated with OVA alone (Supplemental Fig. 4A). This thus adds further evidence

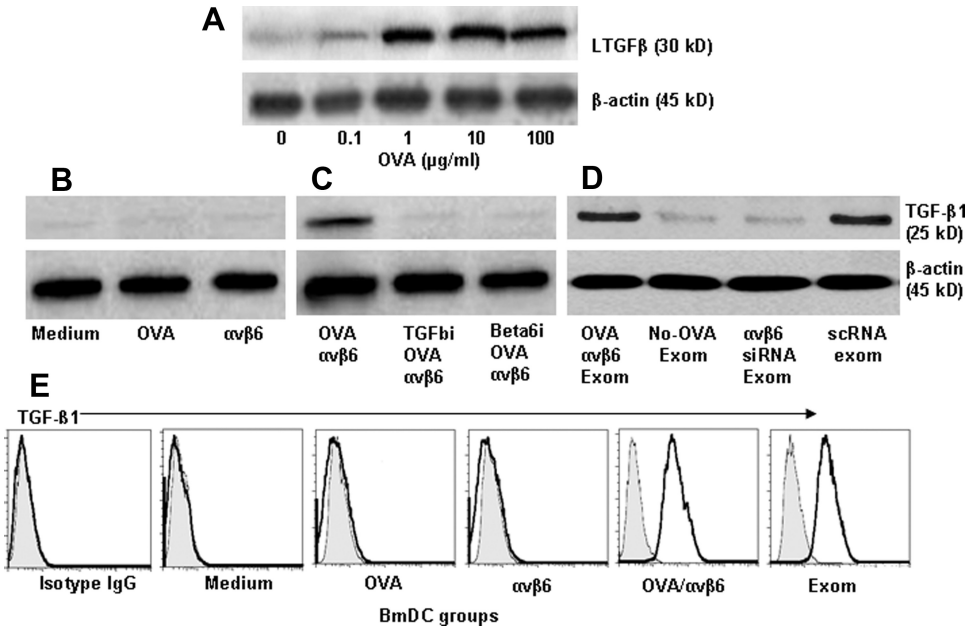

Figure 3. DCs express the TGF-β or LTGFβ upon antigen uptake. (A) Immature BMDCs were cultured in the presence of OVA at graded doses for 24 h. The Western blots show the LAP amount in cellular extracts of BMDCs. (B–D) Immune blots show TGF-β1 in BMDCs. (B) BMDCs were exposed to medium alone, OVA, or αvβ6. (C) BMDCs were exposed to both OVA/αvβ6 or pretreated with anti-TGF-β antibody (TGFbi) or anti-β6 antibody (Beta6i). (D) BMDCs were exposed to OVA/αvβ6-carrying exosomes (Exom), exosomes carrying αvβ6 (No-OVA), or exosomes carrying OVA, no αvβ6 (αvβ6 siRNA; the gene of αvβ6 was knocked down by siRNA). scRNA exom, Exosomes were generated by IEC-treated with small cytoplasmic RNA and OVA. (E) Flow cytometry histograms show TGF-β1$^+$ BMDCs. The BMDCs were treated under the same condition as described in B. The solid histograms represent "no staining cells". The open histograms show TGF-β1$^+$ BMDCs. Each experiment was repeated three times.

that OVA/αvβ6-carrying exosomes have the ability to generate "semi" mature DCs that possess the tolerogenic properties. We also checked whether $CD103^+$ and $CD103^-$ DCs respond to OVA/αvβ6-carrying exosomes differently; the BMDCs in Supplemental Fig. 4A were analyzed further by the gating technique. The results showed that the expression of TGF-β was induced in both subtypes of BMDCs with higher frequency of TGF-β^+ DCs in $CD103^+$ DCs than in $CD103^-$ DCs (Supplemental Fig. 4B).

The above evidence that OVA/αvβ6-carrying, exosome-primed DCs expressed TGF-β in culture indicates that the TGF-β-carrying tolerogenic DCs can be induced in a subepithelial region and contact other immune cells, such as naïve $CD4^+$ T cells, which have the potential to be converted to Tregs [20]. To test the hypothesis, a batch of exosomes was prepared with OVA peptide$_{323–339}$, using the same procedures as above and used to prime BMDCs. OVA-transgenic TCR $CD4^+$ $CD25^-$ T cells were isolated from the spleen of DO11.10 mice and exposed to exosome-primed BMDCs in culture for 72 h. The frequency of the $Foxp3^+$ T cell was then examined by flow cytometry. The data showed that $Foxp3^+$ Tregs were generated in an exosome-primed BMDC number-dependent manner (**Fig. 4A**). On the other hand, OVA alone- or αvβ6 alone-treated BMDCs did not have an apparent effect on the expression of Foxp3 in $CD4^+$ $CD25^-$ T cells (Fig. 4B). The expression of Foxp3 in this experimental system could be blocked by treatment with anti-TGF-β antibody (Fig. 4C, 1) or knocked down the gene of αvβ6 (Fig. 4C, 2).

αvβ6 and antigen-carrying intestinal epithelial exosomes are able to induce functional tolerogenic DCs and Tregs to suppress antigen-specific Th2 responses in the intestine

To gain further insight into the physiological activities of αvβ6/antigen-carrying exosomes, we first tested the effects of such exosomes in naïve mice. To this end, naïve Balb/c mice were injected with αvβ6/OVA-carrying exosomes via the tail vein every other day for four times. After killing the mice on Day 10, $CD11c^+$ TGF-β^+ cells were detected in isolated mononuclear cells from the spleen and intestine (Supplemental Fig. 5). $CD4^+$ $Foxp3^+$ Tregs were also increased in the spleen and intestine in naïve mice and mice sensitized to OVA. However, feeding-sensitized mice with specific antigen OVA did not increase the number of $CD4^+$ $Foxp3^+$ Tregs in intestine and spleen. The induced $CD4^+$ $Foxp3^+$ Tregs exerted strong immune-suppressive function on antigen-specific T cell proliferation (Supplemental Fig. 6). In contrast, $CD4^+CD25^+$ T cells isolated from naïve mice did

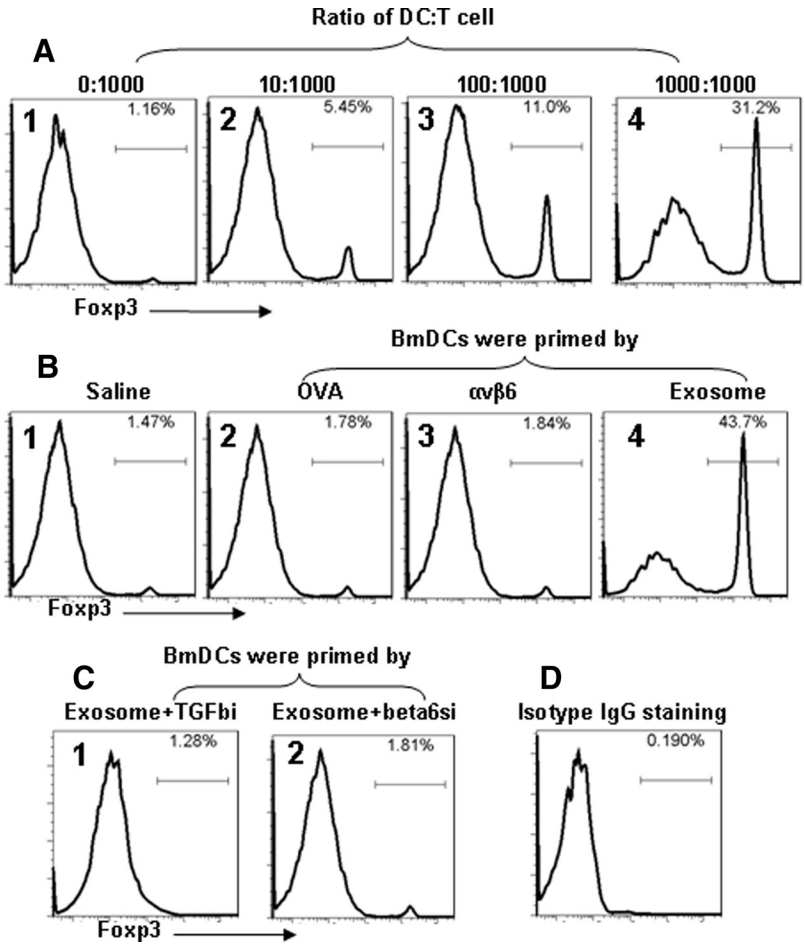

Figure 4. Exosome-primed DCs favor Treg development. DO11.10 $CD4^+CD25^-$ T cells were isolated from the spleen with MACS and cocultured with OVA$_{323–339}$/αvβ6-laden, exosome-primed BMDCs (DC; 10^6 T cell:10^5 DC/well), in addition to the indicated conditions for 72 h. The cells were collected at the end of culture, stained with anti-Foxp3 (the indicator of Treg in this experimental system), and analyzed by flow cytometry. The data are presented as histograms; the gated area indicates the frequency of $Foxp3^+$ T cells. (A1–4) The histograms show the frequency of $Foxp3^+$ Tregs induced by exosome-primed DCs at indicated ratios. (B1–4) The histograms show the frequency of $Foxp3^+$ Tregs in response to saline (B1), OVA alone (B2), αvβ6 alone (B3), or OVA/αvβ6-carrying exosomes (B4). (C1 and 2) These two histograms show the inhibitory effect of anti-TGF-β antibody (C1) or knocking down the gene of αvβ6 (C2) in exosome-producing IECs on the induction of Treg. (D) The histogram shows isotype IgG staining used as a negative control.

not show any antigen-specific, immune-suppressive activity (Supplemental Fig. 6).

To investigate the function of αvβ6/antigen-carrying epithelial exosomes in a relevant disease model, a skewed Th2 response mouse model was developed by following the procedures depicted in **Fig. 5**. Some sensitized mice received the exosomes carrying antigen together, with or without αvβ6, via the tail vein on Days 16, 18, 20, and 22. Mice were then challenged with OVA and killed on Day 33 (Fig. 5A). The OVA-challenged, sensitized mice treated with αvβ6-deficient exosomes or with OVA alone showed high levels of serum OVA-specific IgE (Fig. 5B) and IL-4 (Fig. 5C) and marked OVA-specific Th2 cell proliferation (Fig. 5D); the flow cytometry CFSE dilution assay showed that proliferated cells were IL-4$^+$ cells (data not shown). On the contrary, the mice treated with αvβ6/OVA-carrying exosomes had markedly reduced Th2 immune responses (Fig. 5). To further understand the mechanism, CD11c$^+$ DCs and Tregs were isolated from the spleen and the lamina propria of these mice and stained with the fluorescence-labeled anti-TGF-β antibody. The results of flow cytometry analysis showed significantly less CD11c$^+$ TGF-β$^+$ and Foxp3$^+$ TGF-β$^+$ cells in samples from mice sensitized to antigen (**Fig. 6**, column 2 vs. column 1), which were reduced further by challenge with specific antigen OVA (Fig. 6, column 3). In contrast, pretreatment with αvβ6/OVA-carrying exosomes dramatically increased CD11c$^+$ TGF-β$^+$ and Foxp3$^+$ TGF-β$^+$ cells in the spleen and intestine (Fig. 6, column 4), whereas pretreatment with αvβ6-deficient exosomes carrying antigen did not have such effect (Fig. 6, column 5).

DISCUSSION

Although it is well-known that there are much more tolerogenic DCs in the intestine than in other compartments of the body [2, 3], the mechanisms for generation of tolerogenic DCs remain largely unknown. The present study demonstrates that protein antigens induce DCs to produce the precursor of TGF-β, LTGFβ. The data indicate that the IECs produce the integrin αvβ6 upon uptake of protein antigen, which is entrapped into the same exosomes with protein antigen to be released to the micro milieu, such as the subepithelial region, where exosomes can be captured by DCs. Such process results in the conversion of LTGFβ to the active form, TGF-β, within DCs, thus conferring the DCs with tolerogenic properties. Our study further demonstrates that such αvβ6/OVA-carrying, exosome-primed tolerogenic DCs are capable of driving naïve T cells to become the OVA-specific Tregs. Furthermore, the OVA-specific Treg activation requires contact with αvβ6/OVA-carrying, exosome-primed tolerogenic DCs.

DCs are distributed in the lamina propria immediately below the epithelial layer. This anatomical feature confers to DCs the advantage to capture absorbed foreign antigens before they have the opportunity to contact other immune cells. Therefore, antigen-laden DCs have the privilege to decide the types of subsequent immune response. We have known that there are more tolerogenic DCs in the intestine compared with other body compartments. The mechanism is not fully understood. The present data provide evidence to explain that IEC-derived αvβ6, in synergy with absorbed antigen, has the ability to facilitate the development of tolerogenic DCs in the intestine. The data are in line with previous studies: Travis et al. [6] suggest that integrin αvβ8 plays a role in the induction of oral tolerance; Coombes and Powrie [21] and others [22] indicate that CD103$^+$ DCs are important in the establishment of oral tolerance. CD103 is also an integrin. Collectively, our data and these pioneer studies indicate that some types of integrin can benefit tolerogenic DC development. The present data provide mechanistic information that integrin αvβ6 converts the precursor TGF-β to functional TGF-β in DCs, which converts immature DCs to tolerogenic DCs.

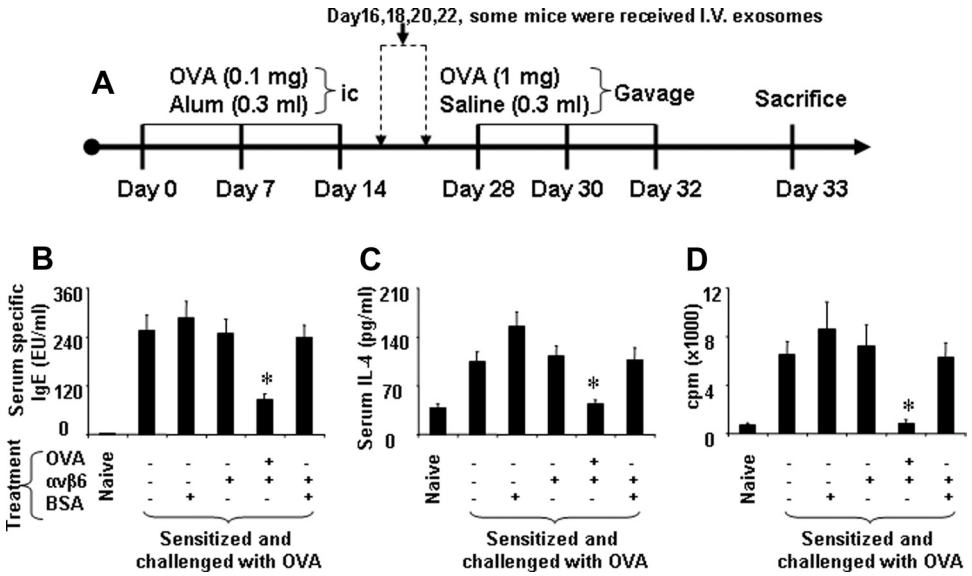

Figure 5. Specific antigen/αvβ6-laden exosomes repress an antigen-specific Th2 response in the intestine. Grouped Balb/c mice (six/group) were sensitized to OVA with alum following the procedures in A. Mice were also treated with exosomes carrying OVA (or BSA) and/or αvβ6 (20 μg/mouse) on Days 16, 18, 20, and 22 via tail-vein injection. ic, subcutaneous injection. (B) Bars indicate serum level of OVA-specific IgE. (C) Bars indicate serum level of IL-4. (D) Bars indicate cpm of [^{3}H] thymidine incorporation in lamina propria mononuclear cell. Data are presented as the means ± SD. *$P < 0.05$, compared with allergic mice, were not treated with exosomes.

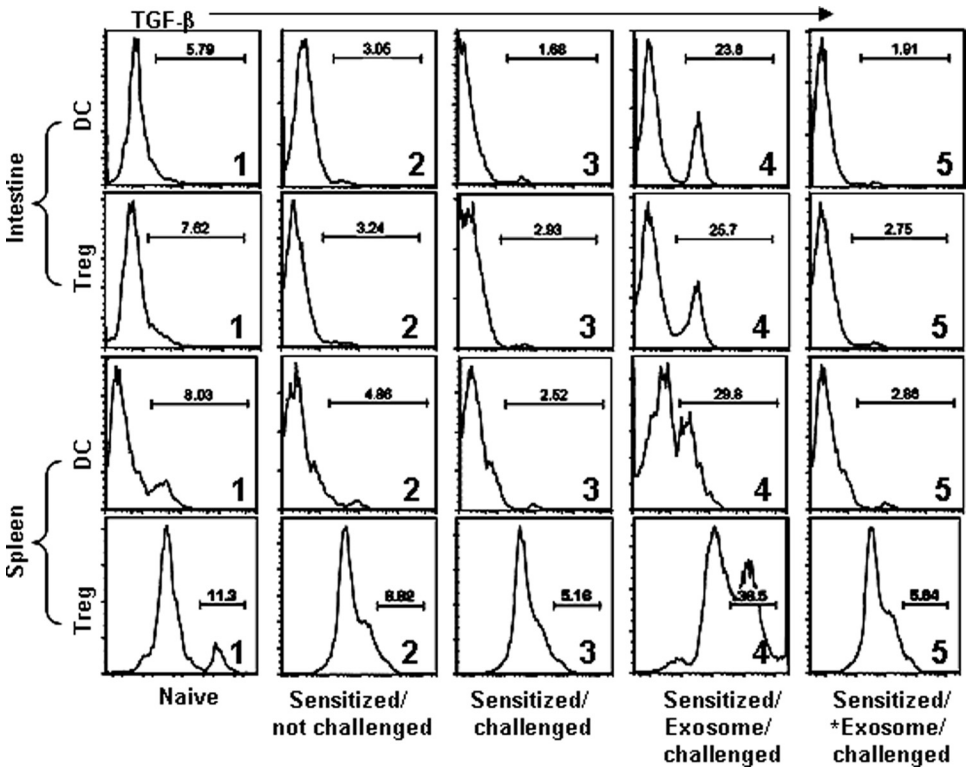

Figure 6. Antigen/αvβ6-carrying exosomes activate Tregs in vivo. Mice (six of each group) were treated with conditions presented below each column of the flow cytometry histograms. Sensitized: mice were sensitized to OVA. Challenged: mice were challenged by specific antigen OVA. Exosome: mice were treated with OVA/αvβ6-carrying exosomes via tail-vein injection. *Exosome: exosomes carrying OVA but no αvβ6. After killing the mice, mononuclear cells were isolated from the spleen and intestine. $CD25^{hi}$ $CD127^{lo}$ cells (Tregs) were further isolated from prepared $CD4^+$ $CD25^+$ T cells and analyzed by flow cytometry to evaluate the rate of TGF-β^+ cells. The histograms show the rate of TGF-β^+ cells.

A number of reports indicate that αvβ6 has an important function to convert LTGFβ to its function form, TGF-β [5]. Loss of αvβ6 results in immune inflammation by the mechanism of impairing the immune-suppressive function of Tregs [5]. The present data add further information to understanding the functions of αvβ6, in addition to converting LTGFβ to TGF-β, which also favors the development of tolerogenic DCs. It is suggested that DCs in the intestine have default tolerogenic properties based on the production of TGF-β [18]. Our study expands the notion by revealing the regulatory mechanism of TGF-β production by DCs. The data show that uptake of protein antigen only increases the expression of the precursor of TGF-β, LTGFβ, in DCs. These DCs actually do not have the tolerogenic properties yet; e.g., they are not able to drive naïve $CD4^+$ T cells to become $CD4^+$ $Foxp3^+$ Tregs, as observed in the present study. The LTGFβ in these DCs requires it to be further converted to TGF-β to gain the tolerogenic properties. Although the mechanisms by which OVA induces LTGFβ in DCs are still unknown, our preliminary data suggest a role by IDO, which was induced in DCs by LPS-free OVA (data not shown). Indeed, IDO blockade was recently found to diminish OVA-induced oral tolerance [23].

Several molecules have been reported to have the capacity to convert LTGFβ to TGF-β, including proteolytic cleavage of LAP by plasmin, thrombin, matrix metalloproteinases, or endoglycosidases; cross-linking by transglutaminase, steroids, or active oxygen species; or binding to thrombospondin-1 or αvβ6 integrin [9]. These molecules dissociate the LTGFβ from the LAP to release TGF-β from the complex. The source and the transport pathway of these molecules need to be taken into account in studies of tolerogenic DC development. Our data show that one of these molecules, αvβ6, can be rapidly up-regulated in IECs upon uptake of protein antigens and suggest IECs to be an important source of αvβ6 in the body. As αvβ6 is expressed on the plasma membrane, it requires a specific transport device to carry it to the destination. Our study reveals that IEC-derived exosomes can fulfill this duty by carrying αvβ6 from epithelial cells to the subepithelial region, where the αvβ6 molecules have opportunity to contact immune cells.

Immature DCs mature quickly upon the uptake of antigens. Once mature, DCs do not further absorb any substances. As shown by the present study, protein antigen and αvβ6 are required to be absorbed into DCs to generate the tolerogenic DC. The data indicate that epithelial cell-derived exosomes are an optimal device to meet this requirement. They entrap specific antigen and αvβ6 into the same exosomes to be absorbed by DCs. As DCs may also express αvβ8, able to convert LTGFβ to TGF-β [6], and contribute to the development of tolerogenic DCs, we also measured the expression of αvβ8 in DCs after exposure to antigen; however, the expression of αvβ8 in DCs was under detection in our experimental system. On the other hand, in contrast to the drastically increased expression of CD80 and CD86 in DCs, induced by exposure to LPS, the exosome-treated DCs only produced mild amounts of CD80 and CD86, indicating that these DCs are partially mature, a condition required in the development of Tregs [24].

It is suggested that exogenous TGF-β can induce naïve $CD4^+$ T cells to develop into $CD4^+$ $Foxp3^+$ Tregs [20, 24, 25]. Tolerogenic DCs can be the regular endogenous

source of TGF-β to be used in the generation of Tregs [3]. The activation of Tregs plays an important role in suppressing other effector T cell responses that are essential to the maintenance of immune homeostasis [26]. TGF-β and/or IL-10 are suggested to be the major effector molecules in Treg-mediated immune suppression [20, 24, 25]. The expression of TGF-β and/or IL-10 on the surface of Tregs is believed to increase upon activation. It is known that TGF-β exists as a precursor, LTGFβ, after synthesis [5]. It requires to be dissociated from the LAP before gaining the immune-regulatory ability. Our data demonstrate that this phenomenon also exists in Treg activation. The increases in the LTGFβ, but not the TGF-β, were found in Tregs upon specific antigen exposure. Apparently, the produced LTGFβ in Tregs still needs to be converted to TGF-β. Thus, the sources of LTGFβ converters are essential to the immune-suppressive function of Tregs. The present study demonstrates that specific antigen/αvβ6-carrying, exosome-pulsed DCs have the capacity to convert LTGFβ to TGF-β in Tregs to render these cells to be functionally capable.

It is proposed that IL-10 also plays a role in the generation of Tregs, such that pulmonary DC-derived IL-10 promotes the generation of IL-10-producing Tr1 cells [27]. However, we did not detect the expression of IL-10 in DCs in our system, which did not increase after uptake of protein antigen or antigen/αvβ6-carrying exosomes. Furthermore, blocking IL-10 did not prevent the production of TGF-β in DCs and Tregs when treated with antigen/αvβ6-carrying exosomes (data not shown). These results suggest an IL-10-independent pathway for Treg generation in the intestine. On the other hand, if coexisting with IL-6, TGF-β-producing DCs may not induce Treg development; instead, they may induce Th17 cell development.

Our study demonstrates further that administration of specific antigen/αvβ6-carrying exosomes could specifically suppress antigen-specific Th2 responses in a mouse model, thus suggesting the therapeutic potential of this strategy for the treatment of antigen-related immune inflammation, such as allergic diseases. The delivered exosomes were found to induce tolerogenic DCs and Tregs in the gut (Supplemental Fig. 5, and not shown), thus helping to restore tolerance toward protein antigen in sensitized animals. Although the mechanisms by which oral OVA challenge induce Th2 allergic inflammation in the gut of OVA-sensitized mice remain largely unclear, our preliminary data indicate that oral, OVA-induced tolerogenic DCs fail to induce conventional Tregs in OVA-sensitized mice. This suggests that the presence of antigen-specific Th2 cells or their molecules at the site of protein antigen exposure curtails the generation of Tregs and thus, favors a skewed Th2 response.

Recent reports indicate that $CD103^+$ DCs also play an important role in the generation of Tregs in the intestine [21, 22]. Naïve DCs up-regulate CD103 and acquire a tolerogenic phenotype. We also examined the expression of TGF-β expression in $CD103^+$ DCs after exposure to αvβ6 and protein antigen-carrying exosomes. Our data show that the frequency of TGF-β^+ DCs is less in $CD103^+$ DCs than in $CD103^-$ DCs; the difference may be because the phagocytosis ability is higher in $CD103^-$ DCs than $CD103^+$ DCs [28].

In summary, the present study reveals a novel pathway by which uptake of protein antigen leads to the integration of αvβ6 and protein antigen to exosomes in IECs and their subsequent release to the vicinity of epithelial cells. Such exosomes favor the development of TGF-β-producing, tolerogenic DCs, which in turn, drives the generation of food antigen-specific Tregs. Re-exposure to specific antigen/αvβ6-carrying, exosome-pulsed DCs plays an important role in turning LTGFß-producing, antigen-specific Tregs to be TGF-β-producers.

AUTHORSHIP

X.C., C-H.S., B-S.F, T-L.L., P-Y.Z, and P.L. were involved in performing experiments and revising the manuscript. X-M.C and Z.X. were involved in experimental design, discussion, and paper writing. P-C.Y. was the principle investigator supervising the project and contributed to project design, data analysis, and paper writing.

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DISCLOSURES

The authors do not have any financial conflict of interest to declare.

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KEY WORDS:
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