B cell–derived exosomes can present allergen peptides and activate allergen-specific T cells to proliferate and produce T_H2-like cytokines

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Background: Exosomes are vesicles of 30 to 100 nm produced by inward budding of endosomal compartments and are released by a range of different cell types. Exosomes from antigen-presenting cells carry immunorelevant molecules like MHC class I and II and costimulatory molecules and thus are suggested to have a role in immune modulation.
Objective: To investigate the role of antigen-presenting cell derived exosomes in allergen presentation and T-cell stimulation.
Methods: Exosomes were isolated from supernatants of B-cell lines derived from patients with birch pollen allergy. The exosomes were characterized with regard to the expression of surface molecules by flow cytometry. Moreover, exosomes were loaded with T-cell–activating peptides from the major birch allergen Bet v 1, and binding was tested with ELISA. Loaded exosomes were used for stimulation of Bet v 1–specific T-cell lines. Cell proliferation and cytokine production were assessed.
Results: The exosomes had a phenotype typical of B cell–derived exosomes with expression of MHC, costimulatory molecules like CD86, tetraspanin proteins such as CD81, and CD19. Furthermore, B cell–derived exosomes bound Bet v 1–derived peptides and subsequently induced a dose-dependent T-cell proliferation. In addition to proliferation, T cells synthesized the cytokines IL-5 and IL-13 in response to peptide-loaded exosomes.
Conclusion: These results demonstrate for the first time that exosomes isolated from B cells can present allergen-derived peptides and thereby induce T-cell proliferation and T_H2-like cytokine production.
Clinical implications: Our data suggest that exosomes from B lymphocytes are an immunostimulatory factor in allergic immune responses. (J Allergy Clin Immunol 2007;120: 1418-24.)

Key words: *Exosomes, Bet v 1, allergy, T cells, B cells*

Exosomes are nanovesicles, 30 to 100 nm in diameter, produced by inward budding of endosomal membranes. They are released extracellularly from a wide range of cell types like dendritic cells (DCs),[1] T and B lymphocytes,[2,3] mast cells,[4] platelets,[5] epithelial cells,[6] and tumor cells.[7] Exosomes from antigen-presenting cells (APCs) bear MHC class I and II, costimulatory molecules like CD54, CD80, and CD86,[8] and they are enriched in tetraspanin proteins like CD63 and CD81.[9] Exosomes have been characterized in clinical samples like bronchoalveolar lavage (BAL),[10] urine,[11] malignant effusions,[12] plasma,[13] and breast milk.[14] The role of exosomes *in vivo* is not known, but it has been shown that exosomes, depending on cell origin, can function as a transport vesicle for removal of unwanted molecules,[15] activate T cells,[16,17] and transport antigen between APCs,[18] and can be of importance in tolerance induction[19] and in inhibiting antitumor responses.[1,20] Exosomes have also been investigated as tools in immunotherapy for cancer,[21] infections,[22] and transplantation.[23]

The mechanisms behind exosome-induced immune stimulation are unknown. Several studies have demonstrated that exosomes can stimulate T cells directly,[16,17] whereas others have shown that exosomes exert their effects through APCs.[18] The role of exosomes in allergic immune responses has previously not been investigated. Allergic diseases are chronic inflammatory disorders in which the immune system is reacting to innocuous antigens in the environment. IgE-mediated allergic diseases affect more than 25% of the children in industrialized countries.[24] Allergen-specific T_H2 cells, activated by APCs presenting allergen-derived peptides to secrete

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Abbreviations used
APC: Antigen-presenting cell
BAL: Bronchoalveolar lavage
CBA: Cytometric bead array
cpm: Counts per minute
DC: Dendritic cell
FITC: Fluorescein isothiocyanate
PE: Phycoerythrin
TCL: T-cell line

T_H2 cytokines like IL-4, IL-5, and IL-13, have a central role in the pathophysiology of allergic diseases.[25]

Our aim was to investigate whether exosomes can bind and present allergen-derived peptides and stimulate allergen-specific T cells. For this purpose, exosomes were isolated from B-cell lines derived from individuals with allergy and analyzed for the presence of membrane molecules important for antigen presentation. Exosomes were loaded with peptides containing relevant T-cell epitopes of the major birch pollen allergen, Bet v 1. Bet v 1–specific T-cell lines (TCLs) were established from individuals with birch pollen allergy and stimulated with unloaded and peptide-loaded exosomes. Proliferation and cytokine responses were assessed.

METHODS

Patients with birch pollen allergy

Individuals with allergy (donors 1, 2, 3, and 4, Table I) with a history of hay fever during early spring, a specific serum IgE ImmunoCAP class >3 to birch pollen and Bet v 1 (Phadia AB, Uppsala, Sweden), and a positive skin prick test reaction (wheal diameter >5 mm) to birch pollen (Soluprick; ALK-Abelló, Hørsholm, Denmark) were included in the study. HLA-typings for HLA-DRB and DQB alleles were performed according to methods described[26] (Table I). The study was approved by the local ethics committee.

Synthesis, biotin labeling, and purification of Bet v 1–derived peptides

Peptides (sequence: Bet v 1 amino acid residues 4-18 [Bet v $1_{4\text{-}18}$]: NYETETTSVIPAARL; Bet v 1 amino acid residues 142-156 [Bet v $1_{142\text{-}156}$]: TLLRAVESLLAHSD) were synthesized using Fmoc (9-fluorenylmethoxycarbonyl)-strategy with HBTU (2-[1H-Benzotriazol-1-yl] 1,1,3,3 tetramethyluronium hexafluorophosphate)-activation (0.1 mmol small-scale cycles) on the Applied Biosystems peptide synthesizer Model 433A (Foster City, Calif) as described.[27]

Purified Bet v 1 peptides were labeled at the amino-terminus with biotin using N-succinimidyl N-(6-[biotinylamino]caproyl)-6-aminocaproate (Fluka Biochemica, Buchs, Switzerland) according to the manufacturer's instructions in a concentration of 250 μg biotin for 1 mg purified peptide. The identity of the biotinylated peptides was checked by mass spectrometry, and they were purified to >90% purity by preparative HPLC (PiChem, Graz, Austria). The endotoxin content of the peptides were <0.0075 ng/mL as measured by Limulus Amebocyte Lysate endochrome assay (Endosafe; Charles River Laboratories, Charleston, SC).

Culturing of EBV-transformed B cells

EBV-transformed B-cell lines from donors 1 and 2 were cultured in complete medium consisting of RPMI-1640 (Gibco; Invitrogen Corp, Paisley, United Kingdom) supplemented with 25 μg/mL gentamicin (Gibco), 10% heat-inactivated FCS (Hyclone, Logan, Utah), 2 mmol/L L-glutamine, 100 IU/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 50 μmol/L β-mercaptoethanol (KEBO-lab, Spånga, Sweden). The medium was exosome-depleted as previously described.[28] The cells were cultured in a 37°C humidified incubator with 6% CO_2.

Exosome preparations

For exosome collection, viable B cells were counted by Trypan blue exclusion and resuspended at a cell density of 3×10^5 cells/mL in complete medium. After 48 hours of incubation, cells were spun down at 300*g*, and the supernatant was used for exosome preparation. Exosomes were purified by differential centrifugation essentially as previously described.[3] The supernatants were centrifuged at 3000*g* for 20 minutes at room temperature and ultracentrifuged at 10,000*g* for 30 minutes at 4°C to remove cell debris. The supernatants were ultracentrifuged at 110,000*g* for 1 hour at 4°C to generate a pellet that has previously been shown to contain exosomes.[1,3,28] The exosomes were washed and resuspended in RPMI or PBS and stored at –80°C. Exosome samples were measured for protein content by using BioRad DC protein assay (Bio-Rad Laboratories, Hercules, Calif) according to the manufacturer's instructions.

Flow cytometry

For flow cytometry analysis, exosomes were adsorbed onto 4.5 μm Ø Dynabeads precoated with anti-MHC class II (Dynal, Oslo, Norway) at a concentration of 5 μg exosomes per 2.8×10^5 dynabeads in PBS containing 0.1% BSA and 0.01% sodium azide overnight at room temperature. The following fluorochrome-labeled mouse mAbs were used for analysis of cells and exosomes: anti–HLA-DR fluorescein isothiocyanate (FITC), anti-MHC class I FITC, anti-CD19 phycoerythrin (PE), anti-CD40 FITC, anti-CD54 PE, anti-CD63 PE, anti-CD80 PE, anti-CD81 PE, and anti-CD86 FITC (Pharmingen/Becton Dickinson, San Jose, Calif) and compared with isotype-matched controls (Becton Dickinson). Samples were analyzed by a FACSCalibur flow cytometer (Becton Dickinson), and the data were analyzed by using CellQuest Pro software (Becton Dickinson). A minimum of 10^4 cells and a minimum of 5×10^3 beads per sample were examined.

Loading of B-cell exosomes with birch peptides

B cell–derived exosomes were loaded with biotinylated or not labeled Bet v 1 peptides, Bet v $1_{4\text{-}18}$, and/or Bet v $1_{142\text{-}156}$. The peptides were loaded to the MHC of the exosomes by direct loading as previously described.[16] Briefly, the exosomes were mixed with the same volume of 0.2 mol/L sodium acetate (pH 5.2) and incubated with 5 μg/mL Bet v $1_{4\text{-}18}$ and/or Bet v $1_{142\text{-}156}$ for 30 minutes at 4°C. The samples were then neutralized to pH 7.0 with 2 mol/L TRIS (pH 11) and incubated for 45 minutes on ice. Unbound peptide was removed by filtering through a 100-kd cutoff filter (Millipore, Bedford, Mass), making the free peptides go through the filter and the exosomes stay on top of the filter. The corresponding amount of birch peptides as loaded on the exosomes were filtrated in parallel and the fraction above the filter, corresponding to the exosome fraction, was used in the functional assays to control for the removal of free peptides. All exosome samples were sterile filtrated through a 0.20-μm filter (Advantec MFS Inc, Dublin, Calif) before use.