

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

Charlotte Admyre, PhD,^a Barbara Bohle, PhD,^b Sara M. Johansson, MSc,^a Margarete Focke-Tejkl, PhD,^b Rudolf Valenta, MD,^b Annika Scheynius, MD, PhD,^a and Susanne Gabrielsson, PhD^a *Stockholm, Sweden, and Vienna, Austria*

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 ■■■■;■■■:■■■-■■■.)

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 induction¹⁹ 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

From ^athe Department of Medicine, Clinical Allergy Research Unit, Karolinska Institutet, Stockholm; and ^bthe Department of Pathophysiology, Division of Immunopathology, Center for Physiology and Pathophysiology and Christian Doppler Laboratory for Allergy Research, Medical University of Vienna.

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Reprint requests: Charlotte Admyre, PhD, Karolinska Institutet, Department of Medicine, Clinical Allergy Research Unit, Karolinska University Hospital L2:04, 171 76 Stockholm, Sweden. E-mail: Charlotte.Admyre@ki.se.

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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

APCs presenting allergen-derived peptides to secrete 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 described²⁶ (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 1₄₋₁₈]: NYETETTSVIPAAARL; Bet v 1 amino acid residues 142-156 [Bet v 1₁₄₂₋₁₅₆]: 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% CO₂.

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 300g, and the supernatant was used for exosome preparation. Exosomes were purified by differential centrifugation essentially as previously described.³ The supernatants were centrifuged at 3000g for 20 minutes at room temperature and ultracentrifuged at 10,000g for 30 minutes at 4°C to remove cell debris. The supernatants were ultracentrifuged at 110,000g 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 (BioRad 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 (Dyna, 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 1₄₋₁₈, and/or Bet v 1₁₄₂₋₁₅₆. 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 1₄₋₁₈ and/or Bet v 1₁₄₂₋₁₅₆ 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.

TABLE I. Descriptive data on the different patients with allergy

Donor	Birch pollen-specific IgE (kU/L)	Bet v 1-specific IgE (kU/L)	Total IgE (kU/L)	HLA phenotype
1	41.8	87.1	430	DRB1*0701/*1501; DRB4*01/5*01; DQB1*02/*06
2	25.4	30.5	92.8	DRB1*0701/*1601; DRB4*01/5*01; DQB1*01/*03
3	10.3	10.2	174	DRB1*0701/*1501; DRB4*01/5*01; DQB1*02/*06
4	27.1	27.8	913	DRB1*0701/*1501; DRB4*01/5*01; DQB1*03/*06

Detection of Bet v 1 peptides on exosomes by ELISA

B cell-derived exosomes were loaded with biotinylated Bet v 1₁₄₂₋₁₅₆ as described and added to ELISA plates (Nunc, Roskilde, Denmark) that had been coated overnight at 4°C with anti-MHC class II mAb (Becton Dickinson) and blocked for 1 hour with 1% BSA (Sigma Aldrich, Steinheim, Germany). After incubation overnight at 4°C, the plates were washed and incubated with a 1:5000 dilution of streptavidin conjugated with horseradish peroxidase (Sigma Aldrich) for 30 minutes at room temperature. After repeated washings, the plates were incubated with the horseradish peroxidase substrate o-phenylenediamine (Sigma Aldrich) for at least 30 minutes, after which the absorbance was detected at 450 nm.

Stimulation of Bet v 1-specific TCLs with peptide-loaded exosomes

Bet v 1-specific TCLs were established as previously described.²⁹ TCLs (5×10^4 /well) were stimulated by using irradiated EBV-transformed B cells (60 Gy) from donor 1 or 2 (3×10^4 cells/well) in round-bottomed 96-well plates (Nunc, Denmark) in duplicate. As negative control, TCL plus irradiated B cells were left in medium alone, and as positive controls, either recombinant (r) Bet v 1 (2 µg/mL) or the mixtures of the Bet v 1-derived 12-mer peptides NYETETTSVIPA and TETTSVIPAAARL (corresponding to amino acid residues 4-18) or TLLRAVESYLLA and RAVESYLLAHSD (corresponding to amino acid residues 142-156: 5 µg/mL and 1 µg/mL of each peptide, respectively) were added. In parallel, TCLs were stimulated with unloaded and peptide-loaded exosome preparations from donors 1 and 2, respectively. After 48 hours, supernatants were collected for cytokine analysis. Cell proliferation was measured by adding [³H]-thymidine (0.5 µCi per well) during the last 16 hours of culture. The results are presented as Δ counts per minute (dpm), which is mean cpm of duplicates in stimulated cultures minus mean cpm of duplicates in negative controls—that is, TCLs incubated with B cells without peptide for B-cell stimulations or TCLs incubated with medium alone for exosome stimulations.

Cytokine analysis

Supernatants from stimulated TCLs were frozen at -20°C until further analysis. The cytometric bead array (CBA) human T_H1/T_H2 kit (Becton Dickinson) was used to assay IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-10 in accordance with the manufacturer's instructions. CBA flex sets were used for analysis of IL-7, IL-9, and IL-13 (Becton Dickinson). The lower limit of detection was 5 pg/mL. Samples were analyzed in duplicate.

Statistics

Wilcoxon matched pair tests were performed by using the Statistica 7.1 software package (StatSoft Scandinavia AB, Uppsala, Sweden). *P* values <.05 were considered statistically significant.

RESULTS

Characterization of B cell-derived exosomes

Exosomes were prepared from the culture supernatants of EBV-transformed B-cell lines from 2 individuals with birch pollen allergy (donors 1 and 2, Table I). For phenotypic characterization, B cells and exosomes from 48-hour B-cell culture supernatants, pelleted by ultracentrifugation and coated onto anti-MHC class II dynabeads, were analyzed by flow cytometry. The B cells expressed MHC classes I and II; the costimulatory molecules CD40, CD80, and CD86; the adhesion molecule CD54; and the B cell-specific marker CD19 (Fig 1, A). Furthermore, B cells also expressed the tetraspanin proteins CD63 and CD81 (Fig 1, A). The B cell-derived exosomes showed expression of MHC classes I and II as well as CD40, CD54, CD63, CD80, CD81, and CD86 (Fig 1, B), a phenotype in accordance with previous studies.⁸ In addition, the exosomes expressed the B cell-specific marker CD19 (Fig 1, B).

B cell-derived exosomes can bind peptides derived from Bet v 1

To investigate whether B cell-derived exosomes can bind Bet v 1-derived peptides, the B cell-derived exosomes were incubated with the biotinylated Bet v 1 peptide Bet v 1₁₄₂₋₁₅₆ by using the acid elution method previously described for loading exosomes with tumor peptides.³⁰ Exosomes loaded with biotinylated Bet v 1₁₄₂₋₁₅₆ gave rise to a significantly higher absorbance signal compared with exosomes not loaded with peptide, biotinylated Bet v 1₁₄₂₋₁₅₆ alone, or PBS when analyzed with ELISA (Fig 2). These data indicate that the biotinylated peptides were present on the exosome surface.

B cell-derived exosomes loaded with Bet v 1 peptides induce specific T-cell proliferation

Bet v 1-specific TCLs generated from donors 1, 3, and 4 (Table I) were incubated with increasing amounts of exosomes either unloaded or loaded with a mixture of Bet v 1₄₋₁₈ and Bet v 1₁₄₂₋₁₅₆. In parallel, T cells were stimulated with Bet v 1 or the respective peptides in the presence of EBV-transformed B cells from donor 1 or donor 2. To confirm that free peptides had been removed from loaded exosomes during the filtration step, the corresponding amount of peptides as used for loading of the exosomes was filtrated in parallel, and the upper fraction corresponding to the exosome fraction was added to the

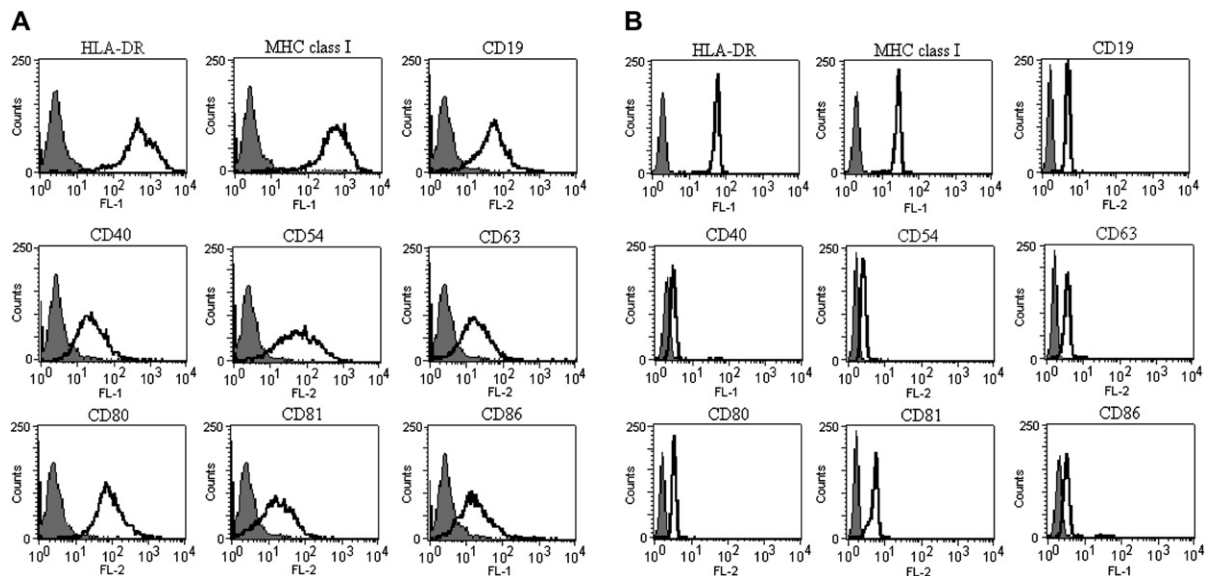


FIG 1. Phenotypic characterization of B cells and their exosomes. EBV-transformed B-cell lines (**A**) or their exosomes coated onto anti-MHC class II beads (**B**) were analyzed for surface molecules by using flow cytometry. All antibodies were compared with isotype-matched controls (gray area). Three repetitive experiments of exosomes and cells from donors 1 and 2 were performed. The data show 1 representative experiment.

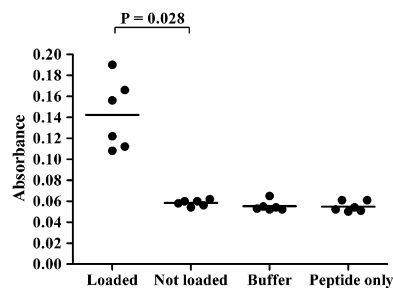


FIG 2. Detection of Bet v 1 peptide on exosomes. Exosomes loaded with biotinylated Bet v 1 peptide, not loaded exosomes, only peptide, or only PBS were added to anti-MHC class II coated plates. The peptides were detected by ELISA. Bars show the mean absorbance from 6 experiments by using exosomes from donor 2. Statistical analysis was performed by using the Wilcoxon matched pairs test.

T cells. Proliferation of Bet v 1-specific T cells was investigated after 48 hours. Birch peptide-loaded exosomes induced a dose-dependent proliferation of T cells (Fig 3) with the strongest responses induced using 50 $\mu\text{g}/\text{mL}$ exosomes (Fig 3). Exosomes not loaded with Bet v 1-derived peptides gave rise to much lower levels of proliferation (Fig 3), showing that the response was antigen-specific. No increased proliferation was seen for the control of free peptide (data not shown), indicating that the proliferation is not a result of the presence of free peptide in the loaded exosome preparation. Bet v 1-specific TCLs responded to stimulation with Bet v 1 or Bet v 1 peptides in the presence of B cells as APCs (Fig 3).

B cell-derived exosomes loaded with Bet v 1-derived peptides induce cytokine production of allergen-specific T cells

Supernatants of stimulated TCLs were analyzed by using CBA. Peptide-loaded exosomes induced a cytokine response similar to that observed on stimulation with peptides presented by the corresponding B-cell lines. Bet v 1-specific TCLs from all 3 donors displayed a typical $\text{T}_\text{H}2$ response characterized by high levels of IL-4, IL-5, and IL-13 and lower levels of IFN- γ and TNF- α (Table II) when stimulated with peptide-presenting B cells. In line with the weaker proliferative response induced by peptide-loaded exosomes compared with B cell-presented peptides, cytokine levels were also lower, showing high levels of IL-5 and IL-13 and low or no levels of IL-4, IFN- γ , and TNF- α (Table II). One exception was TCLs from donor 1, which secreted more IL-13 when stimulated with 50 $\mu\text{g}/\text{mL}$ peptide-loaded exosomes compared with B cells (Fig 3; Table II). Neither Bet v 1-specific TCLs activated with peptide-presenting exosomes nor B cells showed significant production of IL-7 and IL-9 (data not shown). Results from donor 1 showed that autologous and HLA-matched B cells and exosomes induced comparable responses (Table II).

DISCUSSION

The capacity of exosomes to present allergen-derived peptides and to activate allergen-specific T cells was investigated. To obtain sufficient quantities of exosomes, EBV-transformed B cells from 2 individuals with birch pollen allergy with known HLA phenotypes were used as

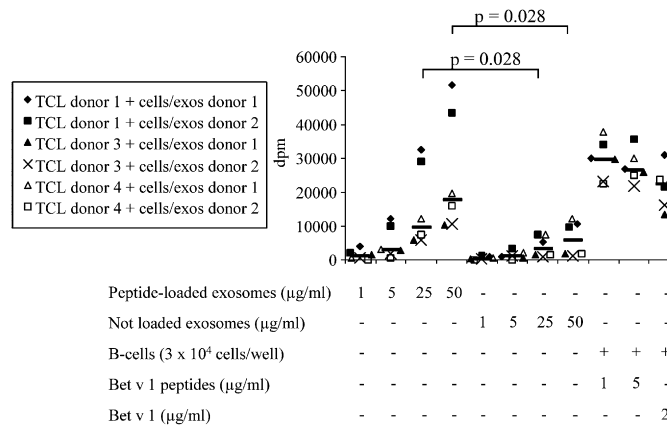


FIG 3. Proliferation of T cells stimulated with Bet v 1 peptide-loaded exosomes (*exos*). Bet v 1-specific TCLs were incubated with Bet v 1 peptide-loaded or not loaded exosomes, with B cells alone or together with Bet v 1 peptides or Bet v 1. Cell proliferation are presented as Δ counts per minute (dpm), or mean cpm of duplicates in stimulated cultures minus mean cpm of duplicates in negative controls—that is, TCLs incubated with B cells without peptide (median, 72,301 cpm; range, 39,508–91,268 cpm) or TCLs incubated with medium alone (median, 344 cpm; range, 261–417 cpm). Bars show median values of all individuals.

TABLE II. Cytokine responses of TCL to Bet v 1 peptides presented by either B cells or exosomes

TCL	Donor	IL-2	IL-4	IL-5	IL-13 (pg/mL)	IL-10	INF- γ	TNF- α
Donor 1	B/P*	1	1007	2612	1238	210	325	498
	E/P*	1	264	86	1269	2756	20.5	39.6
	B/P	2	461	2262	2612	345	153	459
	E/P	2	92	81	1101	2388	15.1	32.4
Donor 3	B/P	1	125	214	1377	5050	29.1	<5
	E/P	1	<5	<5	18	81	<5	<5
	B/P	2	24.6	177	1213	2886	22.6	188
	E/P	2	<5	11.0	113	540	1.5	166
Donor 4	B/P	1	<5	88	2615	4748	23.9	103
	E/P	1	<5	8.9	637	1032	<5	<5
	B/P	2	116	467	1743	3682	32.5	453
	E/P	2	<5	6.0	676	1029	<5	<5

*TCLs were stimulated with B cells plus 5 µg/mL Bet v 1₄₋₁₈ and Bet v 1₁₄₂₋₁₅₆ peptides (*B/P*) or 50 µg/mL peptide-loaded exosomes (*E/P*) derived from either donor 1 or donor 2. Cytokine levels at 48 hours represent mean of duplicates of stimulated cultures minus mean of duplicates from control cultures—that is, B cells plus medium or unloaded exosomes, respectively.

a source for exosomes (Table I). The phenotype of isolated exosomes was in accordance previous reports showing high expression of MHC classes I and II, the tetraspanin proteins CD63 and CD81, and the presence of the costimulatory molecules CD40, CD80, and CD86.^{8,9} In addition, the exosomes in this study expressed the molecule CD19, which together with CD21 and CD81 is part of the B-cell coreceptor.³¹

Exosomes were loaded with 2 distinct peptides derived from the major birch pollen allergen, Bet v 1. Bet v 1₄₋₁₈ corresponds to the amino acid residues 4 to 18 in the N-terminal region, whereas Bet v 1₁₄₂₋₁₅₆ corresponds to amino acid residues 142 to 156 in the C-terminal part of Bet v 1. Both peptides contain relevant T-cell epitopes presented by the MHC class II molecules expressed by the individuals included in this study. Bet v 1₁₄₂₋₁₅₆ is the immunodominant T-cell epitope of Bet v 1.²⁹ Binding of Bet v 1-derived peptides to the surface of the exosomes was confirmed by using ELISA (Fig 2). Stimulation of Bet v

1-specific TCLs with peptide-loaded exosomes induced a dose-dependent proliferation (Fig 3), demonstrating for the first time that B cell-derived exosomes can activate allergen-specific T cells. Furthermore, peptide-loaded exosomes induced the production of IL-5 and IL-13 (Table II), which is typical for Bet v 1-specific T cells.²⁹ The observed variation in the levels of IL-4 and IL-5 or IL-13 in both supernatants from TCLs stimulated with peptide presented by exosomes and B cells may be referred to the possibility that the TCL from the donors are differently sensitive to Bet v 1 stimulation. Proliferative and cytokine responses induced by peptide-loaded exosomes were in general weaker than those induced by peptides presented by EBV-transformed B cells (Fig 3; Table II). However, it is not possible to compare exosomes directly with B cells in our system because with currently available methods, it is not possible to quantify exactly the number of exosomes contained in the used fractions. Nevertheless, the yield of exosomes was very similar between both

donors tested (data not shown), showing that our isolation methods were reliable and reproducible. Moreover, exosomes from both donors induced comparable responses in 3 TCLs, indicating that loading of the exosomes with Bet v 1 peptides worked efficiently and that the quantification of exosomes was reproducible. We here used EBV-transformed B cells as a source of exosomes, and it is possible that exosomes from nontransformed B cells differ to some extent in their effect on T-cell stimulation; however, because of the high number of cells needed, isolation of exosomes from nontransformed B cells was not possible in this study.

It is still under debate how T-cell activation by exosomes is achieved. Some studies indicate that exosomes can stimulate T cells directly,^{3,16,17} whereas others demonstrate that exosomes need the presence of APCs to exert their effect.^{18,32,33} These conflicting results might be a result of differences in the phenotype of the responder cells and/or the exosomes, the exosome dose, or the affinity for the antigen used. In our study, peptide-loaded exosomes activated T cells without addition of APCs. Their high expression of MHC and costimulatory molecules (Fig 1) is probably 1 important factor for this direct effect.

The importance of allergen-peptide loaded exosomes *in vivo* needs still to be investigated. We have previously reported that exosomes that express MHC classes I and II as well as costimulatory molecules are present in BAL fluid from healthy individuals.¹⁰ Our preliminary unpublished data showed that this is also true for patients with allergic asthma, indicating that exosomes are present in the target organ at the site of allergic inflammation.

Furthermore, a previous study has shown that B-cell receptor triggering of a human follicular lymphoma B-cell line leads to increased release of exosomes.³⁴ In individuals with allergy, activation of allergen-specific B cells could possibly lead to increased release of antigen-presenting exosomes with the capacity of activating allergen-specific T_H2 cells. During an allergic response *in vivo*, allergy promoting exosomes might also come from other cellular sources like DCs. Exosomes in BAL fluid are very similar to DC-derived exosomes,¹⁰ and DCs are abundant in the lung.³⁵ DC-derived exosomes have a similar but not identical phenotype to B-cell exosomes⁸ and could therefore give rise to different T-cell responses.

An alternative role for allergen-peptide bearing exosomes *in vivo* is to transport allergen-derived peptides, perhaps together with immune modulatory molecules, between different APCs. A recent publication revealed that exosomes contain mRNA, which can be transported between cells.³⁶ Furthermore, it has been shown that B-cell-derived exosomes express functional integrins capable of anchoring to extracellular matrix and to cell surface adhesion molecules and may be a way of delivering signals at a distance different from that of direct cell-to-cell contact.³⁷ The fact that exosomes express CD55 and CD59, which protect them from complement lysis,³⁸ suggests that they would be stable and survive in the extracellular environment and be able to interact with other cells.

If exosomes have a role in stimulating an allergic response *in vivo*, they may compose a new target for allergy prevention and treatment. However, it is likely that exosomes act as potentiators of immune stimulation and are not the primary mode of T-cell stimulation. Furthermore, complete blocking of exosome release might give adverse effects; hence, this approach requires further knowledge of exosome biology and function. Alternatively, modified exosomes could be used to modulate the allergic immune response to induce a more T-regulatory type of response instead of the allergy-promoting T_H2 response. Exosomes from IL-10-treated DCs and from DCs genetically modified to express Fas ligand have been shown to be able to suppress inflammatory responses in mice,^{39,40} hence such exosomes would be of interest to test in allergic inflammation. Exosome display technology, in which proteins by fusion with the lactadherin C1C2 domain can be targeted to exosomes,⁴¹ makes it possible to manipulate the protein expression of exosomes and tailor-make them for different applications.

In conclusion, this study shows for the first time that exosomes can present allergen-derived peptides and stimulate allergen-specific T cells to proliferate and release cytokines. This finding suggests that APC-derived exosomes may have a contributing role in activation of T cells during allergic immune responses, and future *in vivo* experiments will reveal whether exosomes could constitute a new therapeutic target in antiallergy treatment.

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REFERENCES

- Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* 1998;4:594-600.
- Blanchard N, Lankar D, Faure F, Regnault A, Dumont C, Raposo G, et al. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/zeta complex. *J Immunol* 2002;168:3235-41.
- Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* 1996;183:1161-72.
- Raposo G, Tenza D, Mecheri S, Peronet R, Bonnerot C, Desaymard C. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Mol Biol Cell* 1997;8:2631-45.
- Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* 1999;94:3791-9.
- van Niel G, Raposo G, Candalh C, Boussac M, Hershberg R, Cerf-Bennussan N, et al. Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology* 2001;121:337-49.
- Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, et al. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med* 2001;7:297-303.
- Clayton A, Court J, Navabi H, Adams M, Mason MD, Hobot JA, et al. Analysis of antigen presenting cell derived exosomes, based on immunomagnetic isolation and flow cytometry. *J Immunol Methods* 2001;247:163-74.
- Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ. Selective enrichment of tetraspan proteins on the internal vesicles of

- multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J Biol Chem* 1998;273:20121-7.
10. Admyre C, Grunewald J, Thyberg J, Gripenback S, Tornling G, Eklund A, et al. Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid. *Eur Respir J* 2003;22:578-83.
 11. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A* 2004;101:13368-73.
 12. Andre F, Scharltz NE, Movassagh M, Flament C, Pautier P, Morice P, et al. Malignant effusions and immunogenic tumour-derived exosomes. *Lancet* 2002;360:295-305.
 13. Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. *Int Immunol* 2005;17:879-87.
 14. Admyre C, Johansson SM, Qazi KR, Filen JJ, Lahesmaa R, Norman M, et al. Exosomes with immune modulatory features are present in human breast milk. *J Immunol* 2007;179:1969-78.
 15. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation: association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem* 1987;262:9412-20.
 16. Admyre C, Johansson SM, Paulie S, Gabrielson S. Direct exosome stimulation of peripheral human T cells detected by ELISPOT. *Eur J Immunol* 2006;36:1772-81.
 17. Hwang I, Shen X, Sprent J. Direct stimulation of naive T cells by membrane vesicles from antigen-presenting cells: distinct roles for CD54 and B7 molecules. *Proc Natl Acad Sci U S A* 2003;100:6670-5.
 18. Thery C, Duban L, Segura E, Veron P, Lantz O, Amigorena S. Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat Immunol* 2002;3:1156-62.
 19. Karlsson M, Lundin S, Dahlgren U, Kahu H, Pettersson I, Telemo E. "Tolerosomes" are produced by intestinal epithelial cells. *Eur J Immunol* 2001;31:2892-900.
 20. Chaput N, Scharltz NE, Andre F, Taieb J, Novault S, Bonnaventure P, et al. Exosomes as potent cell-free peptide-based vaccine, II: exosomes in CpG adjuvants efficiently prime naive Tc1 lymphocytes leading to tumor rejection. *J Immunol* 2004;172:2137-46.
 21. Morse MA, Garst J, Osada T, Khan S, Hobeika A, Clay TM, et al. A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J Transl Med* 2005;3:9.
 22. Aline F, Bout D, Amigorena S, Roingard P, Dimier-Poisson I. Toxoplasma gondii antigen-pulsed-dendritic cell-derived exosomes induce a protective immune response against T. gondii infection. *Infect Immun* 2004;72:4127-37.
 23. Peche H, Heslan M, Usal C, Amigorena S, Cuturi MC. Presentation of donor major histocompatibility complex antigens by bone marrow dendritic cell-derived exosomes modulates allograft rejection. *Transplantation* 2003;76:1503-10.
 24. Asher MI, Montefort S, Bjorksten B, Lai CK, Strachan DP, Weiland SK, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* 2006;368:733-43.
 25. Larche M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol* 2006;6:761-71.
 26. Fischer GF, Fae I, Petrasek M, Haas H, Mayr WR. An HLA-DR11 variant (HLA-DRB1*1115) segregating in a family of Turkish origin. *Tissue Antigens* 1995;45:143-4.
 27. Focke M, Linhart B, Hartl A, Wiedermann U, Sperr WR, Valent P, et al. Non-anaphylactic surface-exposed peptides of the major birch pollen allergen, Bet v 1, for preventive vaccination. *Clin Exp Allergy* 2004;34:1525-33.
 28. Thery C, Regnault A, Garin J, Wolfers J, Zitvogel L, Ricciardi-Castagnoli P, et al. Molecular characterization of dendritic cell-derived exosomes: selective accumulation of the heat shock protein hsc73. *J Cell Biol* 1999;147:599-610.
 29. Jahn-Schmid B, Radakovics A, Luttkopf D, Scheurer S, Vieths S, Ebner C, et al. Bet v 1142-156 is the dominant T-cell epitope of the major birch pollen allergen and important for cross-reactivity with Bet v 1-related food allergens. *J Allergy Clin Immunol* 2005;116:213-9.
 30. Hsu DH, Paz P, Villafior G, Rivas A, Mehta-Damani A, Angevin E, et al. Exosomes as a tumor vaccine: enhancing potency through direct loading of antigenic peptides. *J Immunother* 2003;26:440-50.
 31. Rickert RC. Regulation of B lymphocyte activation by complement C3 and the B cell coreceptor complex. *Curr Opin Immunol* 2005;17:237-43.
 32. Vincent-Schneider H, Stumptner-Cuvellette P, Lankar D, Pain S, Raposo G, Benaroch P, et al. Exosomes bearing HLA-DR1 molecules need dendritic cells to efficiently stimulate specific T cells. *Int Immunol* 2002;14:713-22.
 33. Segura E, Nicco C, Lombard B, Veron P, Raposo G, Batteux F, et al. ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T cell priming. *Blood* 2005;105:216-23.
 34. Rialland P, Lankar D, Raposo G, Bonnerot C, Hubert P. BCR-bound antigen is targeted to exosomes in human follicular lymphoma B-cells. *Biol Cell* 2006;98:491-501.
 35. Grayson MH. Lung dendritic cells and the inflammatory response. *Ann Allergy Asthma Immunol* 2006;96:643-51; quiz 52-3, 78.
 36. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;6:654-9.
 37. Clayton A, Turkes A, Dewitt S, Steadman R, Mason MD, Hallett MB. Adhesion and signaling by B cell-derived exosomes: the role of integrins. *FASEB J* 2004;18:977-9.
 38. Clayton A, Harris CL, Court J, Mason MD, Morgan BP. Antigen-presenting cell exosomes are protected from complement-mediated lysis by expression of CD55 and CD59. *Eur J Immunol* 2003;33:522-31.
 39. Kim SH, Lechman ER, Bianco N, Menon R, Keravala A, Nash J, et al. Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. *J Immunol* 2005;174:6440-8.
 40. Kim SH, Bianco N, Menon R, Lechman ER, Shufesky WJ, Morelli AE, et al. Exosomes derived from genetically modified DC expressing FasL are anti-inflammatory and immunosuppressive. *Mol Ther* 2006;13:289-300.
 41. Delcayre A, Estelles A, Sperinde J, Roulon T, Paz P, Aguilar B, et al. Exosome display technology: applications to the development of new diagnostics and therapeutics. *Blood Cells Mol Dis* 2005;35:158-68.