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Biodegradable Polyelectrolyte Microcapsules: Antigen Delivery Tools with Th17 Skewing Activity after Pulmonary Delivery

Stefaan De Koker,* Thomas Naessens,* Bruno G. De Geest,† Pieter Bogaert,* Jo Demeester,† Stefaan De Smedt,‡ and Johan Grooten*†

Because of their large surface area, the lungs appear an attractive route for noninvasive vaccine delivery, harboring the potential to induce local mucosal immune responses in addition to systemic immunity. To evoke adaptive immunity, Ags require the addition of adjuvants that not only enhance the strength of the immune response but also determine the type of response elicited. In this study, we evaluate the adjuvant characteristics of polyelectrolyte microcapsules (PEMs) consisting of the biopolymers dextran-sulfate and poly-l-arginine. PEMs form an entirely new class of microcapsules that are generated by the sequential adsorption of oppositely charged polymers (polyelectrolytes) onto a sacrificial colloidal template, which is subsequently dissolved leaving a hollow microcapsule surrounded by a thin shell. Following intratracheal instillation, PEMs were not only efficiently taken up by APCs but also enhanced their activation status. Pulmonary adaptive immune responses were characterized by the induction of a strongly Th17-polarized response. When compared with a mixture of soluble Ag and empty microcapsules, Ag encapsulation significantly enhanced the strength of this local mucosal response. Given their unique property to selectively generate Th17-polarized immune responses, PEMs may become of significant interest in the development of effective vaccines against fungal and bacterial species. The Journal of Immunology, 2010, 184: 203–211.

The vertebrate immune system is confronted with a wide diversity of pathogens, ranging from viruses to bacteria, fungi, protozoa, and multicellular parasites such as helminths. To successfully combat these distinct pathogen spectra, totally different types of immune defense are required. Intracellular pathogens such as viruses and intracellular bacteria require the induction of IFN-γ-secreting Th1 cells that activate macrophages and stimulate B cells to secrete neutralizing and opsonizing Abs. Th1 cells also promote the generation of cytotoxic T cells that are able to recognize and kill infected cells. In contrast, eradication of extracellular pathogens such as various bacterial species, fungi, and helminths does not require the immune system to attack host cells. Beneficial immune responses against helminths rely on the induction of Th2 responses, promoting IgE isotype switching and the activation of eosinophils and mast cells that subsequently release their toxic granule content and stimulate smooth muscle contraction to expel the parasite. Recently, Th17 cells were described as a new type of CD4 effector cells that mediate a monocyte and neutrophil-driven inflammation. Th17 cells exert their proinflammatory functions mainly by secreting vast amounts of IL-17 and IL-22. These cytokines act on multiple cell types and promote the release of inflammatory cytokines and chemokines that recruit monocytes and neutrophils to the site of infection (1–4). In addition, they stimulate the secretion of antimicrobial peptides by epithelial cells and keratinocytes and appear to enhance epithelial barrier function (5–7). Th17 responses have been linked to the induction of protective immunity against extracellular bacteria and fungi (2, 8–10).

To generate the most effective defense, the innate immune system consequently needs to accurately identify the type of pathogen encountered and to translate this information into the induction of an appropriate adaptive immune response. The main cell type mediating this crucial process is the dendritic cell (DC). DCs are the most potent APCs capable of priming naive T cells. By expressing a vast range of pattern recognition receptors that recognize specific microbial-associated molecular patterns, they are well equipped to sense microbial invasion (11–13). Triggering of different pattern recognition receptors by their respective ligands leads to the expression of both overlapping and unique genes involved in inflammatory and immune responses (14). The distinct cytokine profiles elicited subsequently polarize T cell responses, thereby linking pathogen recognition to the induction of the appropriate CD4 effector T cell response (15, 16).

Modern subunit vaccines are poorly immunogenic by themselves and require the inclusion of immune potentiating adjuvants to generate protective immunity. By interacting with the innate immune system, adjuvants not only enhance the adaptive immune response but also strongly affect its nature. Given the tight link between the type of immune response elicited and the induction of protective responses against a certain pathogen, the polarizing capacities of an adjuvant also largely determine the pathogen spectrum it might be useful against. Besides activating the innate immune system,
adjuvants ideally also enhance Ag uptake and presentation by professional APCs (17). Particulate Ag delivery systems such as polymeric microspheres and liposomes have been reported to generate improved immune responses largely by such mechanism (18–20). In this paper, we have analyzed the adjuvant characteristics of polyelectrolyte microcapsules (PEMs) consisting of two dextran-sulfate/poly-L-arginine bilayers. PEMs form an entirely new class of microparticles that are generated by the electrostatic adsorption of alternating polyelectrolytes of opposite charge onto a sacrificial colloidal template that is subsequently dissolved (21–23). This technique has been reported to enable an efficient encapsulation of proteins under nondenaturing conditions (24–26). In this study, we have applied this layer-by-layer approach to encapsulate OVA as a model inert Ag inside dextran-sulfate/poly-L-arginine PEMs. Microcapsules were delivered to the lungs, which constitute an attractive route for immunization because of their large surface area with many APCs readily accessible. In addition, pulmonary administration might allow the induction of local mucosal immune responses in addition to systemic immunity. Uptake and degradation of the microcapsules by pulmonary APCs were followed over time, as well as the effect of microcapsule administration on the local and lymph node APC activation status. Adaptive immune responses provoked by microcapsule-based immunization were characterized. Finally, by comparing immune responses induced by microcapsule-encapsulated OVA with those induced by a mixture of empty microcapsules and soluble OVA (sOVA), the microcapsules’ Ag carrier effects were discriminated from their inherent immune-activating properties.

Materials and Methods

Preparation of PEMs

Calcium chloride, sodium carbonate, FITC-dextran (2000 kDa), OVA (grade VII, LPS content 0.06 EU/μg as determined by the Limulus amebocyte lysate assay), dextran sulfate (10 kDa), and poly-L-arginine hydrochloride (m.w. >70 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). PEMs were made using calcium carbonate (CaCO₃) microparticles as a sacrificial template. Briefly, CaCO₃ microparticles were synthesized according to Volodkin et al. (24, 25) and subsequently coated by dispersing them in 5 ml of a 2 mg/ml dextran sulfate solution containing 0.5 M NaCl. Microparticles were collected by centrifugation, and residual dextran sulfate was removed by washing twice. Microparticles were stirred in 5 ml of a 1-mg/ml poly-L-arginine solution in 0.5 M NaCl, centrifuged, and washed again. This procedure was repeated until two bilayers of dextran sulfate/poly-L-arginine were deposited. Hollow microcapsules were obtained by dissolution of the CaCO₃ core through incubation of the coated microparticles for 10 min in 10 ml of 0.2 M EDTA solution (pH 5.2). The dissolved ions were then removed by three centrifugation and washings steps. Finally, the microcapsules were resuspended in 1 ml of PBS. The microcapsule concentration was determined by hemocytometry to be 700 × 10⁶ microcapsules/ml. Microcapsules contained <1 EU of LPS/ml as determined by the Limulus amebocyte lysate assay.

To obtain capsules filled with FITC-dextran or OVA, 200 μl of a 5-mg/ml stock solution was added to the CaCl₂ solution before the addition of the stock solution was added to the CaCl₂ solution before the addition of the stock solution was added to the CaCl₂ solution before the addition of the stock solution.

Encapsulation efficiency was determined by measuring the amount of nonencapsulated OVA using the Quik Start Bradford protein assay according to the manufacturer’s instruction.

Mice

Female C57BL/6 mice were purchased from Janvier (Le Genest Saint Isle, France) and housed in a specified pathogen-free facility in microisolation units. All mice were 8–10 wk old at the start of the experiments. All experiments were approved by the local ethical committee for animal experiments.

Bronchoalveolar lavage

Mice were anesthetized with avertin (2.5% in PBS and low endotoxin [LE]; Biowhittaker, Walkersville, MD). Bronchoalveolar lavage (BAL) was performed with Ca²⁺- and Mg²⁺-free HBSS (Invitrogen, Carlsbad, CA), supplemented with 0.05 mM EDTA.

BAL total and differential cell counts

BAL samples were isolated and cells from at least six different mice were counted after lysis of RBCs using ammonium potassium chloride lysis buffer (Biowhittaker). For differential cell counts, cytopsins were prepared using a Shandon cytocentrifuge (Techgen, Zellik, Belgium) and stained with May–Grünewald Giemsa. The percentage of monocytes/macrophages, neutrophils, and eosinophils was determined by counting at least 300 cells.

Flow cytometric analysis

At different time intervals after microcapsule instillation, BALs were performed, and uptake of FITC-dextran–loaded microcapsules by alveolar macrophages was assessed by flow cytometry. Briefly, RBCs were lysed with ammonium potassium chloride lysis buffer (Biowhittaker). Cells were counted and suspended at a concentration of 10⁶/ml. FcRs were blocked by incubation with purified anti-mouse CD16/CD32 (BD Pharmingen, San Diego, CA) for 15 min at 4°C and stained with PerCP-Cy5.5-conjugated antimouse CD11c (BD Pharmingen) for 1 h at 4°C, according to the manufacturer’s instructions. Samples were measured on a FACSCalibur (BD Pharmingen) flow cytometer and analyzed using CellQuest software. PBS-instilled mice were used as negative control. For each time interval, BAL samples from at least three different mice were analyzed.

To analyze the trafficking of PEMs to the mediastinal lymph nodes, FITC-dextran–loaded microcapsules were instilled intratracheally. Four days postinstillation, mice were sacrificed, and single-cell suspensions of mediastinal lymph node cells were prepared. Cells were subsequently stained with the DC marker CD11c–allophycocyanin (BD Pharmingen), and macrophages/DCs were gated on the basis of their scatter profile.

To assess the expression of stimulatory markers induced by microcapsule instillation, mice were instilled with either PBS or 50 million microcapsules of dextran–salt/poly-L-arginine (m.w. >70 kDa) PEMs. Four days after instillation, BAL samples were taken, and single-cell suspensions of draining mediastinal lymph node cells were prepared. Cells were stained with combinations of CD11c–Cy5 (BAL) or CD11c–allophycocyanin (lymph nodes) as described above and either CD40–PE, CD80–PE, CD86–PE, or MHC class II (MHC II)–phycoerythrin (all BD Pharmingen), according to the manufacturer’s instructions. Samples were measured on a FACSCalibur (BD Pharmingen) flow cytometer and analyzed using CellQuest software.

Microscopy

Optical Microscopy. Total and differential BAL cell counts were performed on an Olympus BX51 microscope, equipped with ×4, ×10, ×20, ×40, and ×100 lenses, and a CoolSnap Color camera (from Roper Scientific, Tucson, AZ) were used for bright-field inspection and recording.

Fluorescence Microscopy. FITC-dextran microcapsules were imaged in lung sections after deparaffinization with a Leica SP5 AOBS confocal microscope using the 488-nm Multi Argon laser line. Nuclei were stained with DAPI included in the mounting medium Vectashield and excited with the 405-nm line of an UV diode laser. BAL alveolar macrophages were stained with Pe-Cy5–conjugated anti-mouse CD11c (BD Pharmingen) as described and visualized using the 543-nm HeNe laser line. Nuclei were stained and imaged as for the lung tissue sections.

Vaccination and challenge protocols

PBS-LE (70 μl) containing either 35 μg of sOVA, microcapsule-encapsulated OVA, or a mixture of 35 μg of OVA with 50 × 10⁶ microcapsules was intratracheally applied to the mice on days 0 and 21. Control mice were treated by intratracheal instillation of 70 μl of PBS-LE on days 0 and 21. On days 42 and 43, mice were exposed to an aerosol of 1% OVA using a Jet nebulizer (Vital Signs, Totowa, NJ). BAL was performed 24 h after the last challenge.

Lung tissue CD4 T cell isolation

Lungs were minced and incubated for 30 min at 37°C in RPMI 1640 medium (Invitrogen) containing 150 U/ml collagenase II (Sigma-Aldrich) and 0.02 mg/ml DNase I (Roche Diagnostics, Vilvoorde, Belgium). After washing the cells, CD4⁺ T cells were isolated by the CellMate Biotin Binder Kit according to manufacturer’s protocol (Dynal, Invitrogen, Merelbeke, Belgium).

Real-time quantitative PCR

RNA isolation was performed using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. cDNA was synthesized using a Superscript II Reverse Transcriptase Reagent Kit (Invitrogen). Real-time quantitative PCR (qPCR) was performed on a LightCycler 480 (Roche Molecular Systems) using a qPCR kit for SYBR
Green I (Roche Molecular Systems). Each real-time qPCR amplification was performed in triplicate under the following conditions: a preincubation step at 95°C for 5 min, followed by 50 cycles at 95°C for 10 s and at 60°C for 30 s. The following forward and reverse primers were used: 5'-GCAT CTGCCAATTAGGTCTC-3' and 5'-TGGTGAAGGTGGTGGTTGA-3' (murine CCL2); 5'-ACCAGCTGCCAGTCTGCTG-3' and 5'-AGGCTCCGCC AACATTG-3' (murine Ccl5); 5'-GGACTGCAACAGGCTCTCTCA CAG-3' and 5'-CACCAGCCGCTTTCAAAATAAC-3' (murine TNF-α); 5'-GCCAGCTGCCAGATGTTGCTG-3' and 5'-GGTGGAGGCAGGACA AAGAG-3' (murine RORγt); 5'-GCCAGGCGCGTACGTTGA-3' and 5'-TCAGTGAAGTAAAGGTAACATGCTAATC-3' (murine IFN-γ); 5'-CAGATGTGTTGAC-3' and 5'-AGATAAGTGGGTCTACTAGG-3' (murine IL-5); 5'-GAGACTGGTCCCTCTCA TITICA-3' and 5'-TTGGACCCCTTTACACCTCCTT-3' (murine IL-17); 5'-GGTGCCCTTTAACCCCTCTCA-3' and 5'-TCACAACCTCCTTAG GACCTGTCAGACT-3' (murine IL-10); 5'-CACATTAGCTTGGCTGT TCTT-3' and 5'-TTTTCTGTCGTTGCGTGTTC-3' (murine IL23p19); and 5'-AACCAGGCTCCTTTAG-3' and 5'-GATCTGCGCTCCGT GT-3' (murine IL-12 p55).

mRPL13a mRNA was used as reference housekeeping gene for normalization. For each gene, the relative mRNA expression is presented by means of n-fold induction when compared with PBS-instilled mice.

**Serum Ab titers**

To analyze serum Ab titers, blood samples were collected 3 wk after the second immunization by tail bleeding, and serum was obtained after overnight incubation at 4°C. Ninety-six-well plates (Maxisorp; Nunc, Naperville, IL) were coated with 20 μg/ml OVA and incubated with serial dilutions (1/4) of the serum samples. Dilutions were made in PBS containing 0.1% Tween-20 and 10% FBS. HRP-coupled goat-anti-mouse IgG1 Ab (Southern Biotechnology Associates, Birmingham, AL) and HRP-coupled goat-anti-mouse IgG2c (Southern Biotechnology Associates, Birmingham, AL) were used as detection Abs, according to the manufacturer’s instructions. But plates were coated with 20 μg/ml OVA instead of the capture Ab. ELISA plates were developed with tetramethylbenzidine substrate solution (BD Pharmingen), reaction was stopped with 1 M H2SO4, and optical density was measured at 450 nm.

**Complement activation**

Complement activation was assessed by instilling groups of six mice with either PBS or 50 million empty microcapsules and collecting BAL samples 12 h postinstillation. Concentration of C3a in the BAL was determined by ELISA, using the purified anti-mouse C3a clone I87-1162 (BD Pharmingen) as a capture Ab, and the biotinylated I87-419 clone (BD Pharmingen) as a detection Ab, according to the manufacturer’s instructions. Purified mouse C3a protein (BD Pharmingen) was used as a standard.

**ELISPOT assays**

ELISPOT assays kits (IL-4, IFN-γ, and IL-17) were purchased at R&D Systems (Minneapolis, MN) and used according to the manufacturer’s instructions. In brief, 3 wk after the last immunization, mice were sacrificed and mediastinal lymph nodes were dissected. Single-cell suspensions were prepared and incubated directly on the precoated ELISPOT plates at a concentration of 2.0 × 10^6 cells. Cells were stimulated in the presence of 10 μM of the OVA H2e epitope ISQAVHAAHINEAGR (Anaspec, Fremont, CA) or 10 μM of an irrelevant epitope (influenza M2e epitope SLLEETETIPRNWEGRCNDSSD) for 48 h. As positive and negative controls, cells were incubated with either Con A (Sigma-Aldrich) at 2 μg/ml and without peptide, respectively.

**Results**

**Uptake and degradation of PEMs by pulmonary phagocytic cells**

Dextran-sulfate/poly-l-arginine PEMs were produced as described earlier. In brief, macromolecules of interest were mixed with CaCl2 and Na2CO3, resulting in the formation of macromolecule-loaded CaCO3 microcapsules. These microparticles were coated with alternating layers of dextran-sulfate and poly-l-arginine until two dextran sulfate/poly-l-arginine bilayers were deposited. After dissolution of the CaCO3 with EDTA, a hollow microcapsule with a thin poly-electrolyte shell is obtained (Fig. 1). If the m.w. of the macromolecules is sufficiently large, they remain entrapped inside the microcapsules.

To function as effective Ag delivery systems, PEMs need to be taken up and degraded by pulmonary phagocytic cells. To follow their uptake and degradation, microcapsules encapsulating FITC-dextran were instilled intratracheally. BALs were performed at the indicated time intervals and analyzed by flow cytometry (Fig. 2A). Two days postinstillation, most of the BAL cells expressing the DC/macrophage marker CD11c had taken up microcapsules. Under non-inflammatory conditions, CD11c+ alveolar macrophages represent almost the entire alveolar cellular population, a feature confirmed in the PBS-instilled control group. In the microcapsule-instilled group, also a large CD11c- fraction emerged, indicative for an ongoing inflammatory response. A significant proportion of these CD11c- cells were also microcapsule positive (Fig. 2A, day 2). One week after instillation, the vast majority of the alveolar population consisted of CD11c+ cells containing FITC-dextran–loaded microcapsules (82.2%). A similar picture arose 2 wk following instillation.

The fate of the engulfed microcapsules was further evaluated by confocal microscopy on BAL samples (Fig. 2B) and lung tissue sections (Fig. 2C). Two days postinstillation, FITC–dextran–loaded microcapsules were readily visible inside CD11c+ BAL cells and had largely retained their characteristic spherical shape. At day 7, most of the microcapsules had leaked their content into the cells, although some heavily deformed microcapsule debris could still be detected. Two weeks after instillation, microcapsules had degraded entirely, and FITC–dextran appeared spread throughout the cells. Largely the same observations could be made on lung tissue sections, with most microcapsules being intact at day 2 postinstillation, a mixture of deformed microcapsules and scattered FITC-dextran being visible at day 7, and the further degradation of the microcapsules and complete release of their FITC-dextran content at day 14.

**PEM instillation provokes a local inflammatory response**

As described above, instillation of PEMs results in the emergence of a CD11c+ cellular fraction in the BAL, indicating an ongoing inflammatory response. To further characterize this inflammatory response, total cell numbers recruited and cellular profile of the alveolar infiltrate were analyzed (Fig. 3A).

Two days following microcapsule instillation, a 5-fold increase in total BAL cell numbers was observed. This alveolar infiltrate was dominated by monocytes and neutrophils, with only a minor fraction of eosinophils present. One week after instillation, the neutrophils had largely disappeared, and cell numbers had nearly returned to baseline. At 2 wk postinstillation, the inflammatory response had totally waned.

As alveolar macrophages are one of the first cells encountering and engulfing the microcapsules, PEMs may well exert their transient inflammatory properties by stimulating these alveolar macrophages to release inflammatory mediators. To test this hypothesis, alveolar macrophages were isolated from naive mice and incubated overnight with empty PEMs. LPS treatment was used as a positive control for macrophage activation. As shown in Fig. 3B, phagocytosis of the PEMs by alveolar macrophages did not result in an increased expression of inflammatory cytokines, chemokines, or IL-12 family members, arguing against a direct activation of alveolar macrophages by microcapsule uptake.

Complement activation following administration of biomaterials with charged surfaces is a well described phenomenon. The
complement system not only constitutes a nonspecific biochemical defense mechanism to directly lyse bacteria but also is a potent trigger of innate immune responses and inflammation through the production of the proinflammatory derivatives C3a and C5a. To analyze the dextran-sulfate/poly-L-arginine microcapsules' ability to activate the complement system and hereby trigger inflammatory cell recruitment in the lungs, BAL samples of microcapsule-instilled mice were analyzed for the presence of C3a. As shown in Fig. 3, 12 h postadministration, microcapsule instillation resulted in a drastic increase of the levels of C3a in the BAL fluid when compared with PBS-instilled mice.

**PEM instillation results in an increased APC activation status**

Pulmonary DCs continuously sample the airways and transport internalized Ags and particles to the draining lymph nodes. To address whether dextran-sulfate/poly-L-arginine PEMs get transported to the draining mediastinal lymph nodes, FITC-dextran–loaded microcapsules were instilled, and lymph node suspensions were analyzed by flow cytometry. As depicted in Fig. 4A, a large fraction of mediastinal lymph node DCs had become FITC-dextran positive, demonstrating microcapsule transport to the draining lymph nodes. Furthermore, PEM instillation also resulted in an enhanced activation status of lymph node DCs, characterized by an upregulation of the costimulatory ligands CD80 and CD86. CD40 expression was marginally upregulated, whereas MHC II expression remained unaltered when compared with PBS-instilled mice (Fig. 4B).

Similarly, PEM instillation affected the local alveolar APC activation status. Four days postinstillation, BAL cells of microcapsule-instilled mice expressed increased levels of the IL-12 family member p19, which constitutes a subunit of IL-23. Equally, IL-6 expression was elevated. Most pronounced was the induction of the monocyte chemoattractant CCL2 (MCP-1), which was

**FIGURE 1.** Mixing CaCl₂ and Na₂CO₃ results in the generation of CaCO₃ nanoparticles (gray) (A) that aggregate to form larger CaCO₃ microparticles (B). When performing this procedure in the presence of macromolecules (yellow), macromolecule-loaded CaCO₃ microparticles are obtained (B). These CaCO₃ particles are subsequently coated with alternating layers of dextran-sulfate (blue) and poly-L-arginine (red) (C), followed by the dissolution of CaCO₃ with EDTA, resulting in the generation of a hollow microcapsule surrounded by a polyelectrolyte shell (D).

**FIGURE 2.** A, Flow cytometric analysis of the uptake of FITC-dextran containing PEMs by BAL cells at different time intervals postinstillation. Alveolar macrophages/DCs were stained with CD11c-PE-Cy5. The respective percentages of cells are depicted in each quadrant. B, Confocal images of BAL cells at different time intervals postinstillation of FITC-dextran (green) containing microcapsules. Cells were stained with the macrophage/DC marker CD11c-PE-Cy5 (red). Nuclei were stained with DAPI (blue). C, Confocal images of lung sections at different time intervals after instillation of FITC-dextran (green) containing PEMs. Nuclei were stained with DAPI (blue). Figures show an overlay of differential interphase contrast, green and blue fluorescence, with the right panel being an enlarged picture of the R1 region of the left panel. Microcapsule structures are indicated by full arrows, whereas leaked FITC-dextran is indicated by dotted arrows.
increased 240-fold when compared with PBS-instilled mice (Fig. 4C). In contrast, the expression levels of CXCL1 and 2, two potent neutrophil chemokines, were downregulated following microcapsule instillation. Besides enhancing the expression of a specific inflammatory gene subset, microcapsule instillation also affected the expression pattern of markers involved in T cell interaction (Fig. 4D). Four days postinstillation, flow cytometric analysis revealed the presence of a new cell population expressing intermediate levels of the α-integrin CD11c (Fig. 4D, red). These cells expressed levels of CD40, CD80, and MHC II similar to their CD11c<sup>high</sup> counterparts. In contrast, they displayed an increased expression of the costimulatory B7 family member CD86, pointing toward a possible role of these cells in Ag presentation.

Also within the CD11c<sup>high</sup> population a subpopulation emerged that expressed strongly elevated levels of MHC II along with all costimulatory markers tested (Fig. 4C, blue). These results indicate that as a consequence of PEM instillation, a subpopulation of phagocytes, arises with an increased capacity to present Ags and to stimulate T cells, which might in case of Ag administration lead to the priming of naive T cells.

**PEMs promote adaptive immune responses against encapsulated Ag**

To evaluate the microcapsules’ ability to promote the generation of adaptive immune responses against encapsulated Ags, mice were immunized intratracheally with OVA containing PEMs. Three weeks after a booster immunization, mice were challenged with an OVA aerosol, and the resulting immune response was characterized. Analysis of the cellular alveolar infiltrate revealed that microcapsule-based immunization stimulated vigorous cellular immune responses to subsequent Ag exposure when compared with OVA/PBS-immunized mice (Fig. 5A). These cellular immune responses were characterized by a strong influx of monocytes and, to a lesser extent, of neutrophils and eosinophils.

The characteristics of the elicited local adaptive response were assessed on isolated lung CD4<sup>T</sup> T cells. Using real-time qPCR, the expression of the key transcription factors controlling Th differentiation and the cytokine profile produced were examined (Fig. 5B). Microcapsule immunization resulted in a 4-fold increase in the expression of ROR<sub>γ</sub>T, the recently identified key regulator of Th17 differentiation (27). In addition, also FoxP3, the transcription factor controlling the differentiation of regulatory T cells (Tregs), was increased 3-fold. Expression levels of the Th1 transcription factor T-bet (28) and the Th2 transcription factor GATA-3 (29) remained near baseline. The Th17-polarized response elicited by microcapsule-based immunization was further confirmed by analysis of the cytokine profile expressed by the lung CD4<sup>T</sup> T cells. OVA microcapsule vaccination did, however, result in a striking 32-fold increase in the expression of IL-17, characteristically secreted by Th17 cells. In addition, microcapsule-based immunization also augmented expression levels of the immune regulatory cytokine IL-10.

Finally, to assess the potential of PEMs to induce humoral immune responses after pulmonary delivery, the serum Ab profile of mice primed with OVA-loaded microcapsules was determined. Microcapsule immunization induced Abs of all three isotypes tested, with IgG1 clearly the dominant isotype being produced (Fig. 5C).
Ag encapsulation increases local Th17 responses

Finally, we wanted to address whether Ag encapsulation inside the microcapsules is necessary for the observed adjuvant properties of the microcapsules. To do so, mice were either immunized with microcapsules encapsulating OVA or with a mixture of empty microcapsules and sOVA. Little differences were observed in cellular alveolar recruitment between both immunization regimes in response to OVA aerosol exposure, although eosinophil numbers were significantly more elevated in the empty microcapsules/sOVA-immunized mice (Fig. 6A).

On the level of the local pulmonary Th17 response, marked differences in the strength of these responses could be detected between both immunization regimes. As described above, pulmonary CD4 T cells were isolated following OVA aerosol challenge and examined for their expression of Th differentiation transcription factors and marker cytokines by real-time qPCR. Equally to immunization with microcapsule encapsulated OVA, the mixture of empty microcapsules and sOVA mainly stimulated a Th17-polarized pulmonary response. This response appeared however much fainter when compared with the response induced by PEM-encapsulated OVA, as indicated by the much lower induction levels of RORγT and IL-17 (Fig. 6B). Similarly, but to a lesser extent, induction levels of the Treg transcription factor FoxP3 and the regulatory cytokine IL-10 were less elevated in the empty microcapsule and sOVA group compared with the PEM-encapsulated OVA group.

Using direct ELISPOT assays, Th responses in the draining mediastinal lymph nodes were examined. Three weeks following booster immunizations, mediastinal lymph node cell suspensions were prepared and incubated onto ELISPOT plates in the presence of either the OVA MHC II peptide or an irrelevant peptide (M2e). With the irrelevant peptide, the number of spots did not rise above background level for any of the immunization regimes tested (OVA/PBS, OVA cap or cap + sOVA; data not shown). PEM-encapsulated OVA again elicited a strongly polarized Th17 response, which was approximately twice as high as the response induced by the empty microcapsule and sOVA immunization regimen (Fig. 6C). Remarkably however, empty microcapsule and sOVA immunization also stimulated a Th1 (IFN-γ-producing cells) response, which was low to absent following immunization with encapsulated OVA.

Discussion

Because of their ease of preparation and high encapsulation efficiency (30, 31), dextran-sulfate/poly-l-arginine PEMs appealed as an attractive Ag delivery vehicles for vaccination. In this study, we demonstrate that PEMs not only are efficiently taken up by pulmonary APCs but also alter their functional activation status, thus exerting adjuvant properties strongly polarized toward Th17-supported cellular immune defenses.

Using OVA as a model Ag, we show that dextran-sulfate/poly-l-arginine PEMs possess the capacity to initiate adaptive immune
responses against encapsulated Ags. Challenge of microcapsule-immunized mice with OVA aerosols elicited a fast Ag-driven pulmonary inflammatory response. On the level of CD4 T cells, marked inductions of both RORγT and IL-17 were noticed, pointing toward the presence of a Th17-polarized response. No Th1 responses were observed as we failed to detect any upregulation of T-bet or IFN-γ. Equally, Th2 responses were low to absent, demonstrated by the lack of GATA-3 and IL-4 expression. Besides RORγT and IL-17, also increases in the expression levels of the transcription factor FoxP3 and of the immune regulatory cytokine IL-10 were observed, indicating the induction of Tregs. Considering the strongly opposite biological functions of both Th cell subpopulations, coinduction of the proinflammatory Th17 cells and the immunosuppressive Tregs might appear contradictory. However, both T cell subsets require TGF-β for their differentiation, and their induction appears to be closely interlinked, with Tregs possibly limiting collateral damage to self-tissue inflicted by the proinflammatory Th17 cells (32). As expected from the pronounced Ag-elicted inflammatory response and Th17 activation, microcapsule vaccination also supported a strong humoral response, which was dominated by IgG1 and accompanied by significant levels of IgG2c and IgE. The Th17 cells selectively elicited by microcapsule immunization are most likely the central mediators of the monocyte and neutrophil-driven inflammatory response following Ag challenge. Th17 cells may also be pivotal in the observed humoral immune response. Although little is known about the role of Th17 cells in promoting and skewing Ab responses, general decreases in Ab levels of all isotypes have been reported both in IL-23 (33) and IL-17 (34)-deficient mice, suggesting Th17 cells do affect humoral immunity.

How PEMs mediate their Th17-skewing activities remains currently unknown. Th17 differentiation crucially depends on Ag presentation by activated APCs that produce IL-23 and inflammatory cytokines such as IL-6 and IL-1β (35–39). Although PEMs were unable to directly activate alveolar macrophages ex vivo, they clearly enhanced the activation status of lymph node DCs in vivo as demonstrated by their increased expression of the costimulatory ligands CD80 and CD86. Microcapsule instillation also resulted in the emergence of a local alveolar population expressing elevated levels of MHC II and costimulatory markers. In addition to these increased Ag-presenting abilities, alveolar APCs also expressed elevated levels of the Th17-skewing cytokines IL-6 and IL-23, whereas the level of the Th1-skewing cytokine IL-12 remained baseline. As PEMs fail to directly activate APCs to secrete IL-23, indirect mechanisms such as complement activation might be involved in their immune-activating properties. Although the complement system primarily serves as a nonspecific biochemical defense to opsonize and/or kill pathogens, it also can promote Ag-specific immune responses. Lymphatic transport and complement activation by nanoparticles has been recently exploited by Reddy et al. (40) to activate lymph node DCs and to generate humoral as well as cellular immunity. By interacting with their cognate G protein receptors, the anaphylatoxins C3a and C5a have been reported to modulate the APC activation status (41). Strikingly, we observed strongly elevated levels of the anaphylatoxin C3a in the BAL fluid following microcapsule instillation, pointing toward a possible role of complement activation in the Th17-skewing properties of dextran-sulfate/poly-L-arginine. Up to now, IL-23 production by monocytes and DCs has been reported exclusively in response to microbial-associated molecular patterns, including the TLR ligands peptidoglycan and LPS (42), and fungal β-glucans that trigger the C-type lectin dectin-1 (43). Consequently, it will be important to verify whether the anaphylatoxin C3a, rapidly induced by the microcapsules, constitutes an alternative nonmicrobial trigger for IL-23 production.
Because of their efficient targeting to local pulmonary APCs as well as lymph node DCs, PEMs are clearly interesting Ag delivery vehicles. In addition, PEMs also display inherent immune-activating properties. To separate Ag carrier effects from immune-activating functions, we compared immune responses elicited by immunization with PEM-encapsulated OVA with those elicited by a mixture of sOVA and empty microcapsules. Immunization with encapsulated OVA clearly stimulated a much stronger local Th17 response than the sOVA microcapsule mixture. Although speculative, a prolonged Ag presentation by pulmonary APCs may result in an increased local Th17 proliferation. Indeed, when instilling FITC-dextran–loaded PEMs intratracheally, the microcapsules were still detectable in alveolar and pulmonary phagocytes up to 2 wk after administration. As a result, encapsulated Ag might be presented by the local APC population for a prolonged period of time, thus further stimulating the local mucosal Th17 population, whereas nonencapsulated Ag gets rapidly degraded and cleared from the airways. However, also at the level of the systemic immune response Th17 responses were stronger for encapsulated OVA-immunized mice. Thus, besides the inherent immune-activating properties of PEMs, also their Ag carrier function, promoting a sustained Ag presentation is an important factor in the Th17-skewing activities of the microcapsules. This is further emphasized by the Th1 response elicited by immunization with the sOVA empty microcapsule mixture, which encapsulated OVA largely failed to do, again pinpointing toward important differences between both immunization regimes.

In conclusion, dextran-sulfate/poly-L-arginine PEMs display the unique capacity to selectively promote Th17 responses. Ag encapsulation inside the microcapsules significantly increased the strength and Th17 bias of the local mucosal response compared with a mere mixture of soluble Ag with microcapsules. Although Th17 cells were originally identified as proinflammatory mediators of autoimmune disease (44–46), emerging data suggest they play crucial roles in the immune defense against extracellular pathogens, including bacteria and fungal species, especially at mucosal surfaces (47, 48). Recently, vaccine-induced Th17 cells were shown to improve immune protection also against the intracellular pathogen Mycobacterium tuberculosis (49). As a result, dextran-sulfate/poly-L-arginine microcapsule-based vaccination may become of significant interest in fighting these pathogens. Unraveling the mechanisms underlying the characteristic adjuvant properties of these microcapsules may also provide new insights in how Th17 responses can be initiated by nonmicrobial triggers.

Disclosures
The authors have no financial conflicts of interest.

References