Vaccines

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Surface-Engineered Polyelectrolyte Multilayer Capsules: Synthetic Vaccines Mimicking Microbial Structure and Function**

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While traditional vaccines have dramatically reduced the incidence of many pathogens, no effective vaccines against insidious pathogens like HIV, malaria, and tuberculosis are available today. Two main reasons are thought to underlie this failure. First, immune responses are often directed against immunodominant hypervariable regions, thereby offering pathogens the opportunity to escape through mutation. Second, current adjuvants that were approved for human use mainly stimulate the humoral arm of the immune system but fail to evoke strong cellular immune responses against recombinant antigens in addition to antibody responses.[1,2] Such potent cellular immune responses—which include macrophage-activating CD4 Th1 cells (helper T cells) and cytotoxic CD8 T cells (CTLs) that are capable of killing infected and malignant cells in view of cancer immunotherapy—are now considered crucial to develop the next generation of vaccines.[3]

Vital for the initiation of T-cell responses are the dendritic cells (DCs), which are innate immune cells that engulf pathogens and present antigens as peptides by the major histocompatibility complexes (MHCs) MHCI or MHCII to CD8 and CD4 T cells, respectively.[1] Because DCs have evolved to recognize the particulate nature of bacteria and viruses, they are less well equipped to recognize, internalize, and present soluble antigens. Moreover, while soluble antigens are mainly presented by MHCII to CD4 T cells, antigens in a particulate form are also efficiently cross-presented by MHCI to CD8 T cells.[4] To differentiate into effector cells, T cells also require the presence of inflammatory and polarizing cytokines. These cytokines are typically released by DCs when sensing the presence of microbial-associated molecular patterns (MAMPs; e.g. cell-wall components, microbial nucleic acids…) by so-called pattern-recognition receptors (PRRs); the best characterized PRRs are the Toll-like receptors (TLRs).[8] Nevertheless, mere co-injection of antigen and TLR ligands not even nearly approaches the potency of live pathogens in evoking immunity.[5]

To evoke highly potent immune responses against recombinant antigens, we have designed an entirely synthetic mimic, the morphology of which closely resembles that of a pathogen, by using a layer-by-layer[6] approach (Figure 1 and Figure S1 in the Supporting Information). Previously, we and others have demonstrated that polyelectrolyte multilayer capsules[7,8] can efficiently target antigens to DCs.[9] Importantly, we found that capsules composed of biodegradable polyelectrolytes are well-tolerated in vivo upon both subcutaneous and mucosal administration and that they are not toxic.[9b,c]

In a first step, calcium carbonate microparticles that are co-precipitated with ovalbumin (OVA; used in this study as model protein antigen) are coated in a layer-by-layer (LbL) fashion with two bilayers dextran sulfate (DS) and poly-L-arginine (pLArg) by using electrostatic interactions as the driving force. Hollow $(DS/pLArg)_2$ capsules that encapsulate nearly 50% of the initially added OVA are obtained by dissolving the calcium carbonate core in an aqueous ethylenediaminetetracetic acid (EDTA) solution. Although the polycation poly-L-arginine was deposited as the outermost layer, the zeta potential (Figure 1B) of the resulting capsules proved to be negative, which is likely due to reorganization of the excess of anionic compounds released from the $CaCO_3$ core.[9c]

Subsequently we aimed to engineer the capsule surface with immune-activating properties. For this purpose, TLR ligands based on nucleic acids (ssRNA, dsRNA, and oligonucleotides containing unmethylated CpG motifs) appeared as appealing candidates, because their strongly negative charge should allow electrostatic adsorption to the outer poly-L-arginine capsule layer.[10] Because of their relatively low molecular weight and high stability in vivo, we chose phosphorothioate-stabilized oligodeoxynucleotides containing unmethylated CpG motifs. These oligos are potent triggers of TLR9, thereby activating myeloid and plasmacytoid DCs and B cells in mice. Although TLR9 expression in humans is more restricted (absent on myeloid DCs), CpG-containing oligos are now widely being evaluated as vaccine adjuvants for infectious diseases and therapeutic cancer

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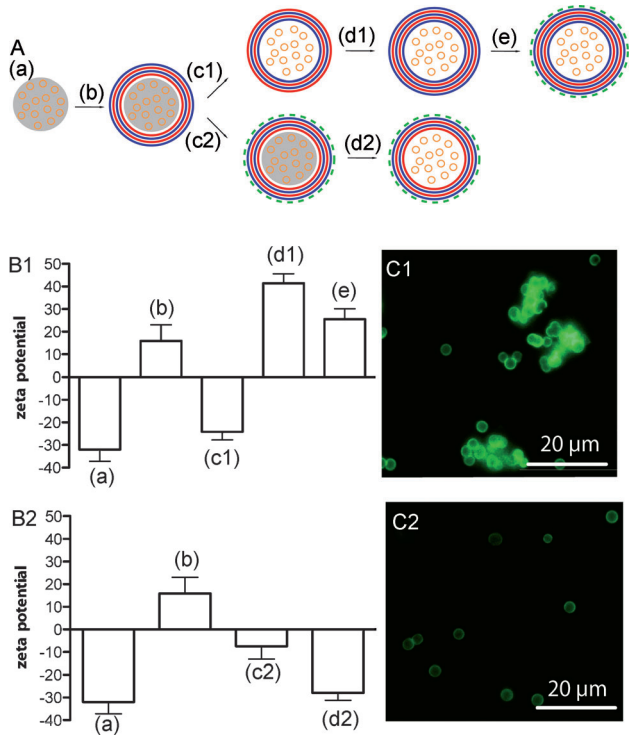

Figure 1. A) Schematic representation of the capsule fabrication; B1, B2) zeta potential values during the capsule fabrication; and C1, C2) fluorescence microscopy images of the vaccine capsules (for visualization purposes, FITC-labeled CpG was used). In a first step (a) $CaCO_3$ (gray) microparticles are loaded with antigen (orange circles) and (b) coated in an LbL fashion. For route 1, the $CaCO_3$ templates are dissolved (c1), which leads to charge reversal (red indicates a negative and blue a positive zeta potential). (d1) An additional layer of pLArg is adsorbed to render the zeta potential positive followed by (e) adsorption of a layer of CpG-containing oligos (dotted green line). The alternative route 2: (c2) a layer of CpG-containing oligos is adsorbed onto LbL-coated $CaCO_3$ microparticles followed by (d2) dissolution of the $CaCO_3$ templates. (B1, C1) and (B2, C2) correspond to route 1 and route 2, respectively.

vaccination because of their capacity to stimulate cellular immunity.[3]

To render the capsule surface cationic, an additional pLArg layer was deposited onto the capsules' surface after removal of the core by treatment with EDTA; the additional pLArg layer reversed the zeta potential to a cationic value (Figure 1 B1) and allowed successful adsorption of CpG-containing oligos. This procedure however induced severe capsule aggregation (Figure 1 C1), which is likely due to insufficient surface coverage of the capsules with CpG-containing oligos as only limited amounts (10 µg CpG-containing oligos per 100 µg OVA) were added to the capsule because of the potent immune-activating properties of the CpG-containing oligo. To cope with these issues, we decided to add the CpG-containing oligos in a last layer during multilayer adsorption (Figure 1 B2). Subsequent addition of EDTA to dissolve the calcium carbonate templates gave non-aggregated capsules (Figure 1 C2) that still retained the vast majority ($(87.2 \pm 0.6)\%$) of the initially added CpG-containing oligos on their surfaces.

In a first series of experiments, we assessed the interactions between capsules and bone-marrow-derived DCs in vitro. Capsules functionalized with CpG-containing oligos were readily internalized by DCs (Figure 2 A). The unfunctionalized capsules induced only minor upregulation of the co-stimulatory markers CD40, CD80, and CD86 and of MHCII (Figure 2 B1) and totally failed to evoke cytokine secretion (Figure 2 B2). In contrast, capsules functionalized with CpG-containing oligos promoted a strong upregulation of co-stimulatory markers combined with a vast secretion of pro-inflammatory and polarizing cytokines. Expression levels of interleukin (IL)-12 and interferon (IFN)-β, which are cytokines that promote Th1 and CTL responses, and of the inflammatory mediators IL-6, tumor-necrosis factor (TNF)-α, monocyte chemotactic protein-1 (MCP-1), CXCL-1, and CXCL-10 raised to a similar extent as those elicited by incubation with CpG-containing oligos in solution, thereby clearly indicating that CpG-containing oligos are fully capable of triggering TLR9, although they are firmly bound to the microcapsules' surface.

As the CpG motif is a potent stimulator of cross-presentation and also promotes MHCII expression by DCs, we have evaluated whether functionalizing capsules with CpG-containing oligos further potentiated the antigen pre-

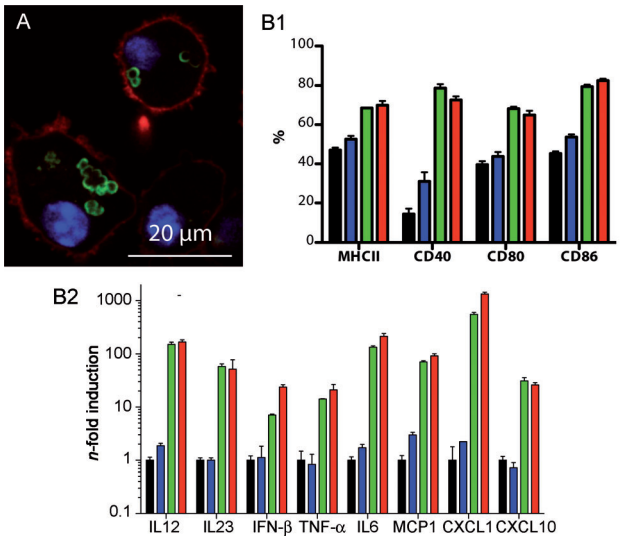

Figure 2. A) Confocal microscopy image of DCs that internalized $(DS/pLArg)_2$ capsules coated with fluorescein isothiocyanate (FITC)-labeled CpG-containing oligos (green). The cell membrane was stained with cholera toxin subunit B conjugated to Alexa Fluor 647 (red) and cell nuclei with 4′,6-diamidino-2-phenylindole (DAPI, blue). B1) Flow-cytometric analysis of DC maturation. Graphs show the percentages of DCs high in the expression of MHCII, CD40, CD80, and CD86 in the presence of phosphate-buffered saline (PBS, black), capsules (blue), CpG-containing oligos in solution (green), and capsules functionalized with CpG-containing oligos (red); B2) real-time quantitative PCR analysis of the expression of polarizing cytokines (IL-12, IL-23, and IFN-β), pro-inflammatory cytokines (IL-6 and TNF-α), and inflammatory chemokines (MCP-1, CXCL-1, and CXCL-10) after a 12 h incubation period of bone-marrow-derived DCs in the presence of the reagents listed under (B1; also the same color code applies). Note that in all experiments (in solution and on the functionalized capsules) equal amounts of CpG-containing oligonucleotides were used.

sentation promoting properties of polyelectrolyte capsules. DCs were pulsed with serial dilutions of the different OVA formulations (OVA in solution, OVA and CpG in solution, encapsulated OVA, and OVA in capsules coated with CpG-containing oligos) and co-cultured with OT-I or OT-II cells. OT-I cells are transgenic CD8 T cells with a T-cell receptor (TCR) specific for the MHCI epitope of OVA, whereas OT-II cells are their CD4 counterparts and have a TCR that is specific for the MHCII epitope of OVA. Proliferation of OT-I or OT-II cells thus reflects the strength of MHCI- or MHCII-mediated presentation of OVA and was analyzed by measuring ^{3}H-thymidine incorporation (Figure 3A). The addition of CpG-containing oligos to OVA in solution strongly increased OT-I cell proliferation, but had little effect on OT-II cell proliferation. In contrast, encapsulation of OVA inside polyelectrolyte capsules strongly increased antigen presentation to both CD4 and CD8 T cells. Coating of microcapsules with CpG-containing oligos tended to slightly further increase antigen presentation, especially at higher OVA concentrations. Although unmodified capsules are almost equally potent as capsules functionalized with CpG-containing oligos, they fail to activate DCs to secrete polarizing cytokines, the secretion of which is the second prerequisite for the differentiation of naïve T cells into effector cells. To analyze the capacity of capsules functionalized with CpG-containing oligos to promote effector-T-cell differentiation, we measured the amount of IFN-γ, the effector cytokine of CD8 T cells and CD4 Th1 cells, in the supernatant of the DC–OT co-cultures. While unmodified capsules only faintly induced IFN-γ, capsules coated with CpG-containing oligos strongly promoted IFN-γ release by both OT-I and OT-II cells (Figure 3B). These effects are not caused by dramatic differences in cell numbers, as OVA capsules are almost equally potent in stimulating T-cell proliferation, but are due to differences in the capacity of proliferating T cells to differentiate into effector T cells. When comparing capsules coated with CpG-containing oligos to a mere mixture of OVA and CpG-containing oligos in solution, little differences in IFN-γ secretion were observed on the level of OT-I cells, but huge increases were noted on the level of OT-II cell proliferation. In this case, the discrepancies between capsules coated with CpG-containing oligos and CpG-containing oligos in solution can be attributed to a vast difference in their ability to stimulate OT-II proliferation, rather than to differences in their capacity to evoke effector-cell differentiation. In conclusion, capsules functionalized with CpG-containing oligos appear to combine the best of both worlds: the capsules strongly promote antigen presentation, and the CpG-containing oligos stimulate effector cell differentiation.

Finally, we have addressed whether synergistic effects in terms of effector-T-cell response and humoral response can be achieved in vivo by co-delivering antigen and CpG-containing oligo in the same particle. First, we verified on tissue sections (Figure S2, the Supporting Information) that also in vivo, CpG-containing oligo remains adsorbed to the capsules' surface. Next, mice were vaccinated with OVA solution, a solution of OVA and CpG-containing oligos, encapsulated OVA, and OVA in capsules coated with CpG-containing oligos by applying a prime-boost scheme with a three-week interval. Three weeks after the booster immunization, the induction of IFN-γ-secreting CD4 and CD8 T cells was measured, and antibody responses were determined. As shown in Figure 4A, vaccination with OVA in capsules and OVA mixed with CpG-containing oligos in solution resulted in a limited increase in the number of IFN-γ-secreting CD4 and CD8 T cells. In contrast, a vast increase in both IFN-γ-secreting CD4 and CD8 T cells was observed after immunization with capsules coated with CpG-containing oligos, thereby clearly demonstrating the synergistic effect of co-delivery of antigen and CpG-containing oligo by polyelectrolyte capsules. On the level of the humoral immune response, OVA in capsules strongly promoted IgG1 responses (Figure 4B1), but failed to evoke robust IgG2c titers (Figure 4B2), which are generally correlated to the strength of the Th1 response evoked. In contrast, mixtures OVA and CpG-containing oligos in solution were more potent in eliciting IgG2c responses, while less strong in inducing IgG1 responses. Two biological phenomena might underlie this discrepancy. First, the CpG-containing oligo might

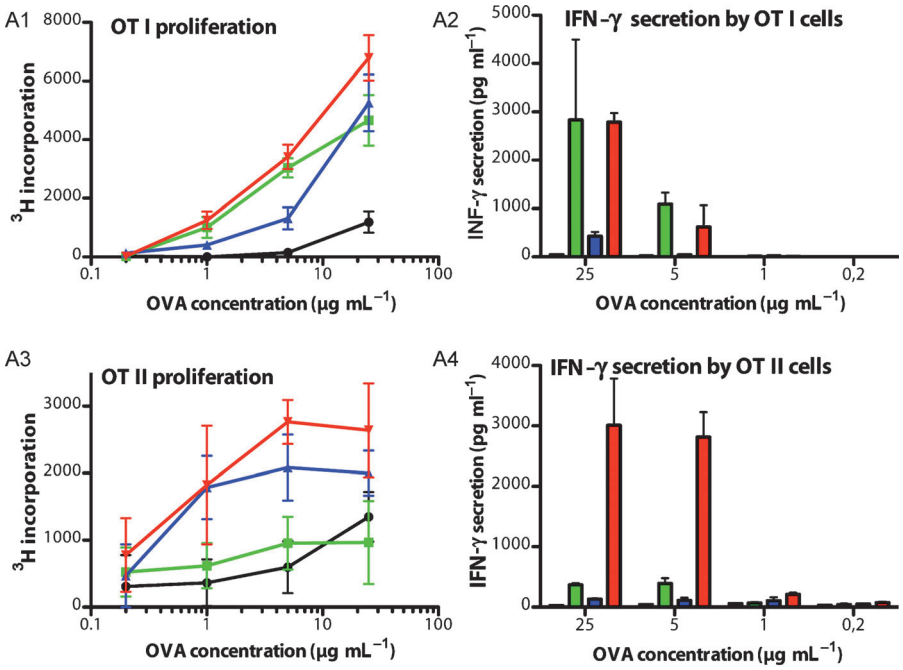

Figure 3. A) Proliferation of and IFN-γ secretion by OVA-specific CD8 T cells (OT I cells; A1, B1) and CD4 T cells (OT II cells; A2, B2) in the presence of OVA in solution (black), encapsulated OVA (blue), OVA and CpG-containing oligos in solution (green), and OVA in capsules functionalized with CpG-containing oligos (red).

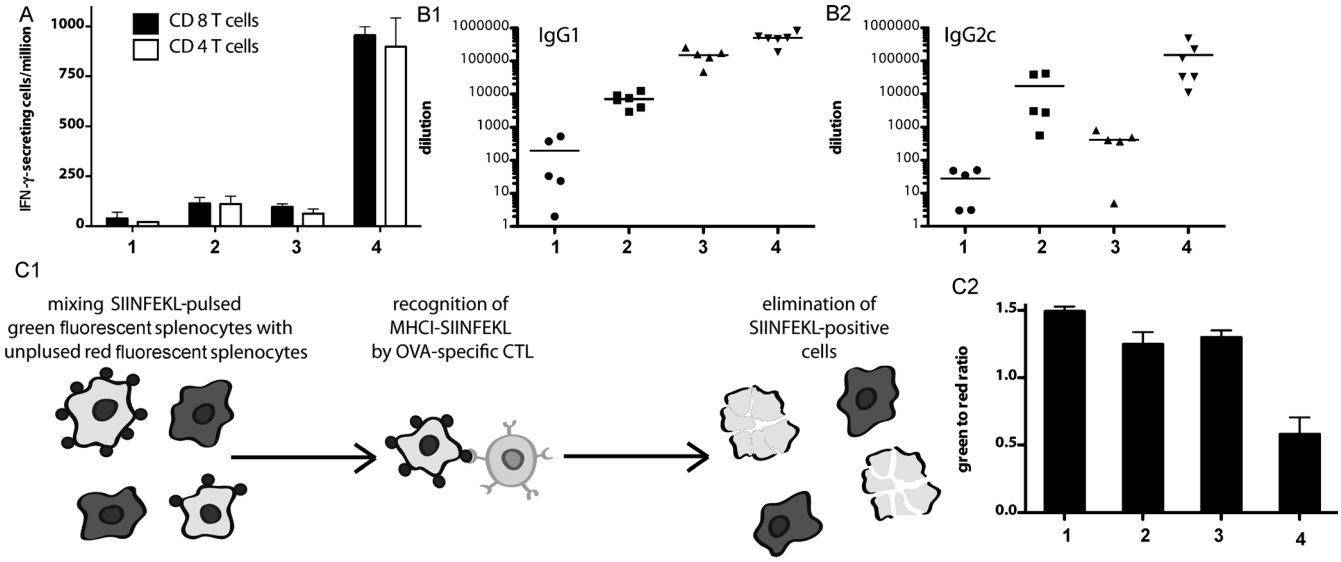

Figure 4. A) Induction of IFN-γ producing, OVA-specific CD4+ and CD8+ T cells as determined by an enzyme-linked immunosorbent spot (ELISPOT) assay with OVA in solution (1), OVA and CpG-containing oligos in solution (2), encapsulated OVA (3), OVA in capsules coated with CpG-containing oligos (4; the same compounds were used in B1, B2, C2). B) Anti-OVA antibodies IgG1 (B1) and IgG2c (B2) titers as determined by an enzyme-linked immunosorbent assay (ELISA) three weeks after the booster subcutaneous immunization. C1) Schematic overview of the protocol applied for the in vivo killing assay. C2) In vivo killing assay, showing the ratio of peptide-pulsed target cells (green fluorescent, light gray with dots in scheme) to unpulsed non-targeted cells (red fluorescent, dark gray in scheme) two days after adoptive transfer to mice immunized with the formulations 1–4.

directly promote isotype switching towards IgG2c by triggering TLR9 on B cells. Second, also IFN-γ secretion by Th1 cells in the lymph node might account for isotype switching towards IgG2c. Importantly, capsules coated with CpG-containing oligos were the most robust inducers of both antibody isotypes, again pointing out the strong benefit of co-delivery of antigen and TLR stimulus by polyelectrolyte capsules.

To address to which extent the evoked CD8 T cells were really "killer" cells capable of lysing cells that present the MHCI epitope of OVA, an in vivo killing assay was performed (Figure 4 C1). For this assay, mice were again immunized with the different formulations according to the prime-boost schedule described earlier. Three weeks after the booster immunization, they received an adoptive transfer of a 1:1 mixture of green fluorescent target cells—being naïve splenocytes pulsed with the MHCI epitope of OVA—and red fluorescent non-target cells—being naïve unpulsed splenocytes. Two days after this adoptive transfer, spleens were dissected, and the ratio of green to red fluorescent cells was determined by flow cytometry. The stronger the evoked cytotoxic-T-cell response, the more green fluorescent target cells are killed, while the red fluorescently labeled non-target cells remain unharmed. As a result, the lower the ratio of green to red fluorescent cells, the more specific killing by cytotoxic T cells has occurred. As shown in Figure 4 C2, only immunization with capsules coated with CpG-containing oligos was capable of inducing significant killing of the target cells.

In summary, we have demonstrated the feasibility of engineering polyelectrolyte multilayer capsules with immune-activating CpG-containing oligonucleotides by optimizing the electrostatic surface properties of the capsules. Immunization with capsules functionalized with CpG-containing oligos proved superior to merely encapsulate OVA or mixtures of soluble antigen and CpG-containing oligos in priming antibody responses and IFN-γ-secreting Th1 and CTL responses. Thereby, polyelectrolyte-based microbial mimics might hold strong potential to develop vaccines against intracellular pathogens such as *Mycobacterium tuberculosis* and HIV, and to also to design therapeutic cancer vaccines.

Experimental Section

Polyelectrolyte capsules: Ovalbumin (OVA; grade VII), dextran sulfate (10 kDa), and poly-L-arginine hydrochloride (Mw > 70 kDa) were purchased from Sigma–Aldrich. CpG-containing oligo (ODN 1826) was purchased from Invivogen. $CaCO_3$ microparticles loaded with OVA and coated with $(DS/pLArg)_2$ were fabricated as reported.[8a,9d] Capsules that were assembled following the procedure c1 in Figure 1 C1 were coated with an additional layer of poly-L-arginine under similar conditions as those used for coating templates with a $CaCO_3$ core. Adsorption of CpG-containing oligos was performed by addition of a solution of CpG-containing oligos (1 mg mL^{-1}, 100 μL) in pure water to 1 mL of either a capsule suspension (procedure c1; Figure 1 C1) or $(DS/pLArg)_2$-coated $CaCO_3$-core templates (procedure c2; Figure 1 C1) under vortexing. The binding efficiency of CpG-containing oligos was measured using FITC-labeled CpG-containing oligos, by measuring the fluorescence in the supernatant after centrifuging the capsules or by measuring the fluorescence of CpG-containing oligos in the supernatant after coating the $(DS/pLArg)_2$-covered $CaCO_3$-core templates as well as after dissolution of these $CaCO_3$-core templates in aqueous EDTA (0.2 M) solution. Complete core removal was verified by optical microscopy under polarized light. Zeta potential of particles coated with polyelectolyte and CpG-containing oligos was measured in pure water using a Malvern Zetasizer NS.

Mice: C57BL/6 mice were obtained from Janvier. OT-I and OT-II transgenic mice (C57BL/6) were purchased from Harlan. Mice were

housed under specific-pathogen-free conditions. All animal experiments were approved by the Local Ethical Committee of Ghent University.

DC preparation: Bone-marrow-derived DCs were generated by flushing tibia and femurs of 2–4 months old mice. After red blood cell lysis, cells were cultured in complete RPMI (Roswell Park Memorial Institute) medium containing 20 ng mL^{-1} GM-CSF (granulocyte macrophage colony-stimulating factor) for 6–8 days.

DC activation in vitro: DC activation was analyzed by incubating DCs (10^6 cells mL^{-1}) with capsules, soluble CpG-containing oligos, or capsules functionalized with CpG-containing oligos in triplicate and analyzed by flow cytometry (The following fluorophore-conjugated antibody reagents from BD Pharmingen were used: MHCII-FITC, CD40-PE, CD80 V450, CD11c-APC and CD86-PE-Cy7; PE = phycoerythrin, V450 = a coumarin dye, APC = allophycocyanin, Cy7 = cyanine dye 7). Data are representative for three independent experiments.

In vitro antigen-presentation assay: Cell suspensions of OVA-specific CD4 and CD8 T cells were prepared from spleen and lymph nodes from OT-II and OT-I mice, respectively. Single cell suspensions were prepared, and CD4 and CD8 T cells were isolated from the suspensions using Dynal mouse CD4/CD8 negative isolation kit (Invitrogen) according to the manufacturers' instructions. DCs were pulsed with serial dilutions of the respective samples for 24 h, washed, counted and subsequently co-cultured with either OT-I or OT-II cells in a 1:10 ratio for 48 h. After 48 h, the supernatant was collected, and the IFN-γ concentration was determined using the murine IFN-γ Flex-Set Cytometric Bead Assay (BD Pharmingen). ^{3}H-Thymidine (1 mCi; Amersham Biosciences) was added for 16 h. Cells were harvested onto lyofilters and ^{3}H-thymidine incorporation was assessed using beta counter. All experiments were independently run in triplicate.

ELISA: Blood samples were collected from the ventral tail vein. Maxisorp (Nunc) plates were coated with OVA (10 μg mL^{-1}) and incubated with serial dilutions of serum. ELISAs were subsequently detected with goat anti-mouse IgG1-HRP (Southern Biotech; HRP = horse-raddish peroxidase) and goat anti-mouse IgG2c-HRP (Southern Biolabs), respectively. Data show antibody titers of individual mice and are representative of three independently performed experiments.

ELISPOT: Splenocytes were harvested three weeks after the booster immunization. Suspensions of 2×10^5 splenocytes were cultured onto IFN-γ ELISPOT plates (Diaclone) in triplicate and restimulated with 5 μg mL^{-1} of either the OVA MHCI epitope peptide SIINFEKL or the OVA MHCII epitope peptide ISQAVHAAHAEINEAGR (both Anaspec). ELISPOTs were developed after a 24 h incubation period. Data are representative for three independent experiments.

CTL assay: Groups of five mice were immunized twice with the different formulations. Splenocytes from naïve mice were pulsed with SIINFEKL peptide (30 μg mL^{-1}) for two hours at 37 °C. Subsequently, these pulsed splenocytes were washed and stained with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes). In parallel, unpulsed splenocytes were stained with 20 μM CellTrackerRed (Molecular Probes). Pulsed and unpulsed splenocytes were mixed in a 1:1 ratio, and 10^7 splenocytes were intravenously injected into immunized mice three weeks after the booster immunization. Two days after adoptive transfer, spleens from receiver mice were isolated, and the ratio of pulsed versus unpulsed transferred target cells was analyzed by flow cytometry for each individual mouse.

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