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Binding, Internalization, and Antigen Presentation of Vaccine-Loaded Nanoengineered Capsules in Blood**

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Standard vaccine technologies based on the induction of antibodies are ineffective in preventing many globally important infectious diseases, including HIV/AIDS.[1] T cell immunity correlates with the control of many chronic infectious diseases, including HIV in humans[2] and SIV in monkeys.[3,4] However, inducing persistent T cell immunity has proven difficult, in part due to problems with prolonged availability of safe antigens to specialized APCs such as dendritic cells (DCs).[5–7] Recent adenovirus vector-based strategies used to induce T cell immunity have actually heightened susceptibility to HIV-1 infection, emphasizing the need for novel, safer, and more effective means to induce immunity.[8–10] Peptides are safe vaccine antigens, however proteases *in vivo* can rapidly degrade peptide-based vaccines and this has limited their utility to date. Our group recently showed that mixing free peptides with macaque monkey blood cells effectively stimulates T cell immunity and prevents AIDS in SIV-infected monkeys.[11,12] However, this technology is limited by the need for high doses of peptides and *ex vivo* mixing of the peptides with blood APCs to prevent rapid degradation of the vaccine antigens. Other effective methods, such as targeting labile vaccine antigens to purified cultured DCs necessitate extensive *ex vivo* isolation of DCs, are also impractical for widespread use.[13,14] Novel technologies to protect peptide antigens for delivery to APCs *in vivo* are required in order to improve HIV vaccine strategies using safe immunostimulatory peptides.

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Nanoengineered capsules composed of sequentially assembled polymer layers hold immense promise for a variety of biomedical applications.[15–18] These capsules are prepared by the layer-by-layer (LbL) deposition of interacting polymers onto a sacrificial colloidal template followed by core removal (Fig. 1). The assembly process allows for nanoscale manipulation of the properties of the capsules, including size, composition, permeability, colloidal stability and surface functionality.[17–21] Alternative approaches to capsule-based delivery systems, such as liposomes[22] and protein-loaded microgels,[23] have been investigated for protein delivery to APCs, however the fine control of capsule properties afforded by LbL assembly and the ability to trigger release of the cargo by means other than changes in pH sets this system apart from other particulate delivery systems, making these capsules promising candidates for the creation of next-generation vaccine carrier systems. For the ultimate application of LbL capsules in vaccine technology, the behavior of the capsules in blood, in particular the interaction with white blood cells (WBCs), the first line of defence and immune response of the body, requires investigation.

In this study, we examined the effect of surface chemistry on the binding of LbL capsules to WBCs in whole human blood, and demonstrate that the surface chemistry can be used to influence the association to APCs. Furthermore, we report, for the first time, the loading of these capsules with an oligopeptide vaccine and demonstrate the delivery of the functionally active cargo to APCs to stimulate an immune response.

Surface chemistry plays a pivotal role in the interaction of materials with living cells, including WBCs. We therefore prepared LbL capsules using a variety of polymers, including synthetic polyelectrolytes (poly(styrene sulfonate), PSS; poly (allylamine hydrochloride), PAH; and thiolatedpoly(methacrylic acid), PMA_{SH}[24,25], polypeptides (poly(L-lysine), PLL; poly (L-glutamic acid); PGA) and DNA.[26,27] PSS/PAH and PLL/PGA are electrostatically assembled polymer pairs, which are typical examples of nondegradable and biodegradable multilayer systems, respectively. Capsules assembled entirely from DNA are held together through specific interaction of the base pairs and are both biodegradable and biocompatible. The PMA_{SH} capsules are a single-component polymer system, consisting of PMA chains stabilized at physiological pH through biodegradable disulfide linkages.[24,25]

As a first step towards the creation of the vaccine carrier vehicles based on LbL capsules, we assessed the efficiency of

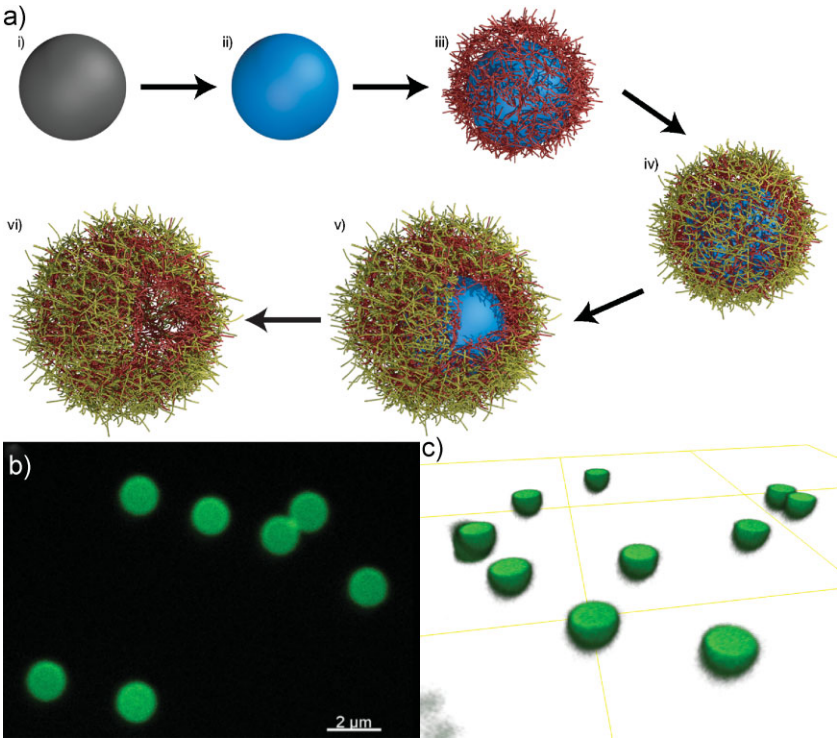

Figure 1. Nanoengineered LbL capsule assembly. a) Schematic of capsule assembly onto a sacrificial colloidal template. The colloidal template (i) is incubated in a solution containing a therapeutic (in this case peptide), which is electrostatically deposited on the surface (shown in blue) (ii). The capsule is assembled by the alternate deposition of interacting polymers (iii and iv) to form a multilayered structure (v). The colloidal template is then removed to form a capsule containing the peptide cargo (vi) (the encapsulated peptide (blue) is omitted for clarity). b) and c) A confocal microscopy cross-section and 3D confocal reconstruction of the PMA_{SH} capsules containing fluorescently labelled peptide, showing the uniform size and loading of the peptide within the capsules.

APCs in whole human blood to bind capsules. Fresh heparin-anticoagulated whole blood (200 μL) from healthy donors was incubated for 1 hour at 37 °C with fluorescently-tagged 1 μm capsules prepared with different surface chemistries. The binding of the capsules to various blood cell populations was then determined by flow cytometry. Red blood cells (RBCs), which make up 99% of the cells in blood, showed minimal binding for all capsule types and capsule concentrations tested (Supporting Information Fig. 1). WBCs were identified by flow cytometry using standard forward and side scatter properties. The primary APC populations of monocytes and DCs were further defined using monoclonal antibodies to CD14 for monocytes and MHC class II (HLA-DR) and lack of expression of markers of other blood cell lineages (i.e., negative for CD3, CD14, CD16, CD19, CD56), for DCs (Supporting Information Fig. 2). The proportion of blood monocytes and dendritic cells associated with fluorescent capsules across the six different capsule surface chemistries was studied at various capsule to WBC ratios (Fig. 2). Capsules with both positively and negatively charged outer layers were investigated, as it was expected that the surface chemistry would affect the interaction with WBCs.

At a 100:1 ratio of capsules to WBCs, all capsules demonstrated a high degree of association with monocytes (>90%, Fig. 2; top panel). Similar behavior was observed with the overall population of WBCs with the exception of capsules with a PSS or DNA outer layer, where only ~30% of the WBCs were associated with the capsules (Fig. 2; top panel). At this high capsule to WBC ratio, between 20 and 50% of DCs associated with the capsules (Fig. 2; top panel). At a lower capsule to WBC ratio of 10:1, the positively charged capsules (PAH or PLL outer layers), and, surprisingly, capsules with a PGA outer layer (negatively charged) exhibited the highest degree of association with the WBCs (Fig. 2; middle panel). This association was more pronounced for the monocytes and to a lesser extent DCs. In contrast, capsules with an outer layer comprised of the negatively charged PSS, DNA or PMA_{SH} demonstrated a lower affinity to WBCs (Fig. 2; middle panel). These data demonstrate that capsules of this size and with varied surface chemistry can effectively interact with WBCs in the complex environment of whole blood. Specifically, although significant numbers of capsules are taken up by granulocytes, sufficient capsules remain available for binding to specialized APC populations, such as DCs. This is of particular interest, as DCs are a rare blood cell population (typically constituting only 0.1% of WBCs), and are critical for the induction of primary immune responses.

At a 1:1 ratio, the WBCs incubated with capsules exhibit only a slight increase in fluorescence compared to the control sample, suggesting that a minor fraction of cells has associated with the engineered capsules (Fig. 2; bottom panel). This implies that a significant proportion of the capsules did not associate with the WBCs. This is an important observation in the context of targeted drug delivery via surface functionalization of the capsules, as such low nonspecific uptake of the capsules will potentially render the capsules available to target cells.

Using flow cytometry to determine the association between the LbL capsules and APCs within whole blood does not distinguish between surface binding and internalization phenomena. Internalization is required for capsule vaccines to efficiently stimulate T cell immunity, since the vaccine antigens need to associate with MHC molecules within the cells. We used PMA_{SH} capsules as a model system to further study cell internalization and antigen presentation. These capsules are stabilized via disulfide linkages, which remain intact in the overall oxidizing environment of the blood but are degraded in reducing conditions similar to those inside living cells, to release the encapsulated cargo.[24,25] These capsules

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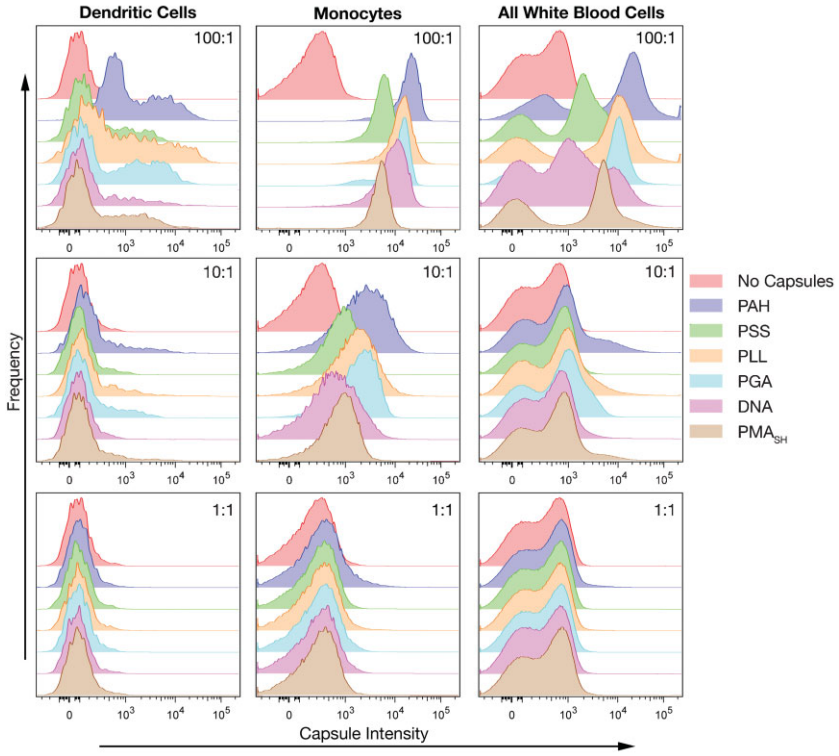

Figure 2. Binding of Rhodamine B-labelled LbL capsules by human white blood cells in fresh whole blood. Association of gated dendritic cells, monocytes and all white blood cells with Rhodamine B-labelled LbL capsules with different outer layer surface chemistries (PAH; poly (allylamine hydrochloride), PSS; poly(styrene sulfonate), PLL; poly(L-lysine), PGA; poly (L-glutamic acid), DNA and PMA_{SH}; poly(methacrylic acid)) incubated at different ratios of capsules to white blood cells (100:1, 10:1 and 1:1), as measured by flow cytometry. Capsules were prepared using the same fluorescent precursor (2 layers of alternating Rhodamine B-labelled PSS and PAH), followed by the addition of 3 or 4 bilayers of the different polymers (PSS/PAH; PLL/PGA; DNA or PMA_{SH}) to result in capsules with different surface chemistries.

are colloidally stable over a range of physiologically relevant experimental conditions (e.g., in the presence of serum proteins) and can be loaded with intact globular proteins[24] and DNA.[28] Here, we studied the internalization of PMA_{SH} capsules by WBCs using imaging flow cytometry. All cell populations (DCs, monocytes, granulocytes) identified as associating with the capsules by conventional flow cytometry also demonstrated the presence of internalized capsules (Fig. 3; a, b, and c). Similar results were observed for all capsules with different surface functionalities tested. Examination of the cells by conventional confocal microscopy also confirmed the presence of capsules within both monocytes and DCs (Fig. 3; d and e).

Following internalization within APCs, the LbL capsules must release their vaccine payload for association with MHC molecules within the cell and presentation on the cell surface to stimulate the cellular arms of the adaptive immune system. To assess the presentation of encapsulated vaccine antigen to responder immune cells, we constructed PMA_{SH} capsules incorporating the 9-amino-acid peptide KP9. The KP9 epitope is amino acids 164–172 of the SIV Gag protein and is presented to monkey (*M. nemestrina*) CD8 T cells via the MHC class I molecule *Mane-A*10*.[29] These monkeys are susceptible to SIV-induced AIDS and KP9-specific CD8 T cells slow the development of progression of infection in this model.[29] The pristine KP9 oligopeptide is a ~1 kDa molecule, which can freely diffuse through the wall of the PMA_{SH} capsules. To achieve encapsulation, the KP9 peptide was modified with a cysteine residue at the N-terminus and covalently attached through a disulfide linkage to the anchoring polymer, PMA_{SH}. As with the capsule layers, cleavage of this disulfide bond inside the cell is expected, hence releasing the KP9. The conjugation was achieved in a two-step process, wherein the thiol groups of PMA_{SH} were first activated to thiol-disulfide exchange using Ellman's reagent, and then used to interact with the KP9 peptide containing a terminal thiol. The resulting polymer-peptide conjugate (KP9-PMA_{SH}) was adsorbed onto amine-functionalized silica template particles and used as the first layer to construct the PMA_{SH} capsules. PMA_{SH} and PVPON layers were then alternately assembled and upon the removal of the template,[28] the KP9-PMA_{SH} conjugate was confined within the PMA_{SH} capsules (Fig. 1).

To assess the presentation of encapsulated KP9 to responder KP9-specific CD8 T cells, we obtained fresh monkey blood from an SIV-infected macaque known to express the MHC class I allele *Mane-A*10* and have a population of circulating CD8 T cells specific for the KP9 epitope. The KP9-specific CD8 T cells are identified by flow cytometry using a fluorescently-labelled tetrameric molecule composed of *Mane-A*10* folded around the KP9 epitope.[30] *In vivo* these CD8 T cells are typically unactivated but are stimulated to express the cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) *ex vivo* if they recognize KP9 in the context of *Mane-A*10* on APCs.[31] We incubated fresh monkey blood with the KP9-loaded PMA_{SH} capsules or control (unloaded) PMA_{SH} capsules. We then identified the KP9-specific CD8 T cells (Supporting Information Fig. 3), and studied their intracellular expression of IFN-γ and TNF-α. Control capsules did not stimulate the KP9-specfic T cells (Fig. 4a), but KP9-loaded capsules stimulated a significant proportion of the KP9-specific T cells to simultaneously express both cytokines (Fig. 4b). KP9 soluble peptide alone was also tested and resulted in T cell stimulation (not shown). This served as a positive control for the assay as it is able to bind directly to MHC-I present on the surface of cells without the requirement of intracellular processing. Activation of T cells was limited to the KP9-specific CD8 T cells, and not any

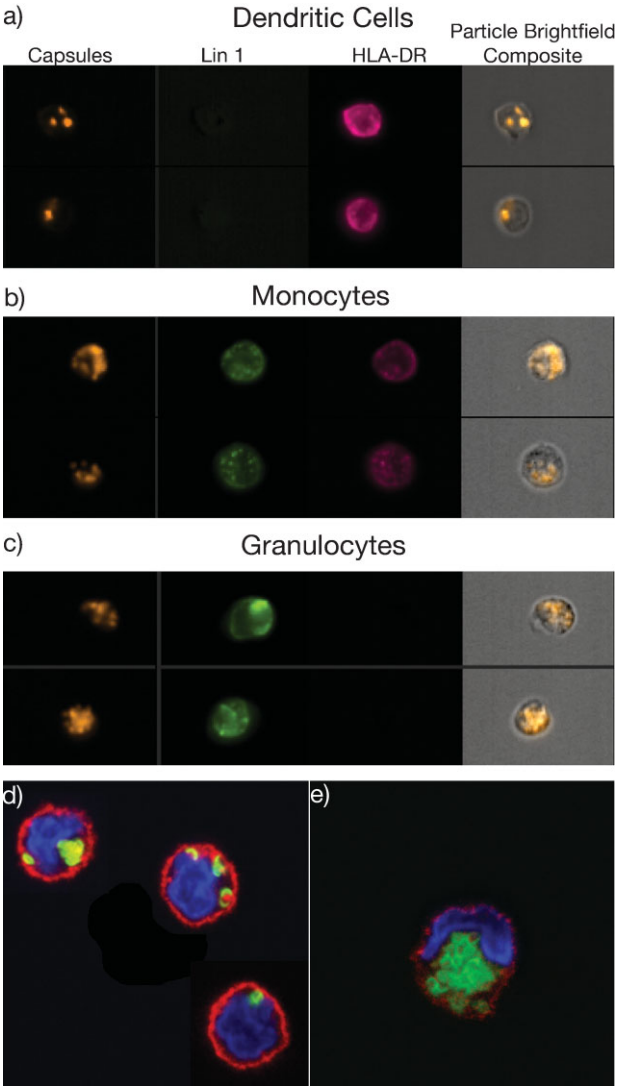

Figure 3. Internalization of Rhodamine B-labelled LbL capsules by white blood cells. Imaging of a) dendritic cells (lin 1^-/HLA-DR^{+++}), b) monocytes (lin 1^+/HLA-DR^+), and c) granulocytes (lin 1^+/HLA-DR^-) with associated Rhodamine B-labelled PMA_{SH} capsules using an AMNIS ImageStream® Flow Cytometer. Columns 1–3 show individual markers for capsules, lin 1 (CD3, CD14, CD16, CD19, CD20, and CD56) and HLA-DR, respectively. The last column is a composite image of brightfield and capsule fluorescence demonstrating internalization of the capsules. d) Confocal cross-section of capsules internalized into dendritic cells and e) monocytes; capsules are labelled green, the cell membrane is labelled red and the cell nucleus is labelled blue.

other T cell populations (Fig. 4c), demonstrating the specificity of the interaction. Furthermore, supernatants derived from the KP9-loaded capsules did not activate the KP9-specific CD8 T cells (Fig. 4d), indicating that the KP9 peptide was retained within the capsules and intracellularly released and processed, presumably through the endoplasmic reticulum, for MHC class I presentation and stimulation of the responding primary CD8 T cells.

In summary, we show here that a variety of LbL nanoengineered capsules efficiently associate with monocytes and DCs, important APC populations, within fresh human blood. Additionally, we have demonstrated that these capsules are internalized by blood APCs. By encapsulating a model HIV vaccine peptide, KP9, within biodeconstructible capsules, we show that KP9 is internalized into the APCs and intracellularly trafficked for presentation with MHC-I to responding primate lymphocytes to elicit an immune response. We expect that these nanoengineered, vaccine-loaded capsules will be highly efficient for the stimulation of immune responses to a wide range of important diseases and will not be limited to the delivery of peptide antigens. Further comparative studies on the ability of vaccine-loaded particles to stimulate immune responses *in vivo* are now warranted.

Experimental

Materials: Silica particles (1 μm diameter) were purchased from MicroParticles GmbH, Germany. 2-(N-Morpholino)ethanesulfonic acid (MES), γ-morpholino-propanesulfonic acid (MOPS), poly(allylamine hydrochloride) (PAH; *Mw* 70000 g mol^{-1}), poly(sodium 4-styrenesulfonate) (PSS; *Mw* 70000 g mol^{-1}), poly(L-lysine) (PLL; *Mw* 45800 g mol^{-1}), poly (L-glutamic acid) (PGA; *Mw* 90600 g mol^{-1}) and poly(vinylpyrrolidone) (PVPON, *Mw* 10000 g mol^{-1}) were purchased from Sigma–Aldrich and were used as received. Poly(methacrylic acid) (PMA; *Mw* 15000 g mol^{-1}) was purchased from Polysciences, Inc. and was used as received. Rhodamine B-labelled PSS (PSS-Rh) was synthesized as outlined previously [32]. PMA_{SH} with 12 and 5 mol % of thiol groups was synthesized from PMA and cystamine dihydrochloride via carbodiimide coupling, as described previously [24]. Oligonucleotides were custom synthesized by Geneworks (Adelaide, South Australia). High purity water with a resistivity greater than 18 MΩ cm was obtained from an inline Millipore RiOs/Origin system (MilliQ water).

LbL Capsule Assembly with Different Surface Chemistries: Fluorescent Capsule Precursor: For the blood binding and internalization experiments, all capsules were prepared from a fluorescent precursor comprised of PSS/PAH assembled onto silica particles. This was to ensure a standardized fluorescence intensity over the range of capsules so that direct comparisons of binding could be made by flow cytometry. To construct the precursor, sacrificial 1 μm diameter silica particles were used for the sequential deposition of PSS-Rh and then PAH to assemble two bilayers. Briefly, PSS-Rh (0.5 mL of 1 mg mL^{-1} in 0.5 M NaCl) was added to an amine-functionalized silica particle suspension in water (0.2 mL of 0.5 wt %). Adsorption was carried out for 20 min, followed by centrifugation (1000 *g* for 1 minute). The supernatant was removed and the particles were washed three times in water. PAH (0.2 mL of 1 mg mL^{-1} in 0.5 M NaCl) was then allowed to adsorb for 20 min and washed as described above. The process was repeated, with alternating layers of PAH or PSS, to result in a film structure of $(PSS\text{-}Rh/PAH)_2$.

PSS/PAH: For capsules with an outer layer of PSS or PAH, three further bilayers of PSS/PAH were deposited onto the fluorescent capsule precursor on the silica particles, as outlined above, resulting in a final film structure of $(PSS\text{-}Rh/PAH)_2/(PSS/PAH)_3$ for the PAH outer layer capsules and $(PSS\text{-}Rh/PAH)_2/(PSS/PAH)_3/PSS$ for the PSS outer layer capsules.

PLL/PGA: For capsules with an outer layer of PLL or PGA, four bilayers of PGA/PLL were deposited onto the fluorescent capsule precursor on the silica particles (using the same procedure and conditions for the deposition of PSS/PAH), resulting in a final film structure of $(PSS\text{-}Rh/PAH)_2/(PGA/PLL)_4$ for the PLL outer layer capsules and $(PSS\text{-}Rh/PAH)_2/(PGA/PLL)_4/PGA$ for the PGA outer layer capsules.

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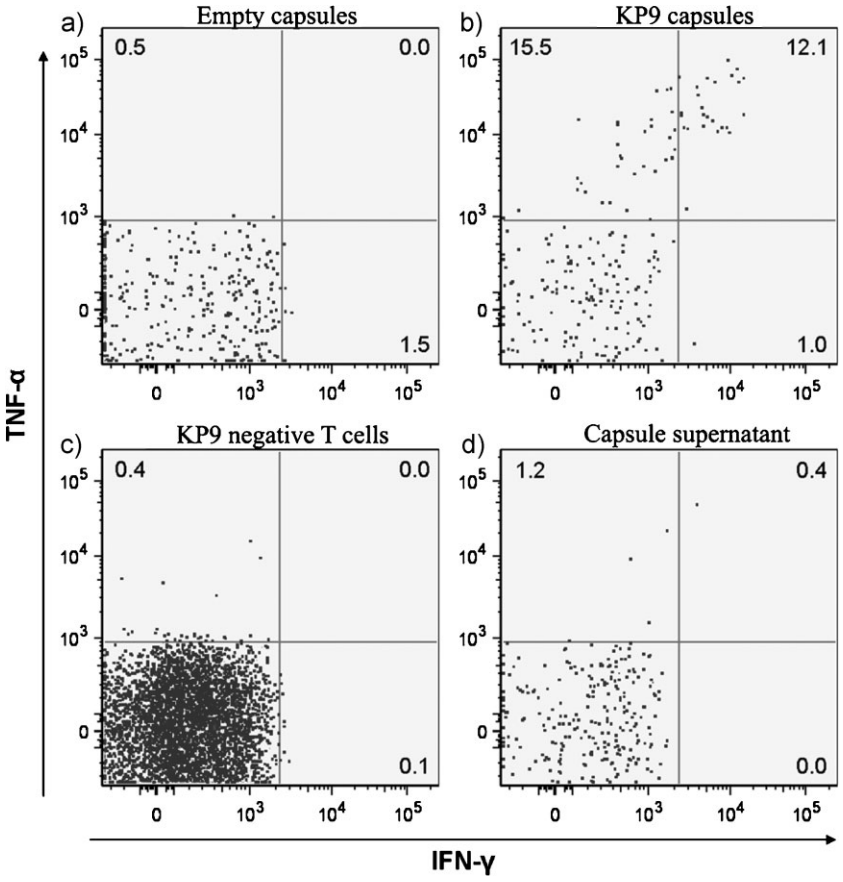

Figure 4. Presentation of LbL encapsulated antigen to activate T cells. T cell activation in response to the encapsulated antigen KP9 was assessed by IFN-γ and TNF-α accumulation in KP9 specific $CD8^+$ T cells in whole blood from an SIV infected *Mane-A*10*$^+$ pigtail macaque. a) Cytokine production of KP9 specific CD8 T cells in blood incubated with empty PMA_{SH} capsules (1000 capsules:1 white blood cell), b) Cytokine production of KP9 specific $CD8^+$ T cells in blood incubated with KP9-loaded PMA_{SH} capsules (50 capsules:1 white blood cell), (c) Cytokine production of non-KP9 specific T cells in blood incubated with KP9-loaded PMA_{SH} capsules (50 capsules:1 white blood cell), d) Cytokine production of KP9 specific $CD8^+$ T cells in blood incubated with supernatant from KP9-loaded PMA_{SH} capsules (equivalent volume to 50 capsules:1 white blood cell).

DNA: For capsules with an outer layer of DNA, a primer $polyT_{30}$ oligomer layer was deposited on the surface of the fluorescent capsule precursor on the silica particles. Subsequently, the particles were alternately incubated in DNA solutions (10 μm in SSC buffer) of $polyA_{15}X_{15}G_{15}$ or $polyT_{15}X_{15}C_{15}$ (where X is GTCAGGAATTCTAGC) and hybridization was allowed to proceed for 20 minutes at room temperature. A total of four bilayers were deposited. The DNA film was then stabilized through cross-linking the film with $X'_{15}X'_{15}X'_{15}$ (where X' is GCTAGAATTCCTGAC) [33]. The final film structure was $(PSS\text{-}Rh/PAH)_2/polyT_{30}/(polyA_{15}X_{15}G_{15}/polyT_{15}X_{15}C_{15})_4$.

PMA_{SH}: For capsules with an outer layer of PMA_{SH} the silica particles with the fluorescent capsule precursor were alternately incubated in PMA_{SH} or PVPON (1 g L^{-1} solution) for 15 min. The particles were washed three times in sodium acetate buffer (20 mM, pH 4) between layers. A total of 4 bilayers were deposited, after which the particles were treated with a solution of chloramine T (2.5 mM) in 2-morpholinoethanesulfonic acid (MES) buffer (10 mM; pH 6) for 1 min, followed by washing with sodium acetate buffer (20 mM, pH 4). On removal of the core (see below), the PVPON is displaced, resulting in a final capsule structure of $(PSS\text{-}Rh/PAH)_2/(PMA_{SH})_4$.

Sacrificial Core Removal: To form capsules, the silica core was dissolved by mixing the particle suspension (200 μL) with HF/NH_4F solution (200 μL, 2:8 M; pH 5) at room temperature. Dissolution of the silica core occurred within 1 min and was visualized *in situ*. The capsules were washed with phosphate buffered saline (PBS) and the concentration of the capsules was determined using a CyFlow Space (Partec GmbH) flow cytometer with absolute volume counting.

KP9-PMA_{SH} Conjugation and Loading in PMA_{SH} Capsules: KP9-PMA_{SH} Conjugate Preparation: The cysteine modified and fluorescently labelled KP9 peptide was reacted with PMA_{SH} (with 5 mol % of thiol groups) to form a KP9-PMA_{SH} conjugate. Briefly, PMA_{SH} (14.6 mg) was fully reduced by incubating with sodium borohydride (1 M, 400 μL) in phosphate buffer (0.1 M; pH 6.5) at room temperature for 1 h. Excess borohydride was neutralized with concentrated hydrochloric acid and the reaction was supplemented with K_2HPO_4 (to a concentration of 0.1 M). The pH was adjusted to 8 and excess Ellman's reagent (10.5 mg, Sigma) was added and the reaction mixture was left overnight. The resulting polymer was isolated via column chromatography using gel filtration and recovered by freeze drying. Thiol-terminated KP9 (0.212 μmol in DMF) was then added to the activated PMA_{SH} polymer (0.5 mg) dissolved in Tris-EDTA buffer (100 μL, 10 mM; pH 7.5). The reaction mixture was incubated at room temperature overnight and the resulting polymer-peptide conjugate was purified twice using a NAP-5 column and recovered via freeze-drying.

Encapsulation of the KP9-PMA_{SH} Conjugate: Amine-functionalized SiO_2 particles (250 μL of a 5 wt % suspension, 1 μm diameter) were washed with sodium acetate buffer (20 mM; pH 5). The KP9-PMA_{SH} conjugate (250 μL of 0.5 g L^{-1}; pH 5) was added to the particles and adsorption was allowed to proceed for 2 h. After this time, the particles were washed with sodium acetate buffer (10 mM; pH 4).

A solution of PMA_{SH} with 12 mol % thiol modification was prepared from the stock solution of PMA_{SH} (50 g L^{-1}) incubated with DTT (0.5 M) in MOPS buffer (20 mM; pH 8) for at least 12 h to ensure separation of the polymer chains. For multilayer assembly, adsorption steps were conducted using PVPON and PMA_{SH} solutions (250 μL of 1 g L^{-1}) in sodium acetate buffer (20 mM; pH 4). Polymers were sequentially added with 15 min incubation time for each layer, until 4 bilayers had been deposited, as described in detail elsewhere [25]. After completion of the multilayer build-up, the particles were exposed to a solution of chloramine T (2.5 mM) in MES buffer (10 mM; pH 6) for 1 min, followed by extensive washing with sodium acetate buffer (20 mM; pH 4). To form hollow capsules, the silica core was dissolved by treatment with HF/NH_4F solution (200 μL of 2:8 M; pH 5), as described above.

Binding and Internalization of LbL Capsules by WBCs: Whole heparinized blood (200 μL) from healthy human volunteers (obtained with informed consent) was incubated with Rhodamine B-labelled capsules (1 μm diameter) of different surface chemistries for 1 h at 37 °C. Cells were surface stained for CD14 (BD Biosciences), HLA-DR (BD Biosciences) and Lineage cocktail 1 (lin 1 (CD3, CD14, CD16, CD19, CD20, and CD56), BD Biosciences) before lysing the red blood cells with FACS lysing solution (BD Biosciences) and fixing the cells with Stabilizing Fixative (BD Biosciences) according to the manufacturer's instructions. Cells were acquired on a FACSCan-

to™ II flow cytometry system and data was analyzed using Flowjo software. Monocytes and DCs were identified by first gating on mononuclear cells according to their scatter characteristics and then identifying DCs as lin $^{-}$/HLA-DR^{+} and monocytes as lin $^{+}$/CD14^{+}. Internalization of the capsules was determined using an ImageStream 100 (Amnis Corp, Seattle) imaging flow cytometer using the same staining protocol as for standard flow cytometry. Confocal microscopy images were obtained using a Leica laser-scanning confocal unit (TCS SP2; Leica, Germany).

Presentation of LbL Encapsulated KP9 to Activate T cells: Whole heparinized blood (100 μL) from an SIV_{mac251} infected *Mane-A*10*$^{+}$ pigtail macaque was incubated at 37 °C (5% CO_2) with either 1 μm diameter KP9-PMA_{SH}-loaded PMA_{SH} capsules (ratio of 50 capsules:1 white blood cell), empty PMA_{SH} capsules (ratio of 1000 capsules:1 white blood cell) or supernatant from the KP9-PMA_{SH}-loaded PMA_{SH} capsules (volume equivalent to 50:1 ratio). All macaque experiments were approved by the University of Melbourne animal ethics committee. Co-stimulatory antibodies (anti-CD28 and anti-CD49d, BD Biosciences) were also added (final concentration 1 μg mL^{-1}). After 3 h brefeldin-A (Sigma) was added (final concentration of 10 μg mL^{-1}) to block secretion of expressed cytokines (except in the case of blood incubated with supernatant, in which brefeldin-A was added at the start of incubation). After a total of 6 h, incubation cells were surface stained for CD3 (BD Biosciences), CD8 (BD Biosciences) and KP9 specific T cells (using *Mane-A*10*/KP9 tetramer). Red blood cells were lysed with FACS lysing solution (BD Biosciences) and permeabilized with FACS Perm-2 solution (BD Biosciences) according to the manufacturer's instructions, before staining for intracellular IFN-γ and TNF-α accumulation. Finally, cells were fixed with formaldehyde (1%) and acquired on a BD FACSCanto™ II flow cytometry system. Data were analyzed using Flowjo software. Cytokine expression from KP9 specific CD8^{+} T cells was analyzed by selecting CD3^{+} cells from the population of mononuclear cells, before gating on the required population from a CD8 versus *Mane-A*10*/KP9 tetramer plot.

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