pH-Triggered Microparticles for Peptide Vaccination[1]

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Improving vaccine delivery to human APCs is a way to increase the CTL response to vaccines. We report the use of a novel pH-triggered microparticle that exploits the ability of APCs to cross-present MHC I-restricted Ags that have been engulfed in the low pH environment of the phagosome. A model MHC class I-restricted peptide Ag from the influenza A matrix protein was encapsulated in spray-dried microparticles composed of dipalmitoylphosphatidylcholine and the pH-sensitive polymethacrylate Eudragit E100. Release of the peptide from the particle was triggered by a drop in pH to the acidity normally found in the phagosome. The particles were efficiently phagocytosed by human monocytes and dendritic cells with minimal cellular toxicity and no functional impairment. Encapsulation of the peptide in the microparticles resulted in efficient presentation of the peptide to CD8$^+$ T cells by human dendritic cells in vitro, and was superior to unencapsulated peptide or peptide encapsulated in an analogous pH-insensitive particle. Vaccination of human HLA-A*0201 transgenic mice with peptide encapsulated in pH-triggering microparticles resulted in priming of CTL responses. These microparticles can be modified to coencapsulate a range of adjuvants along with the Ag of interest. Encapsulation of MHC I epitopes in pH-triggered microparticles increases Ag presentation and may improve CD8$^+$ T cell priming to peptide vaccines against viruses and cancer. *The Journal of Immunology,* **2004, 173: 2578–2585.**

Optimizing the CTL response to vaccines is essential to improve the immunotherapy of cancer and viral diseases (1). CD8$^+$ T cells will only respond to vaccine Ags in vivo if the epitopes contained in the vaccine are presented in the context of MHC I by specialized APCs such as dendritic cells (DCs).[3] The amount of Ag presented at the time of initial encounter between T cell and the APC is a critical factor that dictates the strength of T cell stimulation. Increasing the epitope density decreases the threshold for activation of naive T cells and increases the size of the primary T cell response (2–5). APCs can present soluble exogenous Ags such as those given in vaccines to CD8$^+$ cells by what is known as cross-presentation (6, 7), but the process is inefficient (8–10). Encapsulation of protein Ag in a particulate form that can be phagocytosed by APCs markedly enhances Ag presentation and the resulting CTL response (8–10). Recent work has identified specialized cellular mechanisms by which Ags engulfed in acidic phagosomes directly enter the MHC I pathway (11–13). Targeting vaccine Ags to the phagosome by encapsulating them in microparticles therefore represents a way to improve the presentation of vaccine Ags to CD8$^+$ cells, thereby enhancing the CTL response to peptide/protein vaccines.

Controlled release technology has been used by many investigators to encapsulate vaccine Ags for delivery to APCs. Microparticles made from polymeric biomaterials such as the α-hydroxy acids, including poly(lactic-co-glycolic) acid, have been used extensively (14–16). However, one problem with poly(lactic-co-glycolic) acid microparticles is their slow degradation. Even when those particles are small, and modified to degrade relatively rapidly, they can still be found in situ weeks after injection (17). This slow degradation may lead to suboptimal intracellular delivery of the antigenic payload. One method of overcoming this problem is to make the microparticles from excipients that are pH sensitive (18, 19). Such particles remain intact at the physiological pH of the extracellular fluid, but once taken up by APCs, could disintegrate in the acidic environment of the phagosome (20). The rapid release of the particles' contents directly into an organelle rich in the machinery of Ag presentation (12, 13, 21) should facilitate loading onto MHC I.

We have previously described the generation of phospholipid-based microparticles that have been rendered pH triggerable by incorporation of a polymethacrylate (Eudragit E100 (E100)) as a model pH-sensitive material (19). These particles have properties that are potentially attractive for vaccine delivery. They are typically 2–6 μm in diameter, so they can only be taken up by cells that are capable of phagocytosis (22). They are composed of a variety of inert excipients, typically phospholipids, sugars, proteins, and other macromolecules, and the molecule (drug) of interest. Excipients can be selected that are appropriate for the milieu to which the microparticles will be delivered, thus optimizing biocompatibility (17). Furthermore, the process by which the microparticles are produced, spray drying, allows relatively high loadings of molecules of interest; for example, they can be made to contain 36% (w/w) albumin (23). Injection of microparticles of this type attracts immune cells to the site of injection as part of an acute inflammatory response that could potentiate the T cell response to vaccination (19).

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[3] Abbreviations used in this paper: DC, dendritic cell; DPPC, dipalmitoyl-phosphatidylcholine; poly-HEME, poly hydroxy ethyl methacrylate; poly(I:C), polyinosinic: polycytidylic acid.

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In this study, we describe the use of pH-sensitive microparticles composed of a phospholipid, dipalmitoyl-phosphatidylcholine (DPPC), and the pH-sensitive material E100 (19) as delivery vehicles for peptide Ags. We show pH-dependent release of an MHC I-restricted peptide epitope from influenza A matrix protein, and demonstrate efficient delivery of this epitope to human DCs. Encapsulation of the Ag in pH-triggered particles markedly enhances presentation of the peptide to $CD8^+$ T cells in vitro compared with pH-insensitive particles, and allows priming of CTL responses to the epitope in human HLA-A*0201 transgenic mice.

Materials and Methods

Peptides and other reagents

The 9-aa peptide M58 with the sequence GILGFVFTL was obtained from New England Peptide (Fitchburg, MA) with or without conjugation to the fluorophore AMC (AMC-M58). DPPC was obtained from Avanti Polar Lipids (Alabaster, AL). E100, poly(butyl methacrylate-co-(2-dimethylaminoethyl) methacrylate-co-methyl methacrylate) = 1:2:1, was a gift of Rohm (Darmstadt, Germany). FITC-labeled albumin, rhodamine isothiocyanate (ρ)-labeled lactalbumin, and polyhydroxy ethyl methacrylate (poly-HEME) were obtained from Sigma-Aldrich (St. Louis, MO). Polyinosinic:polycytidylic acid (poly(I:C)) was obtained from Sigma-Aldrich.

Production and characterization of microparticles

Particles containing FITC-albumin or ρ-lactalbumin were made as follows. One hundred milligrams of E100 or poly-HEME, and 400 mg of DPPC were dissolved in 87.5 ml of ethanol. One milligram of either labeled protein in 37.5 ml of water was added dropwise to the ethanol solution. The mixture was then fed into a Buchi 190 bench-top spray drier at the following settings: air flow, 600 Nl/h; inlet temperature, 110°C; aspiration, −18 mbar; solvent flow rate, 12 ml/min. At these settings, the outlet temperature was ~40°C.

Particles containing M58 peptide or AMC-M58 peptide were produced, as follows. M58 peptide was dissolved in acetonitrile:ethanol:water 20:56:24 with 0.1% trifluoroacetic acid, to a peptide concentration of 1 mg/ml. One hundred milligrams of E100 or poly-HEME, and 400 mg of DPPC were dissolved in ethanol, and water was added dropwise until the final volume was 125 ml minus the volume of M58 solution to be added. The pH of the solution was measured as the M58 solution was added. The pH was then adjusted back to initial value with NaOH. The mixture was spray dried, as above.

Particle size and shape determination

The size of particles was determined with a Coulter counter (Coulter Electronics, Luton, U.K.) using a 30-μm orifice. The morphologies of selected particles were assessed by scanning electron microscopy using an AMR-1000 at 10 kV using a gold-palladium conductive coating.

In vitro release of FITC-labeled albumin and M58 peptide

Five-milligram aliquots of particles were suspended in 1 ml of PBS, pH 7.4, and incubated at 37°C. At predetermined time points, the sample was centrifuged (8000 rpm for 4 min), and the supernatant was removed. Samples were resuspended into PBS or, 1.5 h or 4 days after initial suspension, into 100 mM sodium acetate, pH 5. Once suspended in sodium acetate, samples were kept in that solution. The fluorescence in the supernatant was quantitated with a PTI system (Photon Technology International, Lawrenceville, NJ) at the following wavelengths (excitation and emission, respectively): FITC-albumin, 485, 515; AMC-M58, 350, 447.

Donors and cell lines

Leukapheresis products were obtained from healthy blood donors with appropriate consent from the Dana-Farber/Harvard Cancer Center Institutional Review Board (Boston, MA). PBMC were purified by Ficoll density centrifugation and cryopreserved. Immature DCs were generated from plastic-adherent monocytes by culture with IL-4 and GM-CSF, as described (24).

Human T cell lines specific for M58 peptide were generated, as described (24). Clones were generated by plating T cells from lines with peptide-specific cytotoxic activity at 0.3 cells/well with irradiated EBV-lymphoblastoid lines and allogeneic PBMC together with soluble CD3 (OKT3) and IL-2 (100 U/ml; Chiron, Emeryville, CA). Wells with growing clusters were expanded by restimulating with the same combination of allogeneic feeder cells, CD3 Ab, and IL-2 before being screened for cytotoxic activity. The clone used for experiments was $CD8^+$, and stained strongly with an HLA-A*0201-peptide tetramer containing M58 peptide.

*HLA-A*0201 transgenic mice and immunization procedures*

HHD mice express a chimeric human (α1 and α2 chains) and murine (α3 chain) HLA-A*0201 H chain covalently linked to the human β_2-microglobulin L chain. The murine MHC I molecule H-2 D^b has been deleted (25). HHD mice were injected s.c. at the base of the tail with 100 μg of M58 peptide or the corresponding amount of peptide encapsulated in microparticles. No other adjuvant was given. After 7 days, splenocytes from primed HHD mice were harvested and restimulated with peptide-loaded HHD lymphoblasts, as previously described (25). Six days later, cultured cells were tested for cytotoxic activity in a 4-h ^{51}Cr release assay, using as targets either HHD-transfected TAP^- RMA-S cells loaded with M58 or negative control RT Pol 476 (SYNT:EM, Nimes, France) peptides (10 μg/ml).

ELISPOT analysis

ImmunoSpot plates (Cellular Technology, Cleveland, OH) were prepared by precoating with 5 μg/ml anti-IFN-γ Ab (Mabtech, Nacka, Sweden) overnight at 37°C. DCs were loaded overnight with particles containing M58 peptide or with free peptide, harvested, washed, and plated with T cells in varying ratios, and incubated at 37°C for 18 h. After washing, wells were developed, according to the manufacturer's recommendations, and the spots were visualized with a 5-bromo-4-chloro-3-indolyl-phosphate and NBT color development substrate (Bio-Rad, Hercules, CA). An Immunospot Analyzer (Cellular Technology) was used to record and analyze images of wells from developed plates.

Flow cytometry and immunofluorescence microscopy

DCs or PBMCs that had been exposed to varying concentrations of microparticles, FITC-albumin, or poly(I:C) (10 ng/ml) were washed and stained with Abs for relevant surface markers (Beckman Coulter, Gainsville, FL), or with annexin-V (R&D Systems, Minneapolis, MN) using FITC, PE, or PE-Cy7 as fluorophores. Quantification of uptake of FITC-albumin by different cell populations was detemined using flow cytometry, and exclusion of unincorporated particles was done by setting gates on plots of relevant lineage markers vs right-angle light scatter.

For immunofluorescence microscopy, DCs were exposed to rhodamine-albumin particles for 1–16 h (5 μg/ml), fixed with 1% formaldehyde, and permeabilized with Triton X-100 (0.1%). DCs were then stained with Alexa Fluor 488 phalloidin and, in some experiments, 4′,6-diamidino-2-phenylindole, dihydrochloride (both from Molecular Probes, Eugene, OR), according to manufacturer's instructions. Fluorescence microscopy images were acquired using a Zeiss (Oberkochen, Germany) Axiovert microscope, and deconvolution analysis was performed with Openlab Deconvolution Software (Improvision, Lexington, MA).

Time-lapse video microscopy

DCs were harvested and allowed to adhere to 1.5-cm tissue culture plates (Corning-Costar, Acton, MA) overnight, and placed in a chamber connected to a source of 10% CO_2 balanced air. The chamber was placed on a 37°C heating stage. Particles were added to the medium overlying the DCs and allowed to settle for 10 min before the initiation of recording. Images were recorded using an Olympus IX70 microscope connected to a digital camera (Digital Video Camera Company, Austin, TX). Images of selected fields in differential interference contrast were captured with an interval of 30 s over a period of 1 h using QED software with a time-lapse module (QED Imaging, Pittsburgh, PA).

Results

Generation of pH-triggered microparticles containing M58 peptide

Particles containing 0.2% (w/w) FITC-albumin, 0.2% or 2% (w/w) M58 peptide (with and without AMC-M58 peptide), or 20% (w/w) ρ-lactalbumin were generated, as described in *Materials and Methods*, all containing 20% (w/w) E100. In addition, 20% (w/w) poly-HEME particles were produced containing 0.2% (w/w) M58 peptide (with and without AMC-M58 peptide), 0.2% (w/w) FITC-albumin, or 20% (w/w) ρ-lactalbumin. The manufacture process produced a fine powder that was yellow with FITC-albumin, white with M58 or AMC-M58, and bright pink with ρ-lactalbumin. The

powder yield was 20–40% of the total solute. Particles were generally spheroidal (Fig. 1*A*). The median volume-weighted diameters of all particles were in the range of 4–6 μm.

The kinetics of peptide release were studied in vitro (Fig. 1, *B* and *C*). At pH 7.4, release of M58 peptide occurred very slowly from both E100- and poly-HEME-based microparticles. A burst of peptide release occurred from E100 microparticles when the suspending medium was changed to sodium acetate, pH 5 (Fig. 1*B*). In E100 particles, this burst could still be triggered after 4 days in suspension at pH 7.4. Poly-HEME microparticles did not show an increase in peptide release upon immersion in acidic pH, neither shortly after immersion in PBS nor 4 days later (Fig. 1*C*). FITC-albumin containing E100 microparticles were similarly pH responsive to acidic environments (data not shown) (see also Ref. 19).

Uptake of microparticles by DCs and monocytes

To assess the efficiency of particle uptake by different cell populations, PBMCs were cultured overnight with microparticles containing FITC-albumin (Fig. 2), and the relative FITC-fluorescence in T cells, B cells, and monocytes was determined by flow cytometry. The majority of monocytes ($CD14^+$ large cells) were fluorescently labeled with FITC. In contrast, almost none of the T or B cells were FITC labeled.

Microparticles (0.2% (w/w) FITC-albumin, 20% (w/w) E100) were also efficiently engulfed by immature DCs (Fig. 3). Immature, monocyte-derived DCs were prepared using established methods, and their interaction with 20% (w/w) ρ-lactalbumin, 20% (w/w) E100 microparticles was studied by fluorescence microscopy (Fig. 3). DCs were cultured with microparticles for 1–2 h, labeled with a fluorescent phalloidin to delineate the actin cytoskeleton, and then washed thoroughly to remove nonadherent or extracellular particles. After incubation at 37°C, most DCs were associated with one or more microparticles (Fig. 3, *A–C*), and deconvolution analysis of acquired images confirmed that the particles were localized intracellularly, clustered in the perinuclear region of the cells (Fig. 3*G* and supplemental data).[4] DCs were also imaged at later time points, and engulfed particles were still visible in cells 48–72 h after loading (data not shown). However, if DCs were incubated at 4°C (Fig. 3, *D–F*), no particles were visible in association with the cells, suggesting that the uptake of particles was an energy-dependent process. Time-lapse video microscopy was used to visualize the dynamics of this interaction at 37°C. Representative images from a 1-h time course are shown in Fig. 4. Microparticles could be identified as highly refractile objects of subcellular size that were rapidly withdrawn toward the cell body and were engulfed over a period of 15–45 min. These data show that pH-triggered microparticles are preferentially, avidly, and rapidly phagocytosed by professional APCs.

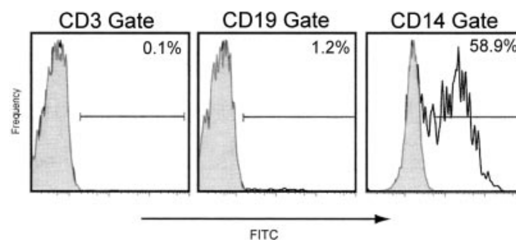


FIGURE 2. Selective uptake of microparticles by human APCs. Human PMBC were cultured in the presence (open histogram) or absence (gray histogram) of FITC-albumin-containing microparticles, and the percentage of cells labeled with FITC was determined using flow cytometry by gating on $CD3^+$ (*left panel*), $CD19^+$ (*middle panel*), or $CD14^+$ cells (*right panel*).

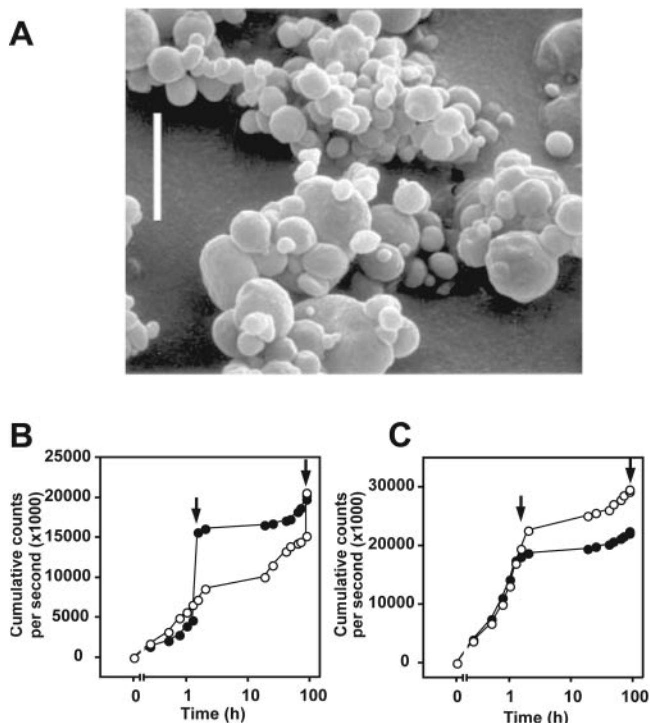


FIGURE 1. E100 microparticles. *A*, Scanning electron micrograph of 20% (w/w) microparticles containing 0.2% (w/w) M58 peptide. The bar represents 5 μm. *B* and *C*, Representative time courses of pH-triggered release of AMC-labeled M58 peptide from 20% (w/w) E100 (*B*) or poly-HEME (*C*) microparticles. Arrows indicate the time point at which the suspending medium was changed from pH 7.4 to pH 5, either 1.5 h (filled symbols) or 4 days (open symbols) after initial placement in suspension.

DC viability, phenotype, and function after particle loading

A theoretical concern about the uptake of microparticles by DCs is that it may cause cytotoxicity or disrupt DC function. We therefore assessed DC viability, maturation, and function following coculture with microparticles. Immature DCs were cocultured with a range of concentrations of 0.2% (w/w) FITC-albumin, 20% (w/w) E100 microparticles overnight (Fig. 5*A*), and the degree of cell death was measured by annexin-V binding. At concentrations of microparticles lower than 10 μg/ml, $<10\%$ of cells were apoptotic (annexin-V positive). At concentrations greater than 10 μg/ml, there was a modest increase in cell death to 20–30%. However, concentrations of microparticles that increased apoptosis in DCs were in excess of those necessary for efficient loading (see below). To assess the microparticles' effect on DC maturation, we measured the expression of CD80, CD86, CD40, and CD83 in DCs cultured with 5 μg/ml 0.2% (w/w) FITC-albumin, 20% (w/w) E100 microparticle (10 μg/ml). The dsRNA complex poly(I:C) was used as a positive control. After 48 h of culture, poly(I:C) induced marked up-regulation of CD80, CD86, and CD40 on the majority of cells, and a subset of cells showed increased expression of CD83 (Fig. 5*B*). In contrast, the expression levels of these surface markers were unchanged by culture with microparticles, suggesting that they did not influence the maturation state of the DCs (Fig. 5*B*). We further assessed the effect of microparticle uptake on APC function by measuring the ability of DCs to stimulate allogeneic T cells following incubation with microparticles (0.2% (w/w) FITC-albumin, 20% (w/w) E100) or with a comparable concentration of soluble FITC-albumin as a control. Fig. 5*C* shows

[4] The on-line version of this article contains supplemental material.

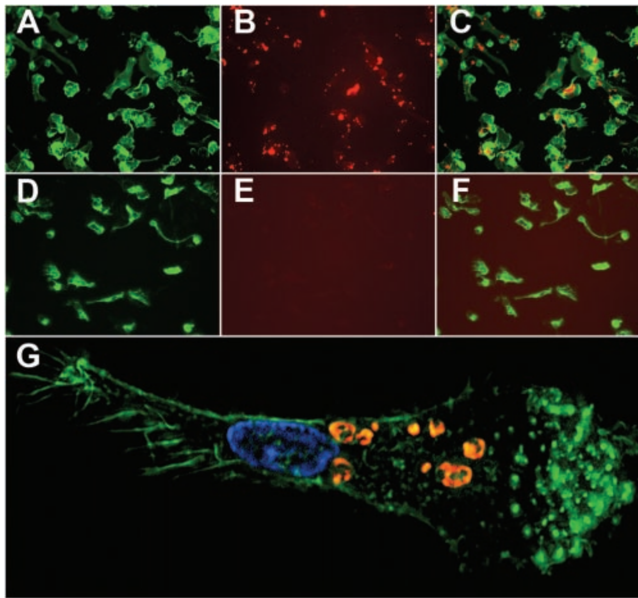


FIGURE 3. Fluorescence microscopy of DCs cultured with microparticles. Human DCs were incubated for 1 h at 37°C (*A–C*) or 4°C (*D–F*) with microparticles containing rhodamine-lactalbumin (red), washed extensively, and then stained to demarkate the actin cytoskeleton (green). Panels show DCs (*A* and *D*), particles (*B* and *E*), or overlaid images (*C* and *F*). *G*, Deconvolution fluorescence microscopy of single DC containing rhodamine-lactalbumin microparticles after incubation at 37°C. Actin cytoskeleton is stained green and the nucleus blue (see supplementary Fig. 1).[4]

that the degree of T cell proliferation elicited by DCs cocultured with 5 μg/ml microparticles was identical with that of control DCs. These data suggest that the uptake of microparticles is not toxic to DCs, and perturbs neither their maturation state nor their ability to stimulate T cells.

Uptake of soluble vs encapsulated FITC-albumin

The avid phagocytosis of microparticles by DCs suggested that they would be more effective at delivering a potential Ag to APCs. We therefore compared the ability of encapsulated protein to enter DCs with that of unencapsulated protein. Immature DCs were cultured in the presence of unencapsulated FITC-albumin or of equivalent concentrations of FITC-albumin as 0.2% (w/w) FITC-albumin, 20% (w/w) E100 microparticles overnight. Flow cytometry revealed that even at low particle concentrations (e.g., 5 μg/ml particle, which corresponds to 10 ng/ml encapsulated FITC-albumin), the majority of DCs were labeled with FITC, up to a maximum of ~80% (Fig. 6*A*). At all concentrations examined, uptake of FITC-albumin was much higher with encapsulated FITC-albumin than with the corresponding concentration of unencapsulated FITC-albumin measured both by percentage of labeled DCs and the fluorescence intensity (Fig. 6*B*). Thus, the phagocytosis of microparticles increased the delivery of encapsulated Ag to DCs.

Peptide Ag presentation by microparticle-loaded human DCs

Improved Ag delivery to DCs is a critical component of Ag presentation. However, to elicit $CD8^+$ T cell responses, phagocytosed Ag must efficiently enter the MHC I presentation pathway. We tested the effect of encapsulation on the ability of DCs to present a peptide epitope, the immunodominant epitope from influenza A matrix protein, to $CD8^+$ T cells. Because wide variations in particle concentrations might influence Ag presentation, we used a fixed concentration of particles and prepared two particle formulations that delivered the peptide concentrations equivalent to 10^{-2} μg/ml or 10^{-3} μg/ml. DCs pulsed with unencapsulated M58 peptide stimulated a peptide-specific HLA-A*0201-restricted T cell clone in a peptide concentration-dependent fashion (Fig. 7). However, at two concentrations (0.2% (w/w) M58, 20% (w/w) E100 microparticles), encapsulated Ag was much more efficient at stimulating a T cell response than the equivalent concentration of soluble peptide. For instance, encapsulated peptide equivalent to a concentration of 10^{-2} μg/ml achieved the same T cell response as that achieved by 1 μg/ml free peptide. This suggests that encapsulating a $CD8^+$ epitope in pH-triggered microparticles markedly increases the presentation of peptide epitopes on MHC I of DCs.

Role of pH triggering in vitro

The contribution of pH triggering to this improved Ag presentation was assessed by comparing peptide delivery to DCs by pH-triggered E100 particles and pH-insensitive microparticles prepared in the same matter, except that E100 was replaced by poly-HEME. Both types of particles were taken up by DCs with equivalent efficacy and were equally nontoxic (data not shown). DCs were cultured overnight in medium containing 5 μg/ml microparticles containing 0.2% (w/w) M58 peptide and either 20% (w/w) of E100 or 20% (w/w) of poly-HEME (Fig. 8). Poly-HEME microparticles elicited very little T cell stimulation. In contrast, pH-triggered microparticles elicited T cell stimulation that was markedly greater than that induced by nontriggering microparticles.

Vaccination using encapsulated peptide Ag

The in vitro results with a $CD8^+$ T cell clone suggested that encapsulation of the peptide in pH-triggered microparticles increased presentation of Ag by MHC I markedly. However, for application as part of a vaccine, encapsulated Ag should also be able to stimulate naive $CD8^+$ T cells. We tested naive $CD8^+$ T cell priming to the M58 epitope by vaccinating HLA-A*0201 transgenic HHD mice. HLA A*0201 transgenic mice such as HHD mice have been used extensively in the study of naive T cell responses to neo-Ags and have an immunodominant response to the M58 epitope from influenza A that is similar to HLA-A*0201-bearing humans (25–35). HHD mice (n = 5 each group) were vaccinated once with

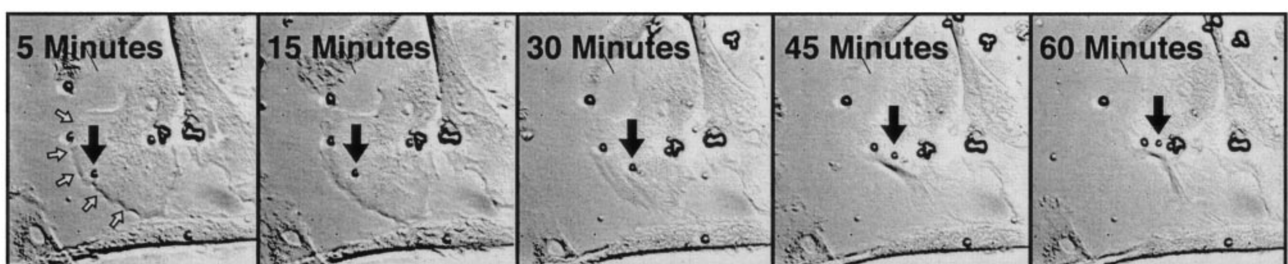


FIGURE 4. Phagocytosis of a microparticle (filled arrow) by an immature DC (leading edge, open arrows) visualized with time-lapse video microscopy. Representative images from the indicated times are shown.

M58 peptide in saline or encapsulated in microparticles. Results shown in Fig. 9 show that encapsulation of M58 allowed the priming of peptide-specific CTL with robust cytotoxic activity significantly greater than that induced by injection of peptide alone. For instance, at an E:T ratio of 30:1, lysis with T cells from particle-vaccinated HHD mice was 42 vs 16% for mice immunized with soluble peptide. Lysis of targets pulsed with an irrelevant peptide by T cells from either group of mice was on average 1.5% and always <8%.

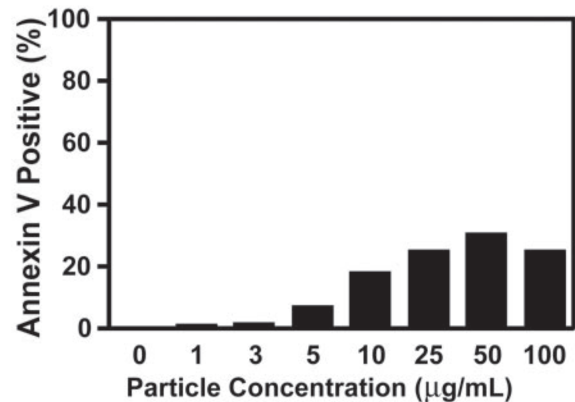
Discussion

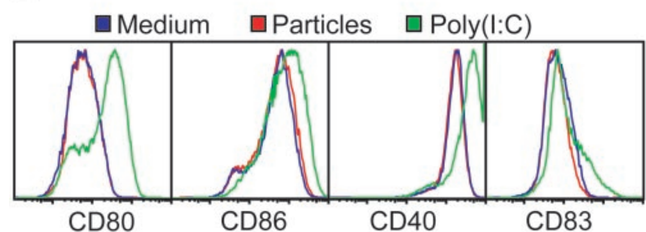
In this study, we show that encapsulation in pH-triggered microparticles markedly increases the delivery of a peptide Ag to the MHC I pathway of human DCs and improves T cell stimulation in vitro and in vivo.

Microparticles composed of 20% (w/w) E100 can encapsulate peptide and protein Ags, and provide both sustained and pH-triggered release of the peptide in vitro. The effect of greatly prolonging the release of peptide from DPPC-based particles at physiological pH (19) is important in that it may take days for all injected particles to be phagocytosed by the cell of interest (17). Because the cell surface-active properties of the polymethacrylates used in this study (E100 and poly-HEME) were not known, but both could potentially have effects on phagocytosis, it was important to document that the particles, whether biologically effective or not, actually entered the cells. pH-triggered microparticles were phagocytosed by DCs efficiently and rapidly. Deconvolution microscopy confirmed their intracellular localization, thus excluding the possibility that the more efficient delivery of encapsulated peptide or protein to the DCs was due to cell surface-adherent microparticles creating high local concentrations at the cell membrane. Our data support the view that these microparticles, having diameters of <10 μm, were taken up by phagocytosis (22). The exact molecular events surrounding microparticle phagocytosis, and whether they are identical for differing particle types, are not completely understood, although the identification and targeting of molecules involved in phagocytosis are an area of active research interest (36).

A concern in designing these pH-triggered particles was whether the polycationic polyamines would be cytotoxic at particle concentrations that were effective (37). In vitro, microparticles did not cause significant apoptosis in DCs after overnight incubation, even though microscopy showed DCs to have engulfed significant numbers of particles per cell. Toxicity was minimal at particle concentrations that effectively loaded peptide and protein into DCs. Moreover, the functional properties and phenotype of DCs loaded with microparticles were not altered.

The delivery of peptide Ag to human DCs by pH-triggered microparticles resulted in robust stimulation of Ag-specific T cells in vitro, which was significantly greater than that caused by nontriggering poly-HEME microparticles, or soluble peptide. DCs loaded with microparticles showed no increase in expression of costimulatory molecules. Thus, the increased T cell stimulation in vitro was not due simply to maturation of the DCs with global enhancement of its ability to activate T cells. Rather, the enhanced T cell stimulation may have been due to increased and possibly prolonged presentation of the Ag. Recent data have shown that the phagosome contains components of the endoplasmic reticulum that are essential for Ag presentation, such as TAP and MHC class I (12, 13, 21). This suggests that the phagosome itself plays a direct role in the cross-presentation of exogenous Ag by MHC class I. Targeting the release of MHC class I Ags directly to the phagosome by pH-triggered microparticles may account, in part, for the increased Ag presentation seen with the microparticles compared with soluble peptide that enters the cell by pinocytosis. This might constitute an advantage over nanoparticulate formulations, such as liposomes, which are small enough to be taken up by pino- or endocytosis (38–41). Although liposomes have been used previously to improve CTL priming in vitro (42, 43), microparticles described in this work are more likely to target phagocytic APCs as they did not enter nonphagocytic cells in detectable amounts.





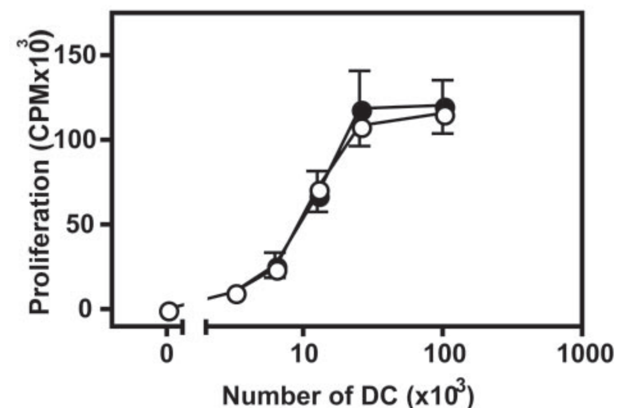


FIGURE 5. Effect of microparticles on DC viability, phenotype, and function. *A*, Apoptosis in DCs that had been cultured overnight with microparticles was assessed by annexin-V staining. Background apoptosis of DCs cultured in medium alone was subtracted. Data are representative of two separate experiments with DC from different donors. *B*, Cell surface expression of markers of activation/maturation on DCs after 48 h in culture with microparticles (red histogram), poly(I:C) (green histogram), or medium control (blue histogram). Results are representative of four experiments with different donors. *C*, Ability of DCs to stimulate allogeneic T cell following culture with FITC-albumin-containing microparticles (●) or with FITC-albumin alone (○) was assessed by [^{3}H]thymidine incoporation. Results show mean and SD of proliferation measured in triplicate for three different T cell donors (50,000 cells/well) cultured for 5 days with the indicated number of DCs per well.

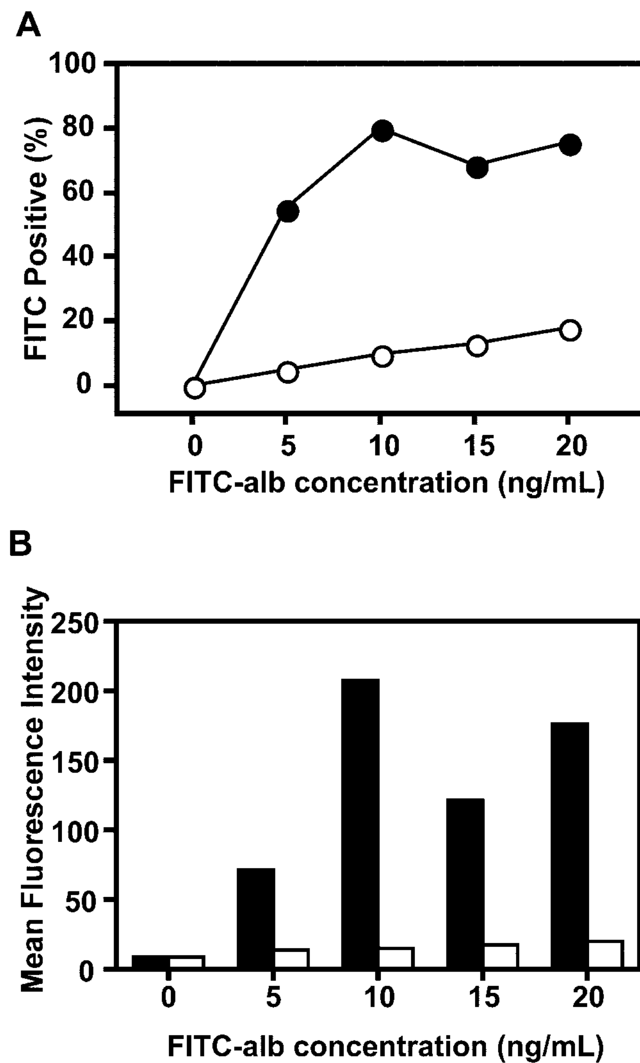


FIGURE 6. Uptake of soluble or microparticle-encapsulated FITC-albumin. DCs were cultured with FITC-albumin containing microparticles (filled symbols/bars) or soluble FITC-albumin (open symbols/bars), and the frequency (*A*) and intensity of fluorescence (*B*) were measured by flow cytometry. Free particles were excluded by gating based on size and CD45 staining. Data are representative of three separate experiments with DCs from different donors.

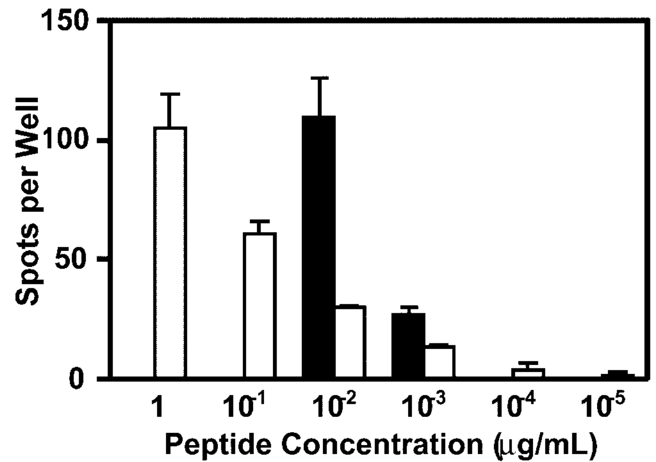


FIGURE 7. Effect of microparticle encapsulation on Ag presentation. HLA-A*0201$^+$ DCs were cultured with unencapsulated MP58 peptide (□) at the concentrations indicated, or with 5 μg/ml microparticles containing 0.2% or 0.02% (w/w) MP58 particles (■). The amount of particle added was calculated to yield concentrations of MP58 peptide equivalent to 10^{-2} or 10^{-3} μg/ml, respectively. DCs were plated at 50,000 cells/well with 5,000 cells of an MP58-specific T cell clone in an IFN-γ ELISPOT assay. Results show the mean and SD of triplicate measurements, and are representative of four different experiments with DCs from different donors.

Although the increased ability of E100 particles to stimulate T cells suggests that the pH-triggering capability is important for Ag presentation, we caution that pH sensitivity is not the only difference between E100 and poly-HEME. Both are polymethacrylates, but they are otherwise quite different molecules. The ideal control would have been a molecule very similar to E100, but not pH triggerable. However, E100 is a copolymer of three different methacrylate monomers, ~50% of which are affected by pH. Because removing pH triggerability would therefore involve altering a large fraction of the monomer units, there could not be a chemically identical (or very similar) molecule that did not pH trigger.

Because increasing the amount of Ag presented by DCs is thought to decrease the activation threshold for naive T cells (2–5, 44, 45), we wished to determine whether the microparticles resulted in improved priming of naive T cells. HHD mice are naive to the M58 epitope, but have an immunodominant T cell response to M58 after immunization with whole influenza virus (27). HHD mice offered the opportunity to evaluate T cell priming in a complex cellular environment that would be as close to the human setting as possible. In vivo, we found that vaccinating HHD mice with particles encapsulating a MHC I epitope resulted in CTL priming, and was much more effective than vaccination with soluble peptide. This finding might not have been predicted by our in vitro data, which showed that phagocytosis of particles by DCs was not associated with activation/maturation of DCs, and by the fact that the vaccine contained no helper epitopes that would have allowed Ag-specific CD4$^+$ cells to activate/mature Ag-loaded DCs. However, like many microparticulate formulations, injection of the pH-triggered microparticles induces transient, mild inflammation at the vaccine site (19). It is possible that local release of inflammatory cytokines and chemokines may have induced activation of APCs. In this setting, the combination of local inflammation and increased Ag presentation on APCs may allow naive T cells to be primed efficiently.

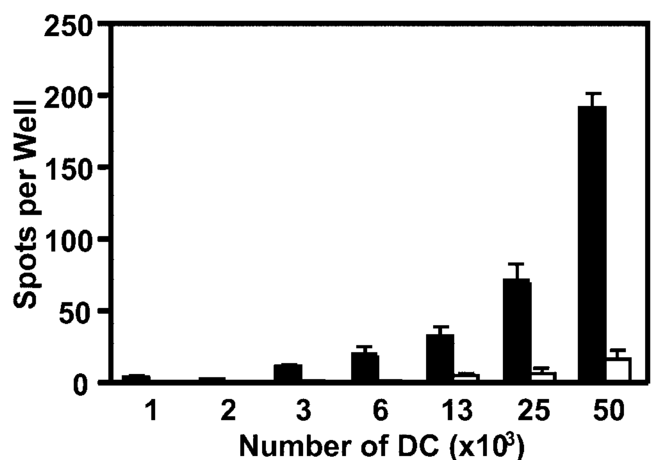


FIGURE 8. Effect of pH triggering on peptide presentation. HLA-A*0201$^+$ DCs were cultured with 5 μg/ml pH-triggerable E100 particles (■) or nontriggerable poly-HEME particles (□) containing 0.2% (w/w) MP58, and then harvested and plated at a range of cells/well with 5000 cells of an MP58-specific T cell clone in an IFN-γ ELISPOT assay.

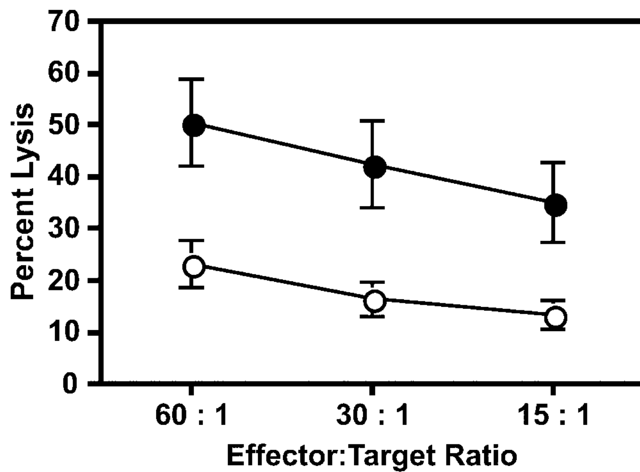


FIGURE 9. Priming of MP58-specific CTL in vivo by vaccination. HHD mice (n = 5 per group) were vaccinated with equivalent amounts of MP58 encapsulated in microparticles (filled symbols) or dissolved in PBS (open symbols), and on day 7 their spleen cells were harvested and restimulated in vitro with 10 μg/ml MP58 peptide. CTL activity was tested 6 days later against ^{51}Cr-labeled RMAS/HHD targets pulsed with MP58 at each of three E:T ratios. CTL activity against targets pulsed with irrelevant peptide was negligible. Results show the mean and SD of results from each group and are representative of three separate experiments.

Although the present formulation of pH-triggered microparticles allowed T cell priming in vivo, the current formulation could be improved for vaccine use if it caused DC maturation. Coencapsulation of immunomodulatory reagents with the Ag of choice should be possible (46). For instance, CpG oligonucleotides are potent activators of the innate immune system, and recent data suggest that their cognate receptor, TLR 9, interacts with CpG-bearing motifs in the endosomal compartment, presumably to permit DCs to scan for DNA from invading microorganisms that have been phagocytosed (12, 13, 21). Coencapsulation of CpG oligonucleotides along with Ag in pH-triggered microparticles would therefore allow the efficient delivery of an activating ligand to a compartment rich in its receptors and may significantly improve the ability of the microparticles to prime a long-lasting T cell response in vivo.

Another interesting related application is made possible by the ease with which particle density can be modified (17), so that formulations could be used for inhalation delivery (47), which might render them useful for induction of airway mucosal immunity. Microparticle production is relatively straightforward once an appropriate formulation has been developed, and is easily amenable to scale-up.

Improving the CD8$^+$ T cell response to vaccines requires the optimization of several factors, including epitope choice, Ag delivery, and DC maturation. pH-triggered microparticles capitalize on the physiology of exogenous Ag entry into the MHC I pathway and improve one critical component of the initiation of the T cell response: Ag presentation. The particles represent a flexible platform on which to base future vaccine designs to elicit CD8$^+$ immunity to cancer and infectious diseases.

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