Micro particles for protein vaccination

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Introduction

Vaccination is the administration of antigens in order to evoke an immune response against an infection or a disease through Antigen Presenting Cells (APCs) such as macrophages and dendritic cells (DCs). Antigens, being mainly protein derivates (peptides) are generated by two major processes: 1. Proteosomal proteolysis of newly synthesized endogenic proteins that are eventually being presented as peptide - major histocompatibility complex class I (pMHC-I) complexes (P. Dubsky et al. 2005) and 2. Internalization of exogenous peptides through phagolysosomes and their manifestation on the cell membrane as peptide - major histocompatibility complex class II (pMHC-II) complexes. Vaccination against infectious agents represents a success of immunology (e.g. Polio, hepatitis B), however, many diseases such as cancer still evade the immune system (A. Stift et al. 2003). Cancer posts many challenges for protein vaccination such as: CTLs activation requires pMHC-I complexes while exogenic administration leads to pMHC-II complexes, weak activation of CTLs (W.M. Ying et al. 2005, J. Banchereau et al. 2000) and short duration of pMHC-I complexes on the cell surface(B. Ludewig et al. 2001). These problems may be solved or reduced through a process of cross-priming, in which internalized exogenous proteins leave the phagosome in order to create pMHC-I complexes rather than pMHC-II complexes thus priming a CTL response. However, this process is extremely inefficient as proteins tend to stay in the phagosomems where they are processed**.** To promote cross-priming and allow possible presentation through pMHC-I, the protein needs to leave actively the phagosome. This can be achieved by using PLGA microspheres which carry the antigen and contain proteins that act as membrane poring agents such as Listeriolysin O (LLO) or Melittin. The aim of the presented research is to develop a polymeric system for the delivery of antigens into APC for cancer immunotherapy.

Materials and Methods

PLGA microspheres production method: Microspheres were prepared using the double-emulsion solvent extraction method as described elsewhere (O. Benny et al 2005). Empty microspheres were prepared in the same way without protein. Labeled microspheres were prepared using 5 uL 6-coumarin.

Morphologic Studies with High Resolution Scanning Electron Microscopy (HRSEM): High Resolution Scanning electron microscopy (Leo 2010) was used to evaluate the shape and surface morphology of PLGA microspheres. After the microspheres were lyophilized, the dried microspheres were mounted on a carbon stub and sputter coated with a thin layer (100-150 Å) of carbon. The surface morphology of the microspheres samples was then visualized under a high resolution scanning electron microscope.

Particle Size Distribution: The particles size distribution was analyzed using a Coulter LS 230 particle size analyzer. Samples were prepared by resuspending 5 to 10 mg microspheres in distilled water. The results were reported as a volume size distribution by a computerized analysis for ideal spheres.

Cells and Cell Culture: Mouse macrophage-like cell line RAW264.7 was maintained in Dulbecco's modified Eagle's Medium (Biological industries, Israel), supplemented with 10%fetal calf serum (FCS) (Gibco, USA), L-glutamine, fungizon and 1%(v/v) penicillin/streptomycin (Biological Industries, Israel). For the generation of monocyte-derived Dendritic cells, PBMC have been isolated from peripheral blood of healthy donors and incubated for 2-3 hours for the adherence of monocyte. After 2-3 hours, non-adherent cells were discarded and adherent cells were washed three times with PBS. Isolated monocytes were cultured with rhGM-CSF (Cytolab, Rehovot, Israel)) 100ng/mL and rhIL-4 100ng/mL for 6 days for the generation of immature dendritic cells (Medium was replaced every 3 days). Mature dendritic cells were generated by adding 200 ng/mL LPS for 2 more days.

Microspheres uptake by cells: Cells (RAW264.7 and iDC) were seeded in a 48-well flat bottom plates ($2*10^5$ cells per well) in a 0.5 mL of growth medium. RAW264.7 cells were allowed to attach overnight. Fluorescent-labeled microspheres in appropriate culture medium were suspended over a bath sonicator for 5 minutes and predetermined amount was added to the culture cells for different time periods. Cells were then washed with PBS and visualized using fluorescent microscope and confocal microscope, and analyzed using FACS. Same experiments were conducted with un-labeled microspheres as control.

Results and Discussion

Morphologic Studies with High Resolution Scanning Electron Microscopy: Targeting of PLGA microspheres into APCs requires that the particles' diameter will be in the range of 1-10um. PLGA microspheres were prepared using different homogenization time and speed. Analysis of one of these preparations is shown in figure 1A. As seen, the Particles diameter range is 1-10 micrometer, with a tail up to 60 micrometer that can represent aggregates. This formulation will be further sieved to achieve a narrower size distribution. HRSEM indicates round shaped, intact, with smooth morphology microspheres as can be seen in Figure 1B.

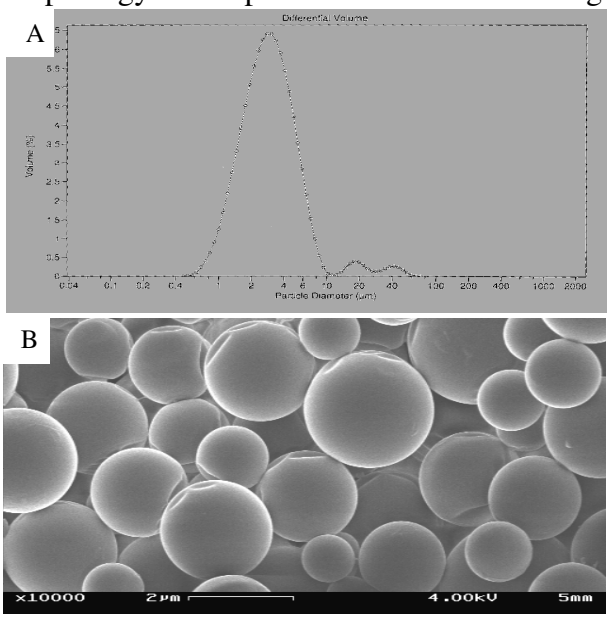

Figure 1:A) Particle size distribution analysis showing particle mean diameter in the range of 0.5-10 um. Preparation: homogenization speed 24000rpm, first homogenization time 1.5 min and 2min for the second Homogenization. B) HRSEM analysis shows intact, round shape with smooth morphology PLGA micro particles. Magnification x10000.

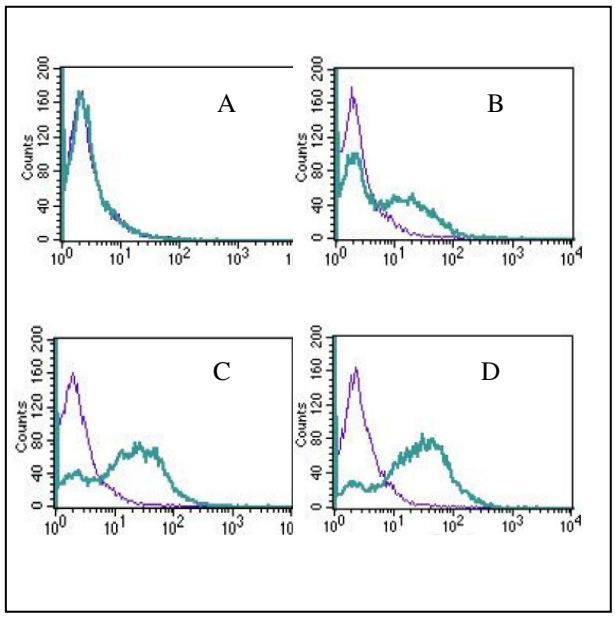

Figure 2: FACS analysis of RAW264.7 cells co-cultured with 6-coumarin labeled microspheres (cyan curve) and un-labeled microspheres (purple curve) for different periods of time. A) 0min B) 30min C) 120 min D) 240min

Microspheres uptake by cells: In order to test the feasibility of APC to actively uptake particles in the range of 1-10 um, 6-coumarin labeled microspheres were co-cultured with RAW264.7 cells and analyzed by FACS (Fig. 2). When the microspheres were added and washed right away (t=0min), no shift in fluorescence was seen compared to cells co-cultured with un-labeled microspheres. This means that the washing procedure did wash all the microspheres that haven't been taken up (A). After 30 min (B), a shift in fluorescence can be seen indicating that after 30 min of incubation, some of the microspheres have been taken up by the cells. This shift is intensified with incubation time (C and D). When analyzed by mean and geometrical mean of the fluorescence shift, the results show increase in fluorescence shift at longer incubation times (Data not shown).

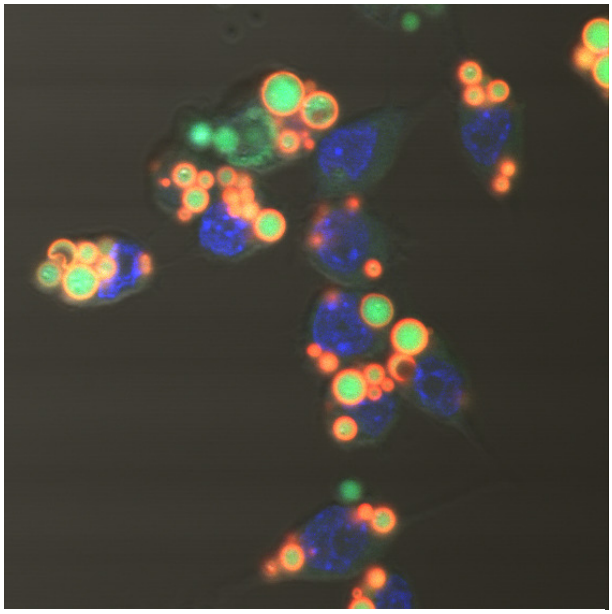

Figure 3: Confocal microscopy image of microspheres uptaken by RAW264.7 macrophages. Microspheres are labeled with 6-coumarin (Green). Cell's nucleus is stained with DAPI (blue). Microspheres are surrounded with red Lyzotracker which stains acid organelles (e.g. phagolysosome).

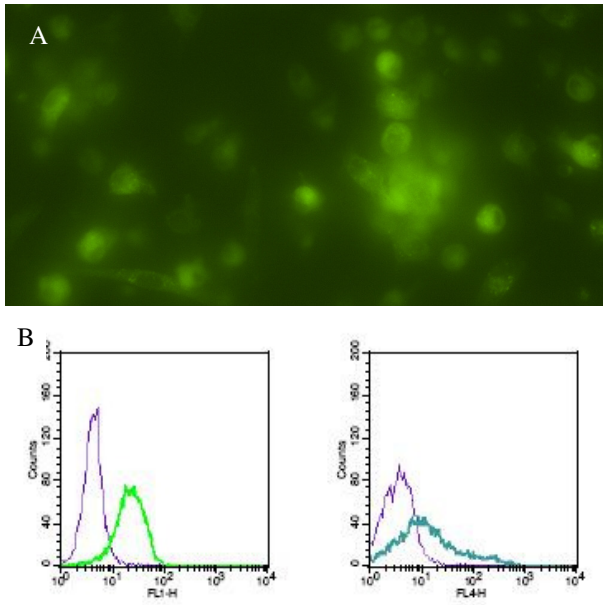

Figure 4:A) FDA staining of immature dendritic cells on day 6 indicates cells are viable B) FACS analysis of mature dendritic cells. Left panel – staining of mature dendritic cells with FITC-antiCD80 primary antibody. Rright panel - staining of mature dendritic cells with APC-antiCD83 primary antibody.

Figure 3 shows confocal microscopy of RAW264.7 cells co-cultured with 6-coumarin labeled microspheres for 4 hours and stained with DAPI and lyzotracker. As can be seen, microspheres are located inside the cells and surrounded by acidic organelle (stained with Lyzotracker), probably, phagolysosome. Furthermore, each cell can uptake more than one particle.

Since the main APC are dendritic cells, our nex aim was to generate monocyte-derived dendritic cells. Figure 4A show fluorescent microscopy image of immature dendritic cells stained with FDA. This data indicates that cells are viable in the culture medium. Figure 4B shows FACS analysis of mature dendritic cells stained with CD80 and CD83 as markers for mature dendritic cells. As can be seen, the mature dendritic cells express these markers.

Next, we investigated the phagocytosis capability of immature dendritic cells. FACS analysis of uptake of microspheres by different types of cells is shown in figure 5**.** As can be seen, iDC uptake microspheres co-cultured for 2 and 4 hours (C1, 2 – indicated by arrow) while other type of cells do not (A1,2 and B1,2). JY cells shows little uptake, probably due to endocytic activity (A1,2). Figure 6 displays fluorescent microscopy image of immature dendritic cells after incubation with 6-coumarin labeled microspheres for 2 hours. As can be seen, Cells are green, indicating the uptake of the labeled microspheres.

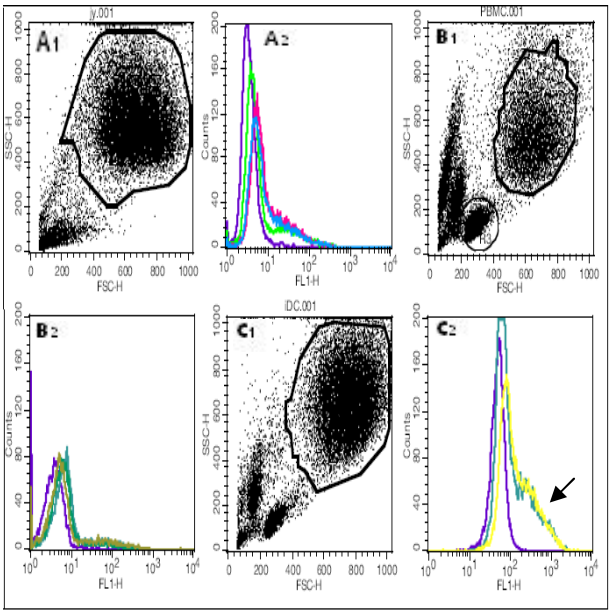

Figure 5 FACS analysis of different types of cells. Cells grown in 6 well plates are incubated with 6-coumarin labeled microspheres. After different periods of time, microspheres are washed and the cells are analyzed by FACS. A1,2 – FACS analysis of JY cells. Purple-omin, cyan-30min, pink-2hr,blue-4hr. B1,2- PBMC. Purple-o min, khaki-2hr, cyan-4hr. C1,2 – immature dendritic cells. purple-o min, yellow-2hr,cyan-4hr.

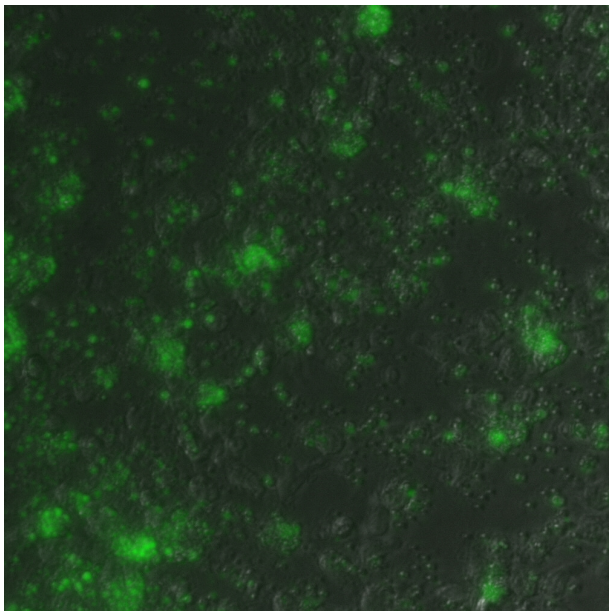

Figure 6: FDA staining of immature dendritic cell on day 6 indicates cells are viable. Original magnification: X20.

Conclusions

Our findings demonstrate that PLGA microspheres may serve as antigen delivery system to APC and specifically to dendritic cells. This system may solve the problems mentioned above and might serve for cancer vaccination procedures. Further examination of antigen presentation and CTL activation is to be tested *in-vitro* and *in-vivo* in the near future.

References

P. Dubsky et al. (2005) *Human Dendritic cell Subsets for Vaccination*. Journal of Clinical Immunology 25(6) 551-572.

W.M. Ying et al. (2005) *PLGA microspheres for improved antigen delivery to dendritic cells as cellular vaccines*. Advenced Drug Delivery Reviews 57(3) 475-482.

J. Banchereau et al. (2000) *Immunobiology of dendritic cells*. Annual Rev immunol 18, 767-811.

B. Ludewig et al. (2001) *Rapid peptide turnover and inefficient presentation of exogenous antigen critically limit the activation of self-reactive CTL by dendritic cells*. J of immuno 166(6) 3678-3687.

A. Stift et al. (2003) *Dendritic cell–based vaccination in solid cancer*. J Clin Oncol 21(1) 135-142.

O. Benny et al. (2005) *Continuous delivery of endogenous inhibitors from poly (lactic-co-glycolic acid) polymeric microspheres inhibits glioma tumor growth*. Clin Can Res 11 768-776.