Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2010, Article ID 894971, 6 pages
doi:10.1155/2010/894971

Research Article

Triggering DTH and CTL Activity by fd Filamentous Bacteriophages: Role of CD4+ T Cells in Memory Responses

Giovanna Del Pozzo,[1] Dina Mascolo,[1] Rossella Sartorius,[2] Alessandra Citro,[1] Pasquale Barba,[1] Luciana D'Apice,[2] and Piergiuseppe De Berardinis[2]

[1] *Institute of Genetics and Biophysics "Adriano Buzzati Traverso", CNR, Via P. Castellino 111, 80131 Naples, Italy*
[2] *Institute of Protein Biochemistry, CNR, Via P. Castellino 111, 80131 Naples, Italy*

Correspondence should be addressed to Piergiuseppe De Berardinis, p.deberardinis@ibp.cnr.it

Received 20 November 2009; Revised 4 February 2010; Accepted 13 February 2010

Academic Editor: Kim Klonowski

The ability of fd bacteriophage particles to trigger different arms of the immune system has been previously shown by us with particular emphasis on the ability of phages to raise CTL responses in vitro and in vivo. Here we show that fd virions in the absence of adjuvants are able to evoke a DTH reaction mediated by antigen specific CD8+ T cells. In addition, we analyzed the induction of CTL responses in mice depleted of CD4+ T cells, and we observed that short-term secondary CTL responses were induced in the absence of CD4+ T cells while induction of long-term memory CTLs required the presence of CD4+ T lymphocytes. These results examine the cellular mechanism at the basis of fd efficiency and provide new elements to further validate the use of fd particles for eliciting and monitoring antigen-specific CTLs.

1. Introduction

The system of antigen delivery by bacteriophage is based on the modification of the phage display technology. The filamentous bacteriophage fd is well understood at both the structural and genetic levels [1]. The capsid contains one major protein pVIII largely alpha helical which forms a shingled tubular array surrounding the single-stranded viral DNA with its N-terminal regions exposed on the viral surface. Given the large number of pVIII proteins (approximately 2700 copies per virion), the peptides displayed on pVIII, even if expressed in a lower copy number on the phage particles, remain densely represented. There is good evidence from immunoassays and NMR spectroscopy that the exposed peptides adopt a stable three-dimensional structure closely resembling that which they exhibit in the wild-type parent proteins [2]. The unexpected observation that fd filamentous bacteriophage particles are taken up and processed by the major histocompatibility (MHC) class I pathway [3] and are able to trigger cytotoxic T lymphocytes (CTLs) [4, 5], suggested that fd virions represent an efficient system for antigen delivery. Here we investigate the use of fd virions that display an epitope recognized by CD8+ T cells to mediate a delayed type hypersensitivity (DTH) reaction, demonstrating the ability of fd bacteriophages to elicit DTH in the absence of adjuvants.

In addition, we analyze the cell subsets involved in the induction of secondary memory CTL responses. Since it is known that coexpression of linked helper T cell and cytotoxic T cell epitopes on the surface of the same antigen-presenting cell (APC) is a requirement for priming a CTL response, initially we designed hybrid bacteriophages that simultaneously display helper and cytotoxic epitopes on the same virion. Using these double-display bacteriophage particles we showed that bacteriophage virions are able to elicit a sustained and antigen-specific CTL response both in vitro and in vivo [4, 5]. However, in further studies we also observed that single hybrid phages displaying a strong CTL epitope ($OVA_{257–264}$) were able to induce an efficient CTL response even in the absence of a T helper exogenous epitope [6]. We hypothesized that the carrier fd may also contain H-2 restricted CD4+ T helper epitopes and that these

latter may exert a sufficient helper function in C57BL/6 mice when strong CTL epitopes, such as $OVA_{257–264}$, are displayed on fd bacteriophages. To better address this issue here we analyze the CTL induction by single-display fd virions in mice depleted of CD4+ T cells.

2. Materials and Methods

2.1. Construction and Purification of Hybrid Bacteriophages. Single hybrid bacteriophages $fdOVA_{257–264}$ were generated by cloning the DNA fragment encoding the $OVA_{257–264}$ (SIINFEKL) MHC H-2^b-restricted cytotoxic peptide (5′-CCGCGGAGGGTTCCATCATCAACTTCGAAAAAC-TGGACGATCCCGCCAAGG-3′) into SacII-StyI-digested fdAMPLAY88 phage genome as previously described [6]. *E. coli* XL1-Blue MRF' Kan cells, transformed with recombinant bacteriophages, produced hybrid phages in the supernatant. The wild type (fdwt) or recombinant virions were harvested from the bacteria culture medium, purified by caesium chloride gradient, and analyzed by SDS-PAGE as described elsewhere [7]. The number of copies of pVIII displaying $OVA_{257–264}$ peptide was determined by N-terminal sequence analysis for each phage preparation, and the concentration of the antigenic peptide on the $fdOVA_{257–264}$ bacteriophages was calculated from the copy number of the modified pVIII in the hybrid virions. The single hybrid phage preparations ($fdOVA_{257–264}$), carrying the $OVA_{257–264}$ peptide, displayed 450–750 recombinant copies of major coat protein pVIII [6].

2.2. Delayed Type Hypersensitivity Test (DTH). A total of 35 mice were sensitized by one injection of 50 μg of the cytotoxic $OVA_{257–264}$ (SIINFEKL) peptide and 140 μg of the T helper $HBVc_{128–140}$ (TPPAYRPPNAPIL) peptide from hepatitis B virus core antigen, emulsified with incomplete Freund's adjuvant (Sigma, Milan, Italy). Eight-week-old female C57BL/6 mice were purchased from Harlan Nossan (Corezzana, Italy) and maintained at the IGB animal facility, according to the Institutional guidelines. Sentinel mice were screened for seropositivity to Sendai virus, Rodent Coronavirus, and Mycoplasma pulmonis by the Murine Immunocomb test (Charles River, Lecco, Italy) and were found to be negative. All experiments with mice were performed in accordance with European Union Laws and guidelines. All animal studies were approved by our institutional review board, and the animal procedures (i.e., immunization and sacrifice) were performed according to rules approved by the ethical committee.

Peptides were purchased from Primm (Milan, Italy). Purity was >95%, as analyzed by HPLC and mass spectrometry. Ten days after sensitization, DTH was performed by injecting the antigen into the right footpad and PBS into the left footpad. Between 10 and 48 hours later the area of swelling was measured using a caliper. DTH reactions were elicited in mice after injection of, respectively, 75 μg $fdOVA_{257–264}$ (displaying 2 μg of $OVA_{257–264}$ peptide), 75 μg of fdwt, 2 μg of $OVA_{257–264}$ synthetic peptide, 75 μg of fdwt plus 2 μg of $OVA_{257–264}$ synthetic peptide, or 100 μg of pure soluble ovalbumin (Sigma, Milan, Italy).

2.3. In Vivo CD4+ Depletion and Mice Immunization. The rat antimouse CD4 mAb GK1.5 (IgG2b) was purified from hybridoma GK1.5 (ATCC TIB-207, Manassas, VA, USA) culture supernatant by affinity chromatography on HiTrap Protein-G column (Amersham-Pharmacia, Freiburg, Germany). Treatment with the depleting anti-CD4 mAb GK1.5 was performed i.p. as previously described [8] on days −3, 1, 3, and every 3 days until the boost, with 200 μg of anti-CD4 mAb. After boost, for assaying long-term responses, the treatment with anti-CD4 mAb was repeated on a weekly basis. Noncompeting FITC-conjugated anti-CD4 mAb (clone RM4-4; BD Pharmingen, Milan, Italy) was used for in vivo CD4+ T cell depletion assessment. The efficiency of CD4+ T cell depletion was controlled by FACS analysis (FACSAria, Becton Dickinson) on spleen cells isolated from representative mice sacrificed at various time points.

The immunizations were performed on days 0 and 15 using 50 μg $fdOVA_{257–264}$ administered either subcutaneously without adjuvant, or intraperitoneally in the presence of poly I:C, according to the following protocol: group a: 50 μg $fdOVA_{257–264}$; group b: 50 μg $fdOVA_{257–264}$ plus anti-CD4 mAb; group c: 50 μg $fdOVA_{257–264}$ plus 10 μg of poly I:C adjuvant; group d: untreated mice. The mice were sacrificed 2 weeks after the boost or two months later.

2.4. Cytotoxicity Assay. Single splenocyte suspensions were prepared by mechanical disruption from either control or immunized mice (responder cells) and were tested for anti-$OVA_{257–264}$ peptide cytotoxic response by the JAM Test [9]. Briefly, responder cells (4×10^6 cells per well) were stimulated in 24-well plates with irradiated syngenic female spleen cells (stimulator cells, 2×10^6 per well), which were prepulsed with $OVA_{257–264}$ peptide at 10 μg/mL, in the presence of IL-2 at 20 U/mL. As a positive control, spleen cells from each mouse were stimulated in parallel cultures with irradiated BALB/c female spleen cells, bearing the H-2^d alloantigen (antiallo response). After 6-7 days cells were harvested and tested for cytotoxic activity against ^{3}H-TdR labelled targets in a 4-hour assay. The anti-$OVA_{257–264}$ peptide responder cells were tested against EL4 target cells (H-2^b, syngenic to C57BL/6 mice) which had been either prepulsed or not with $OVA_{257–264}$ peptide; the anti-allo responder cells were tested against either P815 (H-2^d) or EL4 target cells.

2.5. Elispot Assay. The responder cells were prepared as above from either control or immunized mice. Cells were incubated in anti-IFN-γ mAb precoated multiscreen plates with IL-2 at 20 U/mL and 5×10^5 irradiated syngenic spleen cells, prepulsed with either $OVA_{257–264}$ at 10 μg/mL or medium alone. Responder cells (5×10^5/well) from each mouse were incubated with either medium alone or ConA at 10 ng/mL. After 40 hours of incubation at 37°C, cells were washed and the plates were sequentially incubated with anti-IFN-γ biotinylated mAb, poly-HRP-streptavidin (Endogen, Woburn, MA, USA), and AEC substrate (Sigma, Milan, Italy). IFN-γ transfected TSA cells and the parental

untransfected line were used as controls in each ELISPOT plate, after gamma irradiation. The spots were counted using the ELISPOT reader (A.EL.VIS, Hannover, Germany).

2.6. Statistical Analysis. All statistical analyses were performed using the unpaired Student's *t*-test. Differences were considered statistically significant when $P < .01$.

3. Results

3.1. Use of Single Hybrid Phage fdOVA$_{257–264}$ to Evoke a Delayed Type Hypersensitivity Reaction (DTH). In order to verify if the fd bacteriophages could be used as a vehicle to monitor the presence of antigen-specific CD8+ T cells, we set up an experimental model to reproduce a prior exposure to the antigen. We sensitized C57BL/6 mice injecting them at the base of the tail, with a mixture of OVA$_{257–264}$ cytotoxic peptide, HBVc helper peptide, and incomplete Freund's adjuvant. In this model the development of DTH normally takes 10 days from the time of immunization. DTH reaction was generated injecting 10 mice with recombinant fdOVA$_{257–264}$, 10 mice with wild type fd filamentous bacteriophages (fdwt), five mice with OVA$_{257–264}$ peptide, and five mice with soluble ovalbumin into the right footpad. The left footpad was instead treated with the same volume of PBS. Figure 1(a) shows the measurements of footpad swelling (mean ± SD) at different time points in response to the DTH skin test (value of PBS background was subtracted for each point).

A significant swelling between 24 and 48 hours was induced in the footpad of mice challenged with fd OVA$_{257–264}$ virions. In contrast a DTH reaction was not observed in mice challenged with wild type phage, synthetic OVA$_{257–264}$ peptide or soluble ovalbumin. These results indicate that DTH reaction is triggered by fd virions displaying the OVA$_{257–264}$ epitope and that DTH is thus induced by OVA$_{257–264}$-specific CD8+ T cells.

Figure 1(b) summarizes the mean ± increase of thickness for each mice group at 30 hours. The mice injected with fdOVA$_{257–264}$ show a mean of 0.65 mm of footpad swelling compared with 0.15 mm of footpad swelling of mice injected with fdwt. The groups challenged with the synthetic peptide or with soluble ovalbumin do not show significant swelling, meaning that these are not effective in eliciting the DTH reaction, while, as mentioned above, DTH was induced by administration of fdOVA$_{257–264}$ in the absence of adjuvant. Statistical analysis (Student's *t*-test) revealed that differences between DTH induced by fdOVA$_{257–264}$ with respect to DTH induced with other challenges were significant ($P < .01$, Figure 1(b)). The use of synthetic OVA$_{257–264}$ peptide in the presence of fdwt did not elicit DTH (data not shown). This indicates that DTH was conferred by the antigenic determinant displayed on the phage surface and that induction of DTH by fdOVA$_{257–264}$ virions could not be ascribed to the presence of LPS contaminants in the phage preparations, since the same amount of LPS was present in fdOVA$_{257–264}$ and fdwt samples.

3.2. Use of Single Hybrid Phage FdOVA$_{257–264}$ to Induce CTL Response in Mice Depleted of CD4+ T Cells. Our goal was to determine whether responses to single hybrid bacteriophages carrying a CTL epitope (fdOVA$_{257–264}$) could be generated and maintained in the absence of CD4+ T cells. We administered fdOVA$_{257–264}$ without adjuvant to mice depleted of CD4+ T lymphocytes by anti-CD4 antibody treatment [8] and then measured the secondary cytotoxic response. Specifically, we immunized groups of C57BL/6 mice according to the following protocol: (a) 50 μg fdOVA$_{257–264}$; (b) 50 μg fdOVA$_{257–264}$ plus anti-CD4; (c) 50 μg fdOVA$_{257–264}$ plus poly I:C. Untreated mice were used as control.

Figure 2 summarizes the results obtained with all groups of mice. Figure 2(a) shows the percentage of specific lysis after subtraction of background lysis of unpulsed target, at the responder/target ratio 270 : 1, while Figure 2(b) shows the ELISPOT assay to measure the frequency of IFN-γ producing splenocytes in response to in vitro stimulation with OVA$_{257–264}$ synthetic peptide. The results indicate that the filamentous bacteriophages administered without adjuvant are able to induce both relevant antigen-specific CTLs (mean 29.6% of lysis) and INFγ production (mean 124 Spot Forming Cells (SFC)/million cells) although these responses were lower than those obtained with fdOVA$_{257–264}$ administered in the presence of poly I:C adjuvant (37% lysis and 189 SFC/million cells). When we depleted the CD4+ T lymphocytes, the lytic activity and the IFN-γ production of antigen-specific CD8+ T cells were lower (17% of lytic activity and 94 SFC INFγ/million cells) than those obtained in the presence of CD4+ T cells, but these differences were not statistically significant ($P > .05$). The presence of CTLs in CD4+ T cell depleted mice indicates a secondary expansion, since a single administration of fdOVA$_{257–264}$ particles was not sufficient to induce CTL activity (data not shown).

A group of mice was also immunized with 50 μg of fdOVA$_{257–264}$ without adjuvant and treated with anti-CD4 mAb for two months after boost in order to establish if CD4+ T lymphocytes were necessary for inducing long-term memory cell response. As illustrated in Figures 2(c) and 2(d), we observed no antigen-specific immune response by either cytotoxic test or ELISPOT assay in these mice, in contrast to long-term responses of positive controls. Statistical analysis confirmed significant differences between fdOVA$_{257–264}$ and fdOVA$_{257–264}$ + anti-CD4 mice groups either for the lytic activity ($P = .0004$) or the IFNγ production ($P = .0002$). The efficiency of CD4+ T cells depletion was controlled by FACS analysis of spleen cells isolated from mice treated with anti-CD4 antibodies at different time points: before treatment; day of first immunization; day of boost; two weeks after boost (short-term memory); two months later (long-term memory). The data illustrated in Figure 2(e) show >99% CD4+ T cell depletion in mice treated with anti-CD4 mAb, in agreement with data previously reported in [8]. The percentage of CD8+ spleen cells was similar in mice CD4-depleted and not depleted (data not shown). In conclusion, short-term secondary response following fdOVA$_{257–264}$ immunization is independent from T cell help but long-term secondary response is T helper dependent and

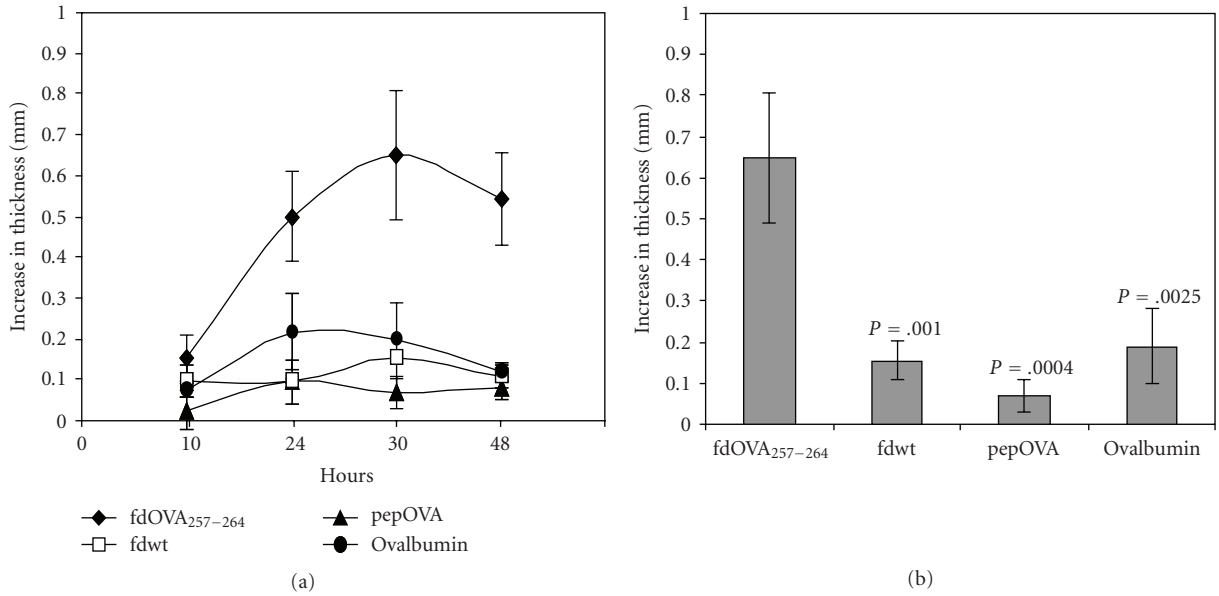

Figure 1: fdOVA$_{257–264}$ administration is able to trigger DTH. Mice were sensitized by one injection of 50 μg of cytotoxic OVA$_{257–264}$ (SIINFEKL) peptide and 140 μg T helper HBVc$_{128–140}$ (TPPAYRPPNAPIL) peptide from hepatitis B virus core antigen, emulsified with incomplete Freund's adjuvant. Ten days after sensitization, DTH was performed by injecting into the right footpad fdOVA$_{257–264}$, fdwt, the synthetic OVA$_{257–264}$ peptide (pepOVA), or soluble ovalbumin. 10–48 hours later the area of swelling was measured using a caliper. (a) Curve response of all mice analyzed. (b) Values at 30 hours of mean thickness ± SD of the different mice groups challenged with the antigens indicated in the abscissa. The *P*-values were calculated by comparing each group with the fdOVA$_{257–264}$ challenged group.

was not observed following fdOVA$_{257–264}$ immunization in mice depleted of CD4+ T lymphocytes.

4. Discussion

Cellular immunity, particularly cytotoxic T lymphocyte activity, plays a role in the clearance of viral infections and in the control of tumor development. Therefore induction of CTL activity is a major goal of vaccine development and its achievement may be determined by the ability of a carrier to deliver antigens into the MHC class I pathway. Here we report a study on the use of fd filamentous bacteriophage as an efficient carrier for delivering CTL epitopes. The bacteriophage fd is a nonpathogenic virus that is not able to infect and replicate in eukaryotic cells. All available evidence indicates that bacteriophage administration is completely harmless to humans [10–12]. In the past few years we have shown that fd bacteriophage particles represent a good antigen delivery system both for T helper and CTL epitopes since these particles intersect both class I and class II pathways [3–5].

In this paper we show that fd particles can also be used to induce DTH reaction, and this may allow the in vivo monitoring of immune responses mediated by antigen-specific CD8+ T cells. In a previously reported study [13], fd virions were used to sensitize mice and then to induce DTH reaction. Here we used fd virions only to challenge the DTH in mice sensitized by the administration of a combination of synthetic helper and CTL peptides. Thus we proved that fd bacteriophages are able to stimulate in the tissue the OVA$_{257–264}$ specific CD8+ T lymphocytes which were primed by a different delivery of the OVA$_{257–264}$ antigenic determinant. DTH reactions in the skin have been observed in many immunotherapy protocols and are often used as an indicator of antitumor immunity and virus vaccine efficacy [14, 15]. In addition, since DTH is relatively straightforward to perform, it may be employed as a preliminary screen for diagnostic virus infection and may serve as an in vivo measure of the lymphocytes trafficking to sites of infection [16].

Here we also analyze the role of CD4+ T cells in the induction by fd virions of CTL memory responses. We previously described that the display of the CTL epitope OVA$_{257–264}$ (SIINFEKL) on the surface of fd virions is sufficient to sustain an MHC class I restricted cytotoxic T lymphocyte response in vivo [6]. Having assessed the in vivo cross-presentation of the OVA$_{257–264}$ epitope displayed on bacteriophages, our aim was to investigate the cellular mechanism that this carrier employs to stimulate CTL induction. The importance of specific CD4+ T cell help for CD8+ T cell mediated immunity is known; however interactions between cross presenting DC, CD4+ T cells and CD8+ T cells in the establishment of immunological memory are still not well defined. A previous study confirmed that T helper cells are required for secondary induction of CTLs in vitro and in vivo, since CD4+ T cell depletion caused the complete disappearance of antigen-specific CTLs after secondary stimulation [17].

In this context our finding is of interest. In fact, we determined that fd particles carrying the OVA$_{257–264}$ peptide

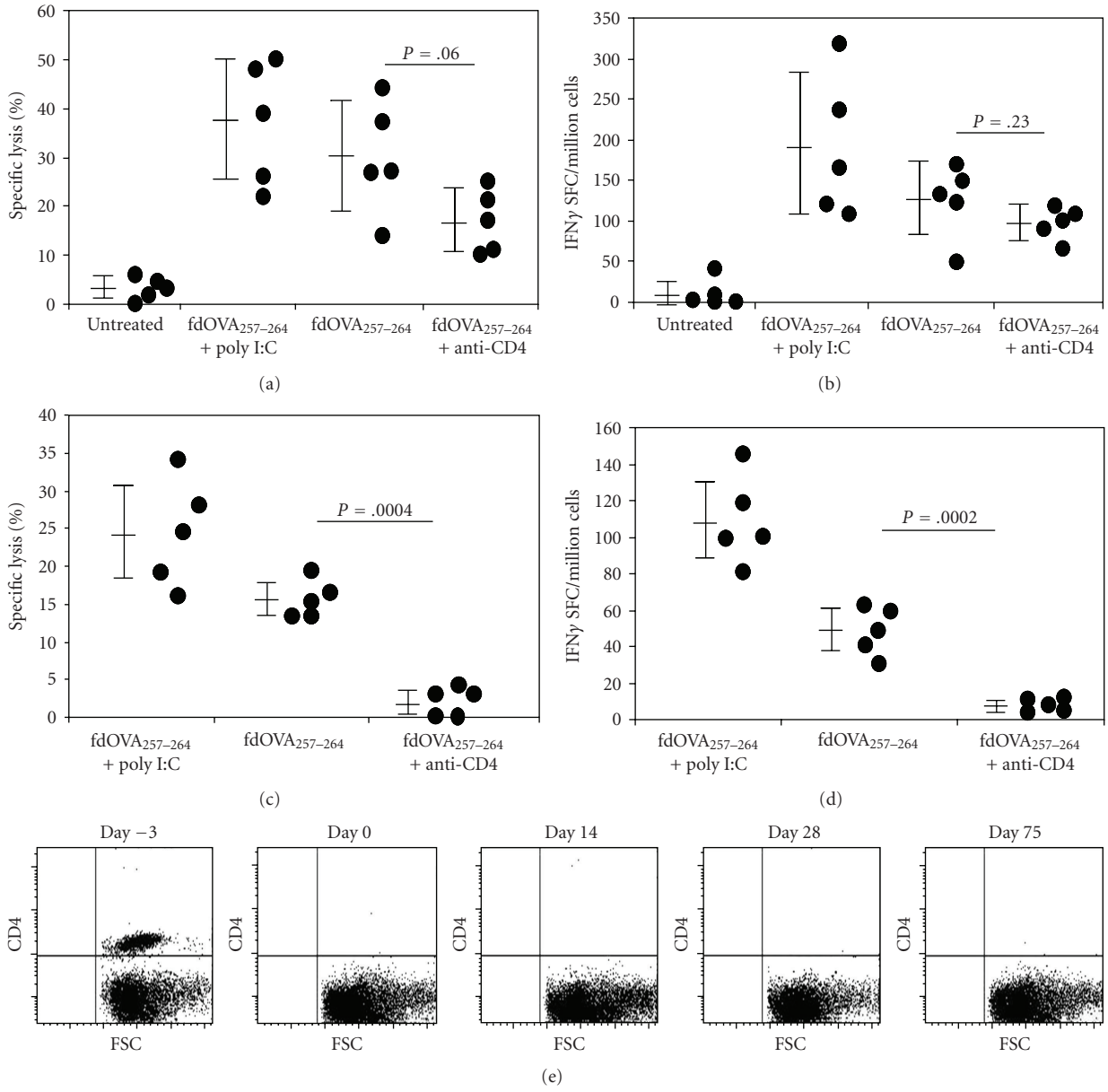

Figure 2: CTL and IFN-γ responses in mice immunized with fd bacteriophage particles in the presence or in the absence of CD4+ T cell help. Mice were immunized by two injections with either $fdOVA_{257–264}$ plus poly I:C or $fdOVA_{257–264}$ particles as indicated in Materials and Methods. Immunizations with $fdOVA_{257–264}$ particles were also performed in mice depleted of CD4+ T cells by treatment with GK1.5 mAb. CTL and IFN-γ productions were assayed after 15 days (a, b) or after two months (c, d) from the last injection. Each symbol represents data obtained from a single mouse. The mean value ± SD for each group is reported. (e) CD4+ T cell depletion was analyzed by cytofluorometry using noncompeting anti-CD4 antibody. The analysis was performed on spleen cells isolated from mice sacrificed at the time points indicated.

are still able to induce secondary expansion of $OVA_{257–264}$-specific CTLs even in the absence of CD4+ T cells. It has been suggested that an alternatively CD4+ Th-independent pathway of CTLs activation may operate via recognition of molecular pattern recognition receptors (PRRs) by DC [18] that in turn initiate signaling cascades activating the DC to provide T cell costimulation and cytokine release. In particular, it has been reported that small amount of IL-2 can be produced also by DC [19], and the addition of exogenous IL-2 to mice depleted of CD4+ T cells restores the secondary expansion of CTL [17]. We plan to investigate in future work the ability of phages to function as PRR agonist, to induce cytokine production, maturation of DCs, and to upregulate their costimolatory molecules.

This study reports that while the presence of CD4+ T cells is not essential to induce a secondary memory response, their presence is instead required to elicit a long-term memory response by fd antigen delivery. This finding should be

taken into consideration in designing fd particles for vaccine strategies.

5. Conclusions

Overall our analysis of the cellular mechanisms employed by fd virions contributes toward defining the potentiality of this safe and inexpensive antigen delivery system, which has been proposed as a subunit vaccine able to improve the antigenicity and the immunogenicity of the antigenic determinants displayed. We show that the fd particles displaying a CTL epitope, and not the synthetic peptide, can be used without adjuvants to activate the antigen-specific CD8+ T cells in the tissue, and to evoke a DTH reaction. This result suggests that fd bacteriophages can be used as a diagnostic tool to in vivo monitor both infections and the presence of antigen-specific CD8+ T cells. Moreover, since the fd system can be applied to vaccine formulation, we have defined the role of CD4+ T cells in the induction of long-term memory responses. We found that the presence of CD4+ T cells is required to sustain long-term induction of CTLs. In this context, in the fd system coactivation of CD4+ T cells can easily be achieved for all types of MHC-restricted antigens by constructing double hybrid bacteriophages displaying helper and CTL epitopes on the same virion.

Acknowledgments

The authors thank Luca D'Orsi of the IGB mouse facility for husbandry of mice and technical assistance and Dr. Laura Pisapia of the IGB FACS facility. This work was supported by a Grant from the Italian Ministry of Research (FIRB Project no. RBLA033WJX).

References

[1] J. Greenwood, A. E. Willis, and R. N. Perham, "Multiple display of foreign peptides on a filamentous bacteriophage: peptides from *Plasmodium falciparum* circumsporozoite protein as antigens," *Journal of Molecular Biology*, vol. 220, no. 4, pp. 821–827, 1991.

[2] R. Jelinek, T. D. Terry, J. J. Gesell, P. Malik, R. N. Perham, and J. J. Opella, "NMR structure of the principal neutralizing determinant of HIV-1 displayed in filamentous bacteriophage coat protein," *Journal of Molecular Biology*, vol. 266, no. 4, pp. 649–655, 1997.

[3] M. Gaubin, C. Fanutti, A. Durrbach, et al., "Processing by APC of filamentous phage displaying antigenic epitopes targets to both HLA class I and class II peptide loading compartments," *DNA Cell Biology*, vol. 22, no. 1, pp. 11–18, 2003.

[4] P. De Berardinis, R. Sartorius, C. Fanutti, R. N. Perham, G. Del Pozzo, and J. Guardiola, "Phage display of peptide epitopes from HIV-1 elicits strong cytolytic responses in vitro and in vivo," *Nature Biotechnology*, vol. 18, no. 8, pp. 873–876, 2000.

[5] R. Sartorius, P. Pisu, L. D'Apice, et al., "The use of filamentous bacteriophage fd to deliver MAGE-A10 or MAGE-A3 HLA-A2 restricted peptides and to induce strong anti-tumor CTL responses," *Journal of Immunology*, vol. 108, no. 6, pp. 3719–3728, 2008.

[6] D. Mascolo, P. Barba, P. De Berardinis, F. Di Rosa, and G. Del Pozzo, "Phage display of a CTL epitope elicits a long-term in vivo cytotoxic response," *FEMS Immunology and Medical Microbiology*, vol. 50, no. 1, pp. 59–66, 2007.

[7] P. Malik and R. N. Perham, "New vectors for peptide display on the surface of filamentous bacteriophage," *Gene*, vol. 171, no. 1, pp. 49–51, 1996.

[8] J. C. Rice and R. P. Bucy, "Differences in the degree of depletion, rate of recovery and the preferential elimination of naïve $CD4^+$ T cells by anti-CD4 monoclonal antibody (GK1.5) in young and aged mice," *The Journal of Immunology*, vol. 154, no. 12, pp. 6644–6654, 1995.

[9] P. Matzinger, "The JAM test. A simple assay for DNA fragmentation and cell death," *Journal of Immunological Methods*, vol. 145, no. 1-2, pp. 185–192, 1991.

[10] A. Sulakvelidze, Z. Alavidze, and J. G. Morris, "Bacteriophage therapy," *Antimicrobial Agents and Chemotherapy*, vol. 45, no. 3, pp. 649–659, 2001.

[11] J. R. Clark and J. B. March, "Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials," *Trends in Biotechnology*, vol. 24, no. 5, pp. 212–218, 2006.

[12] M. Trepel, W. Arap, and R. Pasqualini, "In vivo phage display and vascular heterogeneity: implications for targeted medicine," *Current Opinion in Chemical Biology*, vol. 6, no. 3, pp. 399–404, 2002.

[13] J. Fang, G. Wang, Q. Yang, J. Song, Y. Wang, and L. Wang, "The potential of phage display virions expressing malignant tumor specific antigen MAGE-A1 epitope in murine model," *Vaccine*, vol. 23, no. 40, pp. 4860–4866, 2005.

[14] G. A. Waanders, D. Rimoldi, D. Lienard, et al., "Melanoma-reactive human cytotoxic T lymphocytes derived from skin biopsies of delayed-type hypersensitivity reactions induced by injection of an autologous melanoma cell line," *Clinical Cancer Research*, vol. 3, no. 5, pp. 685–696, 1997.

[15] M. T. Clay, A. C. Obeika, P. J. Mosca, H. K. Lyerly, and M. A. Morse, "Assays for monitoring cellular immune responses to active immunotherapy of cancer," *Clinical Cancer Research*, vol. 7, no. 5, pp. 1127–1135, 2001.

[16] F. M. Gordin, P. M. Hartigan, N. G. Klimas, S. B. Zolla-Pazner, M. S. Simberkoff, and J. D. Hamilton, "Delayed-type hypersensitivity skin tests are an independent predictor of human immunodeficiency virus disease progression. Department of Veterans Affairs Cooperative Study Group," *Journal of Infectious Diseases*, vol. 169, no. 4, pp. 893–897, 1994.

[17] E. M. Janssen, E. E. Lemmens, T. Wolfe, U. Christen, M. G. Von Herrath, and S. P. Schoenberger, "$CD4^+$ T cells are required for secondary expansion and memory in $CD8^+$ T lymphocytes," *Nature*, vol. 421, no. 6925, pp. 852–856, 2003.

[18] S. Akira and K. Takeda, "Toll-like receptor signalling," *Nature Reviews Immunology*, vol. 4, no. 7, pp. 499–511, 2004.

[19] F. Granucci, I. Zanoni, S. Feau, and P. Ricciardi-Castagnoli, "Dendritic cell regulation of immune responses: a new role for interleukin 2 at the intersection of innate and adaptive immunity," *EMBO Journal*, vol. 22, no. 11, pp. 2546–2551, 2003.