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13. ABSTRACT (Maximum 200 Words)

We hypothesize that one can use specific protein or peptide sequences to direct bacteriophage vectors to dendritic cells. We further propose that one can then use such retargeted phage vectors to deliver potentially important antigens to dendritic cells (DC), and that this may allow one to derive vectors capable of eliciting potent immune responses to breast cancer antigens such as her2. These hypotheses are being experimentally tested. During the period covered by this progress report, we have shown that a novel CD40-binding peptide (identified by phage display technology) can be used to selectively enhance the transduction of primary DC by mammalian virus vectors (adenovirus, adeno-associated virus). We have also shown that one can insert a mammalian expression cassette encoding GFP into the genome of T7 phage, and that the resulting genomes encode detectable GFP protein in human cells. Finally, we have constructed modified lambda phage coat proteins which are expected to make it possible to selectively direct lambda phage particles to DC.

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FOREWORD

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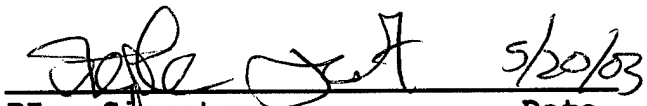

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INTRODUCTION

The overall purpose of this application is develop a novel method for breast cancer vaccine development. The hypothesis which we propose is as follows: that dendritic cell (DC)-targeted bacteriophage vectors expressing a tumor antigen can be used to elicit specific and potent anti-tumor CD8+ T lymphocyte responses. The experiments being performed under the auspices of this grant award are intended to establish proof-of-concept for the approaches set forth in this application, which are expected to have strong potential for clinical translation and for application to other systems (e.g., retargeting of other virus vectors to DC). If successful, these studies could have important implications since phage vectors are simple and inexpensive to produce, highly stable and not hampered by problems of pre-existing immunity (unlike many mammalian viral vector systems).

BODY

Approved Tasks

The following tasks were outlined in the approved statement of work for this grant:

- *Task 1.* Development of phage vectors that target dendritic cells (months 1 - 15)
- *Task 2.* Generation of DC-targeted phage vectors that express an epitope from human HER-2/neu (DC-HER2 phage) (months 13-29)
- *Task 3.* Analysis of the immunogenicity of DC-HER2 phage (months 30-36)

Research Accomplishments associated with the above tasks

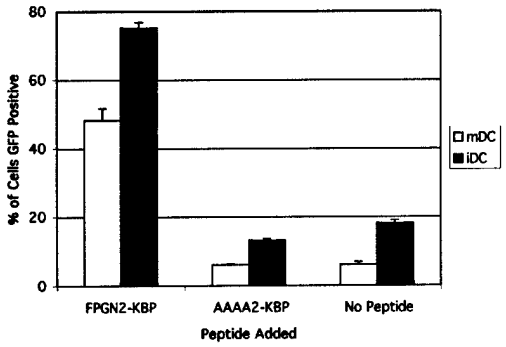
Task 1: Development of phage vectors that target dendritic cells

Retargeting of adenovirus to dendritic cells. Our primary aim in the present set of studies is to derive novel methods for the targeting of phage vectors to mammalian cells. However, it may also be possible to use phage-selected peptides to target mammalian virus vectors to dendritic cells. This could further enhance the utility of the approaches we are taking.

Retargeting of adenovirus vectors using phage-selected peptides. Two peptide phage display libraries were screened using recombinant mouse CD40 – our T7-phage peptide display library (see above) and a commercially available M13-phage library (PhD-12, New England Biolabs). Following biopanning and sequence analysis of randomly selected phage clones amplified after the third round of biopanning, a consensus CD40-binding peptide sequence was identified (sequence = FPGx[N/S]). The ability of one of these FPG-containing peptides to enhance vector-mediated infection of dendritic cells was then analyzed. To do this, a GFP-expressing adenovirus vector (Ad:GFP) was tested for its ability to mediate gene transfer in primary murine dendritic cells (DC), in the presence or absence of a bifunctional peptide that was designed to bind both to the adenovirus fiber protein and also to the CD40 receptor.

DC express only very low levels of the CAR receptor, making them recalcitrant to adenovirus-mediated gene transfer. After addition of the peptides to the Ad:GFP vector, and subsequent incubation with target murine dendritic cells, levels of GFP expression (Fig. 1) were strongly enhanced. Similar findings were also obtained when human DC were used, and when B cells (also CD40+) were examined *(see published paper by Richards et al., Appendix).*

Figure 1: CD40-binding peptide enhances adenovirus infection of murine dendritic cells

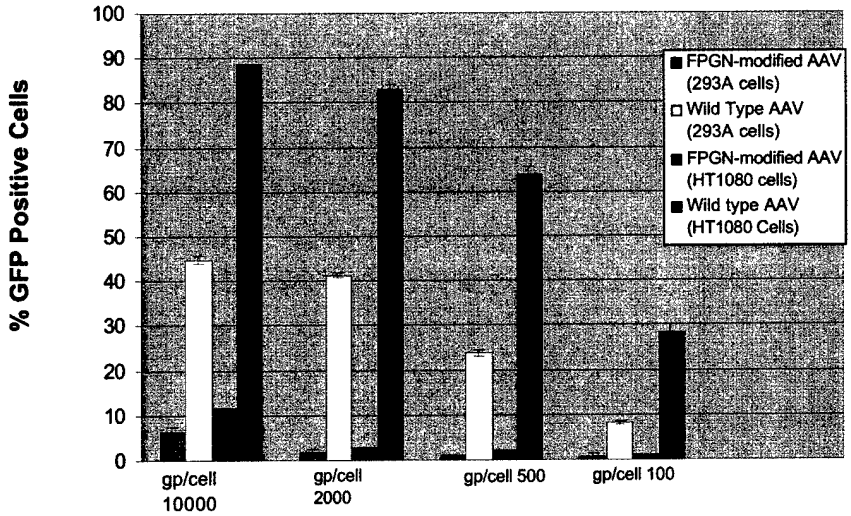


LEGEND (A-C) Immature (day 10) murine BMDCs (10^5) were prepared and exposed for 12 hours to 100 ng/ml LPS to induce maturation. Immature or mature DC were then incubated with 1 x 10^7 pfu AdGFP pre-complexed with FPGN2-KBP or a control peptide (either AAAA2-KBP, an otherwise identical peptide in which the FPGN motif was substituted with AAAA; or FPGNScr-KBP, which contains a scrambled version of the FPGN-containing peptide). 20 hours later GFP expression was examined. (A): Immature DC (filled bars) or mature DC (open bars) were incubated with AdGFP complexed to the indicated peptides at a single fixed concentration (15 μM); (B): BMDC were incubated with AdGFP complexed to either FPGN2-KBP (filled squares or circles, respectively, for immature and mature BMDC) or its scrambled derivative, FPGNScr-KBP (open squares or circles, respectively, for immature and mature BMDC), at a range of concentrations (0-15 μM, as indicated).

Retargeting of adeno-associated virus (AAV) vectors using phage-selected peptides. The ternary complex that is formed between the adenovirus vector and the bifunctional cell-targeting peptide described in the preceding section is likely to be rather unstable and prone to dissociation, in light of the modest (micromolar) binding affinity that short linear peptides typically possess for their ligands. Thus, we decided to use the CD40-binding peptide sequence to genetically modify the capsid of adeno-associated virus serotype 2 (AAV-2). To do this, we inserted the CD40-binding, FPGN-containing motif (see above) into the capsid protein of AAV-2, immediately after amino acid residue 587. We then generated virus stocks that contained a packaged GFP-reporter construct, encapsidated either by wild-type (WT-AAV2) AAV-2 capsid proteins or by the FPGN-peptide-modified AAV-2 capsid (FPGN-AAV2), and analyzed their ability to mediate gene transfer into various mammalian cells.

Insertion of the FPGN peptide after amino-acid 587 in the viral capsid greatly reduced viral infectivity for normal AAV2-susceptible indicator cells (i.e., 293A cells and HT1080 cells; see Figure 2). This is presumably because insertion of the FPGN-containing peptide abolishes the ability of the recombinant AAV capsid to bind to heparan sulfate proteoglycan (HSPG), as previously noted by other workers (*Shi et al., Human Gene Therapy 12:1697, 2001*).

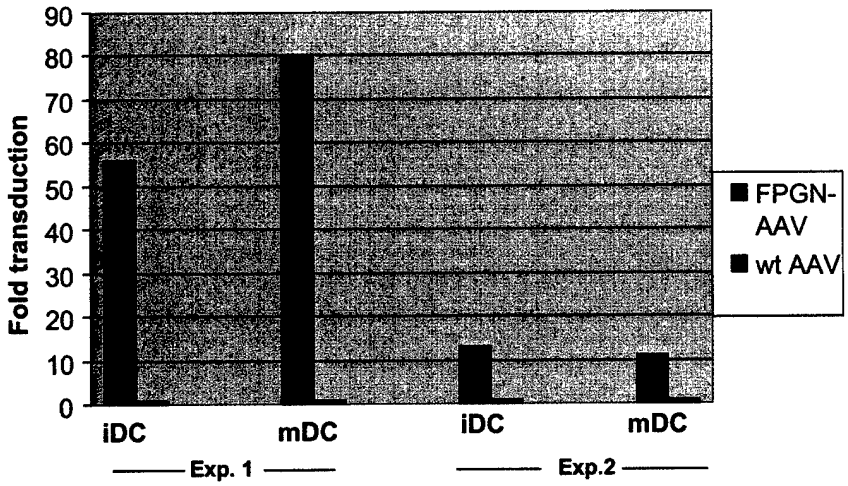
Figure 2: Genetic insertion of the CD40-binding peptide into the capsid of AAV results in decreased transduction efficiency in 293A and HT1080 cell lines



LEGEND. Modified and unmodified AAV encoding GFP was incubated at different genome particles/cell (gp/cell) with either 293A or HT1080 cells for 2 hrs at 37°C. The unbound virus was removed and the cells incubated for 48 hours before performing FACS analysis to quantitate the percent GFP positive cells.

Infection of primary murine dendritic cells (DC) with the modified AAV vector was analyzed next. In this case, the infections were performed using two multiplicities of infection (gp/cell of 1000 and 5000) for both FPGN-AAV and wt AAV. By comparing the relative ratio of gene transfer into primary murine DC versus other cell types (HT1080 cells, 293 cells), we determined that insertion of the CD40-binding peptide into the AAV2 capsid resulted in a 10-60 fold enhancement of the selectivity of AAV2-mediated gene delivery to DC (Figure 3). This strongly suggests that the capsid modification of AAV2 using this peptide may result in improved vectors for DC-directed gene transfer.

Figure 3: Genetic insertion of the CD40-binding peptide into AAV capsid results in selective transduction of primary dendritic cells (DC), versus CD40-negative cells (293A, HT1080)

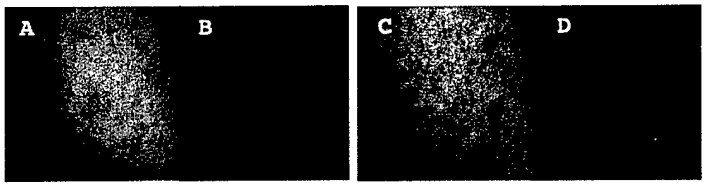


LEGEND. Modified and unmodified AAV encoding GFP was incubated at different genome particles/cell (gp/cell) with either immature DC (iDC) or LPS-matured DC (mDC) cells for 2 hrs at 37°C. The unbound virus was removed and the cells incubated for 48 hours before performing FACS analysis to detect the percent GFP positive cells. Results from two separate experiments are shown.

Current efforts to generate phage vectors that target dendritic cells. In the current project year, we have focussed our efforts on two phage systems – T7 and lambda, in large part because these phage are similar in size and shape to common mammalian viruses.

Construction of modified T7 bacteriophage vectors which contain a mammalian expression cassette encoding GFP. We have inserted a hrGFP expression cassette (CMV promoter, BGH polyA, humanized and hrGFP; Stratagene) into the genome of bacteriophage T7 (Novagen). We then propagated the phage in *E. coli*, and isolated DNA from CsCl density-gradient purified phage particles. This DNA was then used to transfect 293A cells. The results of one such DNA transfection experiment are shown in Figure 4, and confirm that the T7:GFP phage genomes are indeed capable of expressing GFP in mammalian cells.

Figure 4: Expression of hrGFP from T7:GFP genomic DNA in 293 cells



LEGEND: Purified T7 genomic DNA was isolated from phage particles derived from: (i) unmodified T7 (A, B) or (ii) T7 containing an inserted hrGFP mammalian expression cassette. 48 hours later, GFP expression by monitored by fluorescence microscopy (panels B, D); bright field images (A, C) are included for comparison.

Efforts are presently underway to determine whether modified T7 vectors can directly enter mammalian cells, and express the encoded GFP indicator gene. Very preliminary results, shown in Figure 5, suggest that T7:GFP phage which express the CD40-binding, FPGN-containing peptide on their surface can transduce CD40-positive target cells (CD40-transfected SKBR3 cells). However, this result needs to be confirmed by additional experiments.

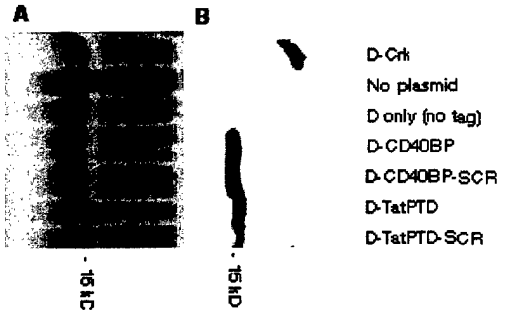
Figure 5: Expression of hrGFP in CD40-transfected SKBR3 cells following exposure to FGPN-peptide-bearing T7:GFP phage particles

LEGEND: 10^{10} phage particles (T7:GFP phage, bearing the CD40-binding, FGPN-containing peptide on its surface) were added to CD40-transfected SKBR3 cells, in the presence of chloroquine. 24 hours later, cells were examined for expression of GFP by light microscopy (right panel; *a brightfield image of the same field of cells is shown in the left panel*)

Development of modified lambda phage vectors. We have initiated efforts to derive retargeted lambda phage vectors, by fusing candidate targeting peptides to the terminus of lambda phage gpD. Our ultimate aim is to generate chimeric lambda phage particles which contain a mixture of wild-type and recombinant D-proteins (D-protein being the major lambda capsid protein). This can be achieved by propagation of wild-type lambda phage, or a lambda reporter phage clone (λ-Zap-GFP; *already constructed*), in a host cell that constitutively (or inducibly) expresses the desired recombinant D-protein. Since the normal lambda phage capsid contains 415 copies of D-protein, and because the D-protein is extremely tolerant of even large protein inserts, it is therefore possible to readily generate hybrid phage particles which contain 10-100 or more copies of recombinant D-protein.

In fact, the construction and expression of D-protein fusions containing the various peptides has already been initiated. Both the Tat-PTD and the CD40-BP have been successfully fused to the D-protein and recombinant clones have been shown to express the expected fusion proteins of interest (Fig. 6).

Figure 6: Expression of lambda D-protein fusions containing peptides of interest



LEGEND: D-protein fusions were constructed, in which the D protein was terminally tagged with a Hisx6 epitope tag, and then fused at the opposite terminus to the indicated peptides (Tat protein transduction domain [PTD] or CD40-binding peptide [CD40], or scrambled [SCR] derivatives thereof) . D-Crk represents a control fusion protein containing the Crk protein. Note that the expected size D-protein::peptide fusions were produced upon IPTG induction of cultures (A; Coomassie-stained gel); these proteins all reacted with a monoclonal antibody specific for the Hisx6 epitope (B; immunoblot), as expected.

Tasks 2, 3: These tasks remain to be initiated. We have made the decision to defer work on the construction of the HER2-expressing phage clones because we believe that the most important phase of the current project relates to the development of modified phage clones which are capable of transducing mammalian cells. Thus, Task 1 has remained our major priority. Since we are now much closer to the development of suitably targeted phage vectors (either T7 or lambda), we intend to proceed with insertion of the HER2 expression cassette and the evaluation of the immunogenicity of HER2-expressing phage in the third year of this project.

KEY RESEARCH ACCOMPLISHMENTS

- Successful modification of existing mammalian virus vectors (adenovirus, adeno-associated virus) using a CD40-binding, FPGN-containing peptide that was identified by phage display. The modified adenovirus and AAV vectors both displayed an enhanced ability to transduce dendritic cells, relative to their unmodified counterparts.

- Generation of T7 vectors which contain a mammalian expression cassette encoding GFP.

- Generation of recombinant lamdba phage coat proteins (modified gD protein) which contain the CD40-binding, FPGN-containing peptide and the protein transduction domain (PTD) from HIV-1 Tat.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations: We have published one paper based on the results summarized in this report, and have also generated two abstracts from this work.

Patents and licenses applied for and/or issued: None

Degrees obtained that are supported by this award: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models, etc: None

Funding applied for based on work supported by this award: None

Employment or research opportunities applied for and/or received on experiences/training supported by this award: Research training for Mr. Casey Maguire and Ms. Heather Lankes was provided. Both are students in the doctoral program at the University of Rochester (UR), and both passed their comprehensive qualifying examination by developing proposals that aimed at breast cancer vaccine development (Ms. Lankes) or vector-targeting to dendritic cells (Mr. Maguire).

CONCLUSIONS

The conclusions which can be drawn from the second year of our experiments are as follows:

1. A previously identified CD40-binding peptide can be successfully used to target mammalian virus vectors (adenovirus, adeno-associated virus) to primary dendritic cells.

2. Recombinant T7 bacteriophage clones which contain a mammalian expression cassette encoding a readily-assayable indicator gene (green fluorescent protein; GFP) can be generated, and the inserted indicator gene can be expressed following introduction of phage DNA into human cells.

3. Recombinant lambda phage coat protein (gD protein) can be efficiently produced, bearing the novel CD40-binding peptide that we have identified (see #1) or bearing a short protein transduction domain (PTD) peptide from HIV-1 Tat. This will make it possible to derive retargeted lambda phage particles, which may have the ability to transduce primary dendritic cells (see #1).

"So What Section"

The knowledge gained from these experiments advances the basic goals of this grant application, and brings us closer to being able to test our underlying hypothesis, that genetically modified bacteriophage vectors may represent a useful system for permitting gene (antigen) transfer to dendritic cells, for purposes of breast cancer vaccine development.

BIBLIOGRAPHY (PUBLICATIONS)

These materials are all also included as Appendices

Manuscripts:

Richards J., Abend, J., Miller, M., Chakraborty-Sett, S., Dewhurst, S., and Whetter, L. A peptide containing a novel FPGN CD40-binding sequence enhances adenoviral infection of murine and human dendritic cells. **Eur. J. Biochem**. 270: 2287-2294, 2003.

Abstracts:

J. Richards, M. Miller, J. Abend, L. Whetter, S. Dewhurst. Targeting of viral and phage-based vectors to dendritic cells for breast cancer vaccine development. Poster Presentation at the Era of Hope Breast Cancer Meeting, September 2002 (Orlando, FL).

Maguire C., Ramirez, S., Richards, J., S. Dewhurst. Targeted Gene Transfer to Murine Dendritic Cells using Adeno-associated virus-2 with a Novel CD40-binding Peptide displayed on its Capsid. Poster Presentation at the American Society for Gene Therapy Meeting, June 2003.

APPENDIX MATERIALS

Award Number:	DAMD17-01-1-0384
TITLE:	Dendritic Cell-Targeted Phage Vectors for Breast Cancer Vaccine Development
PRINCIPAL INVESTIGATOR:	Stephen Dewhurst, Ph.D.
CONTRACTING ORGANIZATION:	University of Rochester Medical Center Rochester, New York 14642
REPORT DATE:	June 2003
TYPE OF REPORT:	Annual

List of Materials Appended

Published Paper:

Richards J., Abend, J., Miller, M., Chakraborty-Sett, S., Dewhurst, S., and Whetter, L. A peptide containing a novel FPGN CD40-binding sequence enhances adenoviral infection of murine and human dendritic cells. **Eur. J. Biochem**. 270: 2287-2294, 2003.

- 8 pages

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J. Richards, M. Miller, J. Abend, L. Whetter, S. Dewhurst. Targeting of viral and phage-based vectors to dendritic cells for breast cancer vaccine development. Poster Presentation at the Era of Hope Breast Cancer Meeting, September 2002 (Orlando, FL).

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- 1 page

ABSTRACT

J. Richards, M. Miller, J. Abend, L. Whetter, S. Dewhurst. Targeting of viral and phage-based vectors to dendritic cells for breast cancer vaccine development. Poster Presentation at the Era of Hope Breast Cancer Meeting, September 2002 (Orlando, FL).

Abstract: To improve the safety and efficacy of viral vectors for breast cancer vaccine development and other applications, it is advantageous to exercise control over the target cell tropism of the vectors. To this end, we are using phage display technology to select for receptor-specific peptide and small protein ligands, which have the ability to engage internalizing or endocytosing cell surface receptors present on primary dendritic cells (DC). CD40 has emerged as a useful model target for these experiments, since it is expressed on DC, and also because the conjugation of an anti-CD40 monoclonal antibody to the surface of adenovirus particles has been shown to result in enhanced infection of DC. Identification of CD40-binding peptides offers the possibility of improving this system, by genetically incorporating the novel targeting peptides into the genome of the virus or phage-based vector. Phage-derived peptide sequences selected for binding to CD40 were found to contain an FPG consensus sequence. These sequences were then used to design bifunctional peptides, capable of simultaneously binding to the adenovirus particle and to the CD40 molecule. These peptides were able to strongly enhance infection of murine and human DC by adenovirus vectors. Experiments are presently in progress to examine whether these same CD40-binding peptide sequences can be used to allow bacteriophage vectors (T7, phage lambda) to infect DC, and to express an indicator gene in these cells (GFP, luciferase). Overall, these studies are intended to determine whether it is possible to develop efficient phage-based DNA delivery systems for DC; vectors of this kind may have significant utility for breast cancer vaccine delivery and other applications.

ABSTRACT

Maguire C., Ramirez, S., Richards, J., S. Dewhurst. Targeted Gene Transfer to Murine Dendritic Cells using Adeno-associated virus-2 with a Novel CD40-binding Peptide displayed on its Capsid. Poster Presentation at the American Society for Gene Therapy Meeting, June 2003.

Abstract: Dendritic cells (DC) play a crucial role in antigen presentation, and in the development of immune responses to infectious agents and vaccines. As a consequence, there is considerable interest in the development of novel approaches to the delivery of immunogens to DC, with a view to developing improved vaccines. Antigen delivery to DC can be achieved by several methods, which include the use of recombinant virus vectors that encode the immunogen of interest. To efficiently deliver virus vectors to DC, it would be desirable to target the virus to a receptor that is selectively expressed on DC, as compared to other cell types. One molecule which has been used for this purpose is CD40. CD40 is expressed on antigen-presenting cells (DC, B cells) and on endothelial cells, but not on most other cell types; it is also important in regulating cellular activation.. Our laboratory has recently utilized phage display peptide libraries in order to identify a consensus peptide sequence which interacts with both human and murine CD40. A bifunctional peptide containing this CD40-binding motif as well as an adenovirus fiber-binding domain was then synthesized, and shown to enhance adenovirally-mediated gene delivery to primary murine DC and to CD40-positive B cell lines, following non-covalent attachment of the bifunctional peptide to the surface of the virus. This established proof-of-principle support for the notion that the phage-selected CD40-binding protein could be used to modify the host cell tropism of a mammalian virus vector. However, the ternary complex that is formed between the adenovirus and the bifunctional cell-targeting peptide is likely to be rather unstable and prone to dissociation, in light of the modest (micromolar) binding affinity that short linear peptides typically possess for their ligands. Thus, we decided to use the CD40-binding peptide sequence to genetically modify the capsid of adeno-associated virus serotype 2 (AAV-2). We then generated virus stocks that contained a packaged GFP-reporter construct, encapsidated either by wild-type (WT-AAV2) AAV-2 capsid proteins or by the CD40-modified AAV-2 capsid (CD40-AAV2), and analyzed their ability to mediate gene transfer into various mammalian cells. By comparing the relative ratio of gene transfer into primary murine DC versus other cell types (HT1080 cells, 293 cells), we determined that insertion of the CD40-binding peptide into the AAV2 capsid resulted in a 10-60 fold enhancement of the selectivity of AAV2-mediated gene delivery to DC. This strongly suggests that the capsid modification of AAV2 using this peptide may result in improved vectors for DC-directed gene transfer.

Eur. J. Biochem. **270**, 2287–2294 (2003) © FEBS 2003 doi:10.1046/j.1432-1033.2003.03596.x

A peptide containing a novel FPGN CD40-binding sequence enhances adenoviral infection of murine and human dendritic cells

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Department of Microbiology and Immunology, University of Rochester, NY, USA

CD40 is a receptor with numerous functions in the activation of antigen presenting cells (APCs), particularly dendritic cells (DC). Using phage display technology, we identified linear peptides containing a novel FPGN/S consensus sequence that enhances the binding of phage to a purified murine CD40-immunoglobulin (Ig) fusion protein (CD40-Ig), but not to Ig alone. To examine the ability the FPGN/S peptides to enhance adenoviral infection of CD40-positive cells, we used bifunctional peptides consisting of an FPGN-containing peptide covalently linked to an adenoviral knob-binding peptide (KBP). One of these, FPGN2-KBP, was able to enhance adenoviral infection of both murine and human DCs in a dose-dependent manner. FPGN2-KBP also improved infection of murine B cell blasts, a murine B lymphoma cell line (L10A), and immortalized human B cells. To demonstrate that enhancement of adenoviral infection depended on the presence of CD40, we analyzed infection of the breast cancer line, SKBR3, that does not express CD40 or the adenovirus cellular receptor, CAR. Infection of SKBR3 cells was enhanced by FPGN2-KBP following transient transfection with a plasmid vector that expresses murine CD40, but not when the cells were mock-transfected. In conclusion, we have isolated a peptide that binds to murine CD40, and promotes the uptake of adenoviruses into CD40-expressing cells of both murine and human origin, suggesting that it may have potential applications for antigen delivery to CD40-positive antigen-presenting cells.

Keywords: CD40; phage display; DC; adenovirus.

CD40 is a transmembrane receptor in the tumor necrosis factor receptor family and was characterized first by its expression on solid tumors, then on B lymphocytes. CD40 expression is highest in antigen presenting cells such as DC, monocyte/macrophages and B cells. Cross-linking of CD40, following its interaction with CD40 ligand (CD40L) on activated helper T lymphocytes, induces activation and maturation of antigen presenting cells [1]. In immature dendritic cells (DC), this is characterized by up-regulation of costimulatory molecules (MHC class II, CD40, CD80, and CD86), increased migration to lymph nodes and secretion of interleukin-12 and other pro-inflammatory cytokines [2–4]. On B cells, CD40 engagement prolongs survival and promotes their differentiation into memory cells [5,6]. Genetic defects that result in dysfunctional CD40–CD40L interactions lead to 'hyper–IgM syndrome', that is characterized by immunodeficiency, an absence of circulating IgG antibodies, and lack of germinal center formation in the lymph nodes [7,8].

As DC express high levels of CD40, CD40 represents a potential target for vaccine delivery to DC. Covalently linked bispecific antibodies that bind to CD40 and adenoviral fiber knob have been shown to enhance infection of murine DC [9]. These vectors also have been found to induce maturation of DC, presumably through CD40 cross-linking [10]. CD40-induced maturation is essential for the appropriate stimulation of T helper cells and for the generation of a vigorous cell-mediated immune response [3,11]. As adenovirus vectors conjugated to bispecific CD40 antibodies both infect DC and induce their maturation, CD40 represents a promising target for adenoviral-mediated vaccine delivery.

CD40 is also expressed on some tumors, and has been implicated in tumor immune evasion and angiogenesis [12–14]. High CD40 expression has been found on melanoma, lung and other tumors and was correlated with a poor prognosis [15–17]. A single-chain variable region from an anti-CD40 monoclonal antibody that was linked to the *Pseudomonas* exotoxin, PE40, selectively killed B lymphoma cells [18,19], suggesting that CD40 on malignant cells can be a target for tumor therapy. High CD40 expression has also been observed in atherosclerotic vessels, tumor endothelium and in rejected allograft tissue, suggesting that CD40 targeting could have other therapeutic uses as well [1].

Phage display technology allows for selection of target-specific peptides from combinatorial peptide libraries displayed on the surface of bacteriophage M13 [20]. Phage display peptide libraries have been used previously to select

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Abbreviations: AdV-GFP, adenovirus type 5 expressing green fluorescent protein; CAR, coxsackie-adenovirus receptor; DC, dendritic cell(s); EBV, Epstein–Barr virus; GFP, green fluorescent protein; KBP, adenovirus fiber knob-binding peptide; LPS, lipopolysaccharide; m.o.i., multiplicity of infection.

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for peptides that bind to cell surface receptors such as transferrin and the tumor antigen HER2/neu, and these peptides have been used to modify the tropism of viral or phage vectors [21,22]. In this study, we used phage peptide display to identify a peptide, ATYSEFPGNLKP, that binds to CD40 and enhances adenoviral infection of mouse and human DC and B lineage cells.

Materials and methods

Cell lines

Epstein–Barr virus- (EBV-) transformed human B cells were provided by X. Jin (University of Rochester, NY, USA) and L10A cells were provided by A. Bottaro (University of Rochester). In some experiments, EBV-transformed human B cells were treated with 5 $\mu g \cdot mL^{-1}$ lipopolysaccharide (LPS) to enhance adenovirus infection. SKBR3 human breast cancer cells were obtained from American Type Culture Collection; these cells do not express either CD40 or the primary adenovirus receptor (CAR; our unpublished data). All cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Sigma), 0.5 $U \cdot L^{-1}$ penicillin/streptomycin, and 2 mM L-glutamine (Gibco-BRL).

Recombinant adenovirus that expresses GFP

A recombinant adenovirus that expresses jellyfish green fluourescent protein (AdV-GFP) was constructed with reagents obtained from B. Vogelstein (Johns Hopkins University, MD, USA [23]). Briefly, pAd-Track-CMV was linearized and cotransformed into *Escherichia coli* BJ5183 cells with the adenoviral backbone plasmid, pAd-Easy-1. Recombinants were selected for kanamycin resistance and recombination was confirmed by restriction endonuclease analysis. Linearized recombinant plasmid DNA was transfected into QBI-293 A cells (Quantum Biotechnologies, Inc., Montréal, Quebec, Canada) under agarose overlay. Plaques exhibiting green fluorescence under UV phase-contrast microscopy were harvested and subjected to three rounds of plaque purification. Virus stocks were expanded and purified by cesium chloride gradient, followed by extensive dialysis, and resuspended in phosphate buffered saline. Virus stocks were prepared using only endotoxin-free materials and there was no detectable endotoxin in the final preparation as assessed by E-Toxate assay (Sigma).

Human DC

DC were derived from human blood using a modification of established methods [24]. CD14-positive cells were isolated from peripheral blood mononuclear cells using MACS separation columns (Miltenyi, Cologne, Germany). Cells were cultured in RPMI containing 1% autologous plasma, 1 $ng \cdot mL^{-1}$ GM-CSF, 20 $ng \cdot mL^{-1}$ IL-4 (R & D Systems, Minneapolis, MN, USA) and penicillin/streptomycin. Media were replenished at 3 and 6 days and immature DC were harvested after 8 days. Day 8 human DC were positive for CD40, CD80 and CD86 and negative for CD14, as assessed by flow cytometry (data not shown).

Murine DC

Mouse DC were prepared from bone marrow of BALB/c mice according to the method described by Lutz *et al.* [25]. Cells were plated at a density of 2×10^6 cells per 100 mm dish and cultured in RPMI supplemented with 10% FBS and 10% culture supernatant from a murine GM/CSF-expressing cell line (a gift from A. Livingstone, University of Rochester, NY, USA). Media were replenished at day 3 and day 6, and nonadherent cells were harvested at day 10. In some experiments, LPS (Sigma) was added at 100 $ng \cdot mL^{-1}$ for 12 h to induce maturation. Day 10 DC were positive for CD40, CD11c, and MHCII by flow cytometry. LPS treatment increased the expression of CD40 and MHCII (data not shown).

Murine B cell blasts

Spleens were harvested from BALB/c mice and were ground between the frosted ends of two glass microscope slides to release cells from the splenic capsule. Resulting cells were rinsed twice in RPMI-1640 medium, and cultured in RPMI-1640 medium supplemented with 5 $\mu g \cdot mL^{-1}$ LPS and 7 $\mu g \cdot mL^{-1}$ of dextran sulfate (Amresco; Solon, OH, USA) for 2–3 days to allow for blast cell formation.

Biopanning of PhD-12 library against murine CD40

A PhD-12 phage library was prepared and expanded according to manufacturer's directions (New England Biolabs). A single well of a six-well sterile tissue culture plate (Falcon) was coated with CD40-Ig (a gift from Dr David Gray [26]), at a concentration of 100 $\mu g \cdot mL^{-1}$ in NaCl/Tris buffer and incubated overnight at 4 °C in a humidified container with gentle agitation. The plate was rinsed three times with NaCl/Tris +0.1% [v/v] Tween-20 and blocked with 1 mL of 5% phage blocking reagent (Novagen, Madison, WI, USA) for 1 h at room temperature followed by five rinses with TBST. Ten microliter (1.5×10^{11} phage) in 90 µL 5% blocking reagent were added to the coated well and incubated for 1 h at room temperature with gentle agitation. Unbound phage were removed by washing ten times with NaCl/Tris +0.1% [v/v] Tween-20. Bound phage were eluted with 100 µL of 0.2 M glycine-HCl (pH 2.2) containing 1 $mg \cdot mL^{-1}$ BSA at room temperature with gentle agitation. The eluted phage were neutralized immediately with 15 µL of 1 M Tris/HCl (pH 9.1). The phage were amplified using the *E. coli* ER2738 host strain (New England Biolabs, Inc.) and subjected to two additional rounds of biopanning and amplification. Upon completion of three rounds of biopanning, individual phage clones were selected, amplified and purified by precipitating with 20% PEG-8000 in 2.5 M NaCl. The phage DNA was isolated using 100 µL iodide buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA, 4 M NaI], and precipitated with 250 µL 100% ethanol. Phage DNA was sequenced using the −28 gIII sequencing primer (New England Biolabs, Inc.).

Phage Binding Assay

Microtiter wells were coated with either CD40-Ig (mouse CD40) or Human IgG1 lambda (Sigma) at a concentration

of 10 $\mu g \cdot mL^{-1}$ in TBS buffer and were incubated overnight at 4 °C in a humidified container with gentle agitation. The plate was warmed to room temperature and excess target was removed. The wells were blocked for 1 h at room temperature with 5% phage blocking reagent (Novagen). The plate was rinsed five times with TBST and dilutions of 1×10^9, 1×10^8 and 1×10^7 of the phage clones were added and allowed to bind for 1 h at room temperature. After washing with TBST, to remove unbound phage, bound phage were eluted with 100 μL of 0.2 M glycine/HCl (pH 2.2) containing 1 $mg \cdot mL^{-1}$ BSA at room temperature with gentle agitation. The eluted phage were neutralized immediately with 15 μL of 1 M Tris/HCl (pH 9.1) and the volume was brought to 1 mL, prior to determination of phage titers by limiting dilution.

AdV-GFP infections

Bifunctional adenoviral-binding peptides containing CD40-binding peptide sequences linked to the adenoviral knob binding peptide, KBP (RAIVfrvqwlrryfvngsrSGGG) as described by Hong *et al.* [27], were obtained from Alpha Diagnostic (San Antonio, TX, USA). Control peptides included a peptide in which AAAA was substituted for the FPGN motif (AAAA2-KBP), or in which the CD40-binding peptide sequence was 'scrambled' randomly (FPGNScr-KBP).

For DC and B cells, peptide and adenovirus were mixed in a final volume of 20 μL in complete cell medium for 30 min at room temperature [the final peptide concentration used ranged from 0 (controls) to 15 μM, and adenovirus was added at a concentration consistent with the final desired multiplicity of infection (m.o.i.) for the experiment]. Peptide-adenovirus complexes were then added to 80 μL of cells in a 48-well plate and GFP fluorescence was assessed by FACS analysis at 20 h postinfection. In some experiments, SKBR3 cells at 90–95% confluence in a 6-well plate were transfected with 4 μg of pRSV-mCD40 (gift of G. Bishop, University of Iowa) using Lipofectamine 2000 (Invitrogen) in the presence of FBS. Media was replaced after 8 h, and cells were infected at 24 h post-transfection for 1 h with 20 μL precomplexed AdV-GFP (5×10^7 adenovirus and FPGN2-KBP or FPGNScr-KBP) adenovirus at a total volume of 1 mL (final peptide concentration, 10 μM), after which media was replaced. GFP fluorescence was assessed by FACS analysis at 20 h postinfection. Pictures were taken on an Olympus CK40 fluorescence microscope (Olympus, Tokyo, Japan) using QIMAGE PRO software (Digital Domain, Inc., Sykesville, MD, USA).

Results

Phage display clones selected for CD40-Ig binding contain a novel FPGN consensus sequence. After three rounds of biopanning using the PhD-12 random peptide display library, five clones (PCP1-PCP5) were selected for sequencing. Three of the five clones contained the sequence FPGN/S while a fourth clone contained FPPS. The fifth displayed a sequence that did not have any apparent consensus with the other four (Fig. 1A). When these phage clones were assayed individually for binding to CD40-Ig, only those clones containing FPGN or FPGS bound to CD40-Ig above background binding to BSA; none of the clones bound to IgG1 above background. Between 0.01%–0.1% of applied FPG-containing phage was recovered from CD40-Ig after one hour of binding, regardless of the input titer (Fig. 1B).

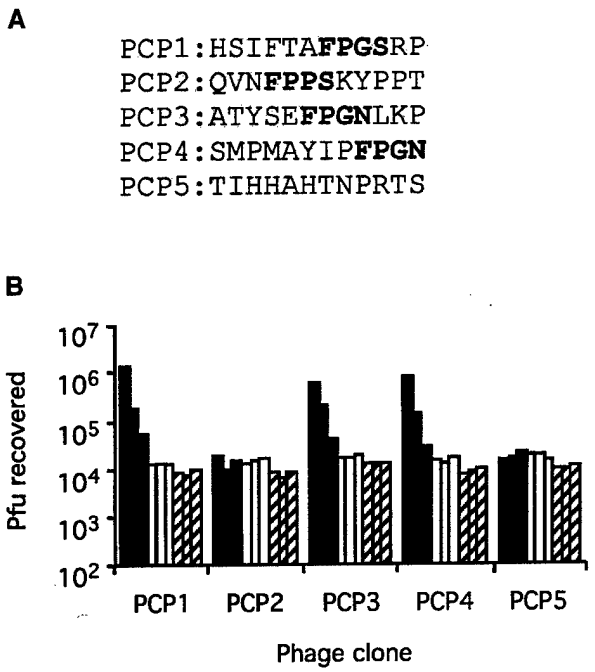

Fig. 1. Phage clones selected for binding to CD40-Ig contain a novel FPGN/S consensus sequence; only phage clones containing this sequence bound specifically to CD40-Ig. (A) Following three rounds of biopanning, sequencing of five phage clones was sufficient to identify a consensus sequence. (B) Purified phage clones were allowed to bind to purified CD40-Ig (filled bars), human IgG1 isotype control (open bars), or BSA (patterned bars) for 1 h at decreasing titers of 10^9, 10^8, and 10^7 p.f.u. Samples were then washed, eluted with acidic glycine, and titered; the results are shown (note that the first bar in each set of three corresponds to 10^9 pfu of input phage, with subsequent bars denoting the serial 10-fold decreases in phage input).

FPGN-containing peptides facilitate the uptake of adenovirus into CD40-expressing cells

To test the ability of CD40-binding peptides in facilitating adenovirus entry into CD40-expressing cells, we used a method in which a bifunctional peptide containing the peptide of interest is covalently linked to a peptide that binds to the adenoviral knob protein (KBP) [27]. This method can promote the internalization of adenoviruses by improving binding to alternate receptors on cells that do not express the high-affinity adenovirus receptor, CAR. For our studies, we used a recombinant adenovirus that expresses the jellyfish green fluorescent protein (GFP) to permit analysis of infected cells by flow cytometry.

We initially selected one FPGS and one FPGN-containing peptide (PCP1 and PCP3, respectively) for further analysis. These two peptides were chosen because the consensus sequence is centrally located in

'the randomized insert peptide. Thus, we predicted that the phage insert sequence could be used to create a bifunctional adenovirus-binding peptide, with a reasonable expectation that the putative CD40-binding region would be 'isolated' from any structural or steric effects due to an adjacent motif such as the fiber-binding domain. Our data revealed that bifunctional peptides based on both the PCP1 and the PCP3 peptide (Fig. 1A) enhanced infection of DC (data not shown), but one (PCP1) also caused significant cytotoxicity in the cultures, for reasons that are uncertain. We therefore focused the bulk of our efforts on bifunctional peptides which incorporated the sequences derived from the PCP3 insert. All subsequent experiments were performed using bifunctional peptides derived from the PCP3 insert sequence; these peptides are refered to hereafter as FGPN2-KBP (PCP3 insert linked to the fiber-binding domain), FGPNScr-KBP (scrambled version of the PCP3 insert linked to KBP) or AAAA2-KBP (identical to FGPN2-KBP, except that the FPGN motif was replaced by four alanines; see Materials and methods).

To confirm the specificity of FPGN2-KBP for CD40, we evaluated infection of SKBR3 cells; these cells do not express CD40 or CAR (data not shown) and are not readily transduced by wild-type adenovirus type 5 vectors. The cells were transfected with a plasmid that expresses murine CD40 (pRSVmCD40) and infected with peptide/adenovirus complexes. At the time of infection (24 h post-transfection), the cells were 44% positive for murine CD40 vs. 2.4% background staining of mock-transfected cells (data not shown). At 20 h postinfection, 43.7% of CD40-transfected cells were positive for GFP, while only 12.6% of mock-transfected cells were GFP-positive (Fig. 2), demonstrating that FPGN2-KBP-mediated adenovirus infection is improved upon the expression of CD40 on the cell surface to enhance adenovirus infectivity.

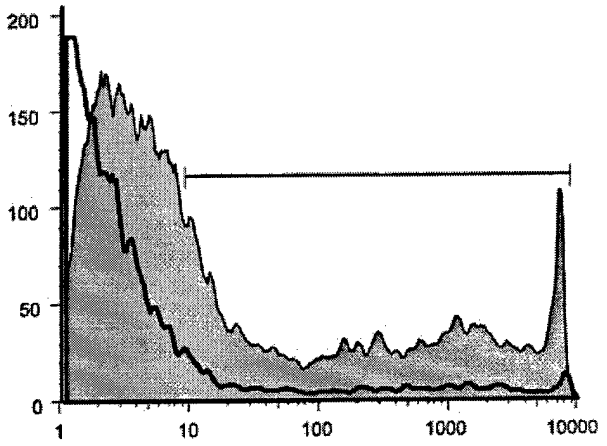

Fig. 2. Adenovirus complexed with FPGN2-KBP preferentially transduced SKBR3 cells transiently transfected with murine CD40. SKBR3 cells were transiently transfected with pRSV-mCD40. At 24 h post-transfection, cells were infected with 5×10^8 pfu AdV-GFP precomplexed with FPGN2-KBP (10 μM). Media was replaced 1 h later, and GFP expression was assessed 20 h post-transduction by FACS analysis. The figure shows a comparison of log fluorescent GFP expression for 10^4 mock-transfected (bold line) vs. CD40-transfected (filled) cells. The gate shown represents GFP-positive cells as determined by fluorescence of uninfected cells. Some 43.7% of CD40 transfected cells were determined to be GFP positive vs. 12.6% of mock-transfected cells.

FPGN2-KBP enhancement of adenovirus infection of DC requires the FPGN motif and is dose-dependent

To explore the potential for FPGN2-KBP to promote antigen delivery to DC, murine bone marrow-derived DC were cultured overnight with or without LPS (to induce maturation and up-regulate CD40 expression). FPGN2-KBP enhanced infection of both immature and mature murine DC (42% and 57% above levels obtained in the absence of peptide, respectively, for immature and mature DC; Fig. 3A). The difference was statistically significant (Tukey test, $P < 0.01$). Infection of cells by AdV-GFP complexed to the AAAA2-KBP peptide was at levels similar to those obtained in the absence of peptide, indicating that the FPGN motif is essential. To eliminate the possibility that nonsequence specific amino acid interactions could be contributing to CD40 binding, we also obtained a peptide in which the amino acid sequence of FPGN2 was scrambled (FKEAGSPYTLPN-KBP or FPGN2scr-KBP). A range of different peptide concentrations (5, 10 and 15 μM) was evaluated, using AdV-GFP at a fixed m.o.i. (100 p.f.u.·cell^{-1}). There was a statistically significant enhancement of adenovirus infection at each of the concentrations tested and a positive relationship between dose and number of GFP-expressing cells for both immature and mature murine DC (Fig. 3B). In contrast, the scrambled peptide, FPGNScr-KBP, did not enhance adenovirus infection. The number of GFP-positive cells was consistently higher with immature DC than with mature DC, regardless of whether or not AdV-GFP infection was enhanced by the addition of peptide. At the highest peptide concentration tested (15 μM), 78% of the immature DC expressed GFP when infected using FPGN2-KBP compared with 21% when infected using FPGNScr control peptide (a 3.7-fold increase). Similarly, the FPGN2-KBP peptide enhanced AdV-GFP infection of mature DC from a baseline level of 15% GFP-positive cells (with FPGNScr control peptide) to a level of 66% (4.4-fold enhancement). The enhancement of infection by FPGN2-KBP was readily visualized under fluorescence microscopy (Fig. 3C).

FPGN2-KBP enhances AdV-GFP infection of human DC, as well as mouse and human B cells

To examine whether the FPGN CD40-binding peptide cross-reacts with human CD40, human DC were derived from CD14-positive blood monocytes after 7 days of culture in the presence of IL-4 and GM/CSF. On day 8, the cells were infected with AdV-GFP (m.o.i., 100), either in the absence of peptide or in the presence of 10 μM FPGN2-KBP or FPGNScr-KBP. At 20 h postinfection, flow cytometry analysis showed that 39% of cells were GFP-positive when infected in the presence of FPGN2-KBP, compared with only 7.9% in the presence of FPGNScr-KBP or 8.7% with AdV-GFP alone (Fig. 4).

As CD40 is also present on B cells, we evaluated FPGN2-KBP enhancement of adenovirus infection of a human

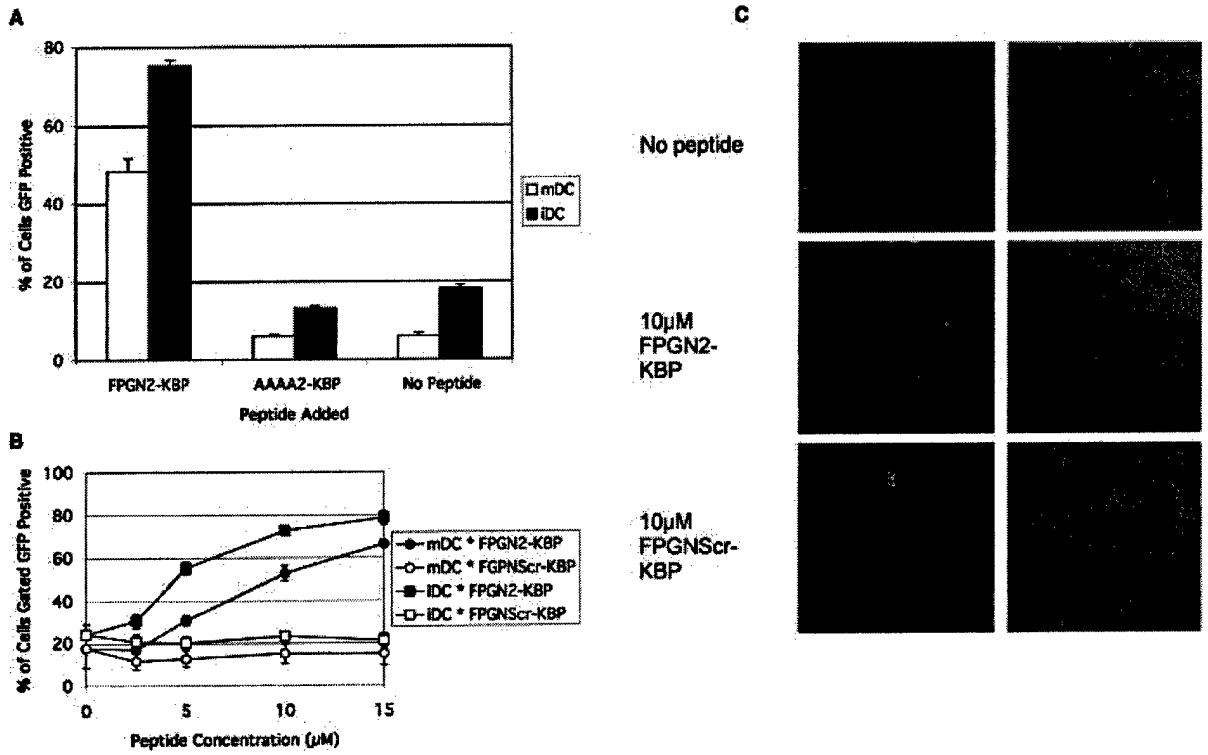

Fig. 3. FPGN2-KBP enhances AdV-GFP infection of murine BMDCs. (A–C) Immature (day 10) murine BMDCs (10^5) were prepared and exposed for 12 h to 100 ng·mL^{-1} LPS to induce maturation. Immature or mature DC were then incubated with 1×10^7 pfu AdV-GFP precomplexed with FPGN2-KBP or a control peptide (either AAAA2-KBP, an otherwise identical peptide in which the FPGN motif was substituted with AAAA; or FPGNScr-KBP, that contains a scrambled version of the FPGN-containing peptide). GFP expression was examined 20 h later. (A) Immature DC (filled bars) or mature DC (open bars) were incubated with AdV-GFP complexed to the indicated peptides at a single fixed concentration (15 μM). (B) BMDC were incubated with AdV-GFP complexed to either FPGN2-KBP (filled squares or circles, respectively, for immature and mature BMDC) or its scrambled derivative, FPGNScr-KBP (open squares or circles, respectively, for immature and mature BMDC), at a range of concentrations (0–15 μM, as indicated). (A,B) GFP was detected by FACS analysis; cells were gated as GFP-positive based on fluorescence of uninfected cells, and the percentage of GFP positive cells is shown. Error bars represent the standard deviation of triplicate infections. Results shown are representative of three experiments. In both immature and mature BMDC, the number of GFP-positive cells infected in the presence of FPGN2-KBP was significantly greater than cells infected in the presence of AAAA2-KBP or FGPNScr-KBP, as determined using analysis of variance followed by a Tukey test, $P < 0.01$. (C) Pictures (400 × magnification) of immature BMDC infected as described above with AdV-GFP complexed with 10 μM FPGN2-KBP, 10 μM FPGNScr-KBP, or no peptide at 16 h postinfection. Fields were selected randomly for similar cell density using bright field visualization (right hand panels); GFP fluorescence is shown in the left hand panels.

EBV-immortalized B cell line (LCL), a murine lymphoma line (L10A), and primary murine splenocyte-derived B cell blasts (Blasts). Each of these cell types required the use of a different m.o.i., based on preliminary analysis of its relative susceptibility to adenovirus infection (data not shown). The m.o.i. selected were 1000 for EBV-immortalized B cells, 2600 for L10A, and 100 for B cell LPS-blasts. FPGN2-KBP (10 μM) enhanced infection of each of these cell types, although the percentage of infected cells varied widely.

Human EBV-immortalized B cells infected with AdV-GFP alone at a m.o.i. of 1000 yielded 1.3% GFP-positive cells. This was unaltered by the addition of the scrambled control peptide, but it was increased to 7.3% when with FPGN2-KBP (5.6-fold enhancement; Fig. 4). In contrast, L10A cells, presumably due to their low expression of CAR and adenoviral coreceptor αv integrin (data not shown), were highly resistant to infection with adenovirus; infection with unmodified AdV-GFP was virtually undetectable even at an m.o.i. of 2600 (% GFP positive cells was ≈0.2%, identical to the background level of fluorescence measured as in the absence of added adenovirus). In the presence of FPGNScr, adenovirus infection was also nearly absent (≈ 0.2%). In the presence of FPGN2, however, AdV-GFP infection of L10A cells became detectable (≈ 1%; Fig. 4). Finally, in primary murine B cell LPS-blasts, FPGN2-KBP enhanced infection from 3% (no peptide or in the presence of scrambled peptide) to 13% with an m.o.i. of 100 (a 4.4-fold enhancement; Fig. 4).

Discussion

In this study we have identified novel peptides containing an FPGN/S consensus sequence using phage-display technology, and shown that phage bearing these peptides bind to a CD40-Ig fusion protein but not to Ig alone. When linked to a adenoviral knob-binding peptide (KBP), one of these peptides, FPGN2, was able to complex with adenovirus in such a way as to greatly enhance the infectivity of

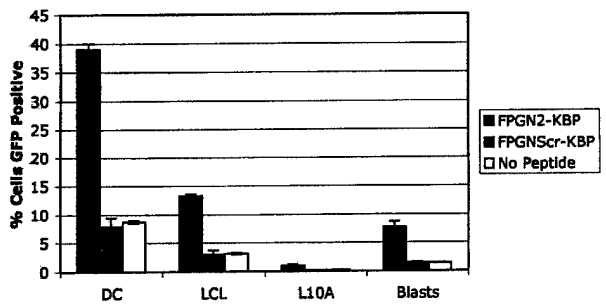

Fig. 4. FPGN2-KBP enhances AdV-GFP infection of human DC, as well as human and murine B cells. DC, LCL, L10A, Blasts: these labels refer, respectively, to day 8 human DC, EBV-immortalized human B cells, murine B lymphoma L10A cells and murine LPS blasts. Cells (10^5) were infected with AdV-GFP precomplexed with FPGN2-KBP (10 μM), FPGNScr-KPB (10 μM) or no peptide; m.o.i. used for infection were 100 (DC, Blasts), 1000 (LCL), or 2600 (L10A). In all cases, GFP expression was assessed 20 h postinfection by FACS analysis. Propidium iodide (PI) negative (viable) cells were gated as GFP-positive based on fluorescence of uninfected cells. The percentage of GFP positive cells is indicated for cells infected with AdV-GFP/FPGN2-KBP, AdV-GFP/FPGNScr-KBP, or in the absence of peptide. Error bars represent the standard deviation of triplicate infections. Results shown are representative of three experiments. In all cases, the number of GFP-positive cells infected by AdV-GFP in the presence of FPGN2-KBP was significantly greater than for cells infected with AdV-GFP complexed to FPGNScr-KBP or cells infected with AdV-GFP in the absence of peptide; statistical significance was determined using analysis of variance followed by a Tukey test, $P < 0.01$.

adenovirus for CD40-expressing cells. This enhancement was dependent on amino acid content as well as sequence, as peptides in which FPGN was replaced with AAAA, or in which the entire peptide sequence was scrambled, were ineffective. Furthermore, infectivity was not enhanced by FPGN2-KBP in the absence of CD40, as demonstrated with the use of a CD40/CAR-negative cell line, SKBR3. However, when SKBR3 cells were transfected with a CD40 expression plasmid, adenovirus infection was enhanced with FPGN2-KBP at levels similar to those obtained in DC. Collectively, these data suggest that the major effect of the FPGN2-KBP peptide is to enhance adenovirus binding to target cells that are deficient in, or express low levels of CAR.

For vaccine delivery with viral vectors, it may be useful for optimal T cell activation to infect immature DC in such a way that DC maturation (including migration to the local lymph node and increased expression of MHC class II, costimulatory molecules, and inflammatory cytokines) coincides with antigen expression. In this context, the present system may prove advantageous, particularly because adenovirus infection itself has been shown to enhance the maturation of DCs [28–30]. Therefore, the ability of the FPGN2-KBP peptide to enhance infection of immature DC by E1-deleted adenovirus type5-based vectors may be useful for future studies, including approaches that rely on adenovirally mediated delivery of immunogens for vaccination [31].

We were surprised that the FPGN2-KBP peptide did not have a greater effect in enhancement of adenoviral-mediated GFP expression in mature DC than in immature DC, as DC maturation significantly increases cell surface expression of CD40. In fact, the amount of enhancement mediated by FPGN2-KBP in immature DC was similar to that of other cell types tested (≈ fourfold), suggesting that the level of CD40 on these cells was not limiting. Thus, we tentatively conclude that other factors may influence the efficiency of adenovirally mediated gene expression in mature vs. immature DC, including the expression of adenovirus coreceptors (αv integrins), the efficiency of viral uptake and uncoating (in intracellular environments that possess marked differences in their proteasomal machinery and cytoskeleton) and the availability of nuclear transcription factors.

As adenovirus vectors retargeted to CD40 by bifunctional antibodies have been shown previously to infect immature DC, induce their maturation and initiate a potent immune response to antigen [9,10], CD40-binding peptides represent a promising development of viral vaccine delivery. Furthermore, in light of the high levels of CD40 expression on many tumors, and its up-regulation in inflammatory disorders such as atherosclerosis and Alzheimer's disease, CD40 is a potential target for gene therapy and targeted drug delivery. The use of phage-selected, cell surface ligand-binding peptides for targeted drug delivery has been established by the work of Arap and colleagues, who showed that tumor-specific peptides linked to doxorubicin exhibit enhanced tumoricidal and antiangiogenic activity with reduced adverse effects, compared to doxorubicin alone [32]. Therefore, peptide-mediated delivery of therapeutic agents directly to the sites of CD40 up-regulation should be possible, particularly in conditions such as atherosclerosis and angiogenesis where the target cells (endothelia) are accessible to agents introduced into the circulation.

It is uncertain whether the present approach to adenovirus-targeting (i.e. the use of bifunctional peptides) will prove useful for *in vivo* applications such as vaccine delivery. Although our data provide strong proof of principle support for the notion that a novel CD40-binding peptide can be used to enhance adenovirus infection of DC, it is possible that bifunctional peptides might become detached from the virus in an *in vivo* setting – particularly because of the generally low (micromolar) binding affinity of short peptides for their ligands. This may explain why previous studies using bifunctional peptides for adenovirus targetting have been performed exclusively *in vitro* (like the studies reported here) [27,33]. Thus, it may be necessary to introduce directly the novel CD40-binding peptide into the adenovirus fiber protein in order to successfully utilize this peptide for DC-targeting *in vivo*; future studies will be needed to address this question.

In summary, we have used phage display technology to isolate a novel CD40-binding peptide that has no detectable homology to CD40 ligand (data not shown) and that enhances adenoviral infection of CD40-positive cells of both human and murine origin, including DC and B cells resistant to infection with an unmodified recombinant adenovirus type-5 based vector. This peptide may have a use in vaccine delivery or gene therapy, and the ability to use the same CD40-targeting peptide for murine and human applications should provide an important advantage in translation of experimental findings to a clinical setting.

Acknowledgements

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