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Molecular imaging of T4 phage in mammalian tissues and cells

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Abbreviations: FPLC, fast protein liquid chromatography; GFP, green fluorescent protein; i.v., intravenously; IIET, Institute of Immunology and Experimental Therapy; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, luria broth; M, marker; PCR, polymerase chain reaction; RTD, routine test dilution; SPF, specific pathogen free

Advances in phage therapy encourage scientific interest in interactions of phages with human and animal organisms. This has created a need for developing tools that facilitate studies of phage circulation and deposition in tissues and cells.

Here we propose a new green fluorescent protein (GFP)-based method for T4 phage molecular imaging in living systems. The method employs decoration of a phage capsid with GFP fused to the N-terminus of Hoc protein by in vivo phage display. Fluorescent phages were positively assessed as regards their applicability for detection inside living mammalian cells (by phagocytosis) and tissues (filtering and retention by lymph nodes and spleen). Molecular imaging provides innovative techniques that have brought substantial progress in life sciences. We propose it as a useful tool for studies of phage biology.

Introduction

Advances in phage therapy[1-3] encourage scientific interest in interactions of phages with human and animal organisms. Phages are postulated to be an important alternative to the insufficient antibacterial drugs arsenal. This has prompted questions on detailed recognition of phage interactions with mammalian cells and tissues. Both investigations of phage biology and applications of bacteriophages in medicine have induced a need for developing tools that facilitate studies of phage circulation and deposition in tissues and cells.

Molecular imaging is potentially a highly useful tool for the investigation of phages in living systems. Molecular imaging comprises a wide spectrum of techniques that have brought substantial progress in life sciences. It utilizes various biological particles engaged in production or conversion of light. Among these, the green fluorescent protein (GFP) is a popular fluorescent marker, which was discovered in studies of jellyfish *Aequorea victoria*. Due to the ability of autocatalytic formation of the chromophore, GFP and GFP-like proteins have enabled novel approaches in live cell imaging, including whole body imaging and dynamic tracking.[4] Thus, furnishing a phage with GFP will initiate creative methods of molecular imaging in bacteriophage studies.

Decorating a phage capsid with foreign peptides or proteins can be done by phage display technique, which has become a standard in biotechnology. Phage display relies on fusing a foreign protein to a selected phage structural protein. Fusion may result from introduction of a foreign gene to the phage genome.[5] However, it can also be done by incorporation of fusion proteins expressed in bacteria: in vitro as purified compounds[6] or in vivo by propagation of a phage in bacteria that express a desired fusion which is further incorporated into new phage capsids.[7-9] The second method might be favorable in biological studies, since it does not require generation of genetically modified organisms (GMOs) by introducing foreign elements to phage genomes. A large bank of T4 mutants have already been constructed and exploited to facilitate mutating lytic phages; among that mutants there are ones without decorating (non-essential) capsid proteins: gpHoc or gpSoc. Deletions of that proteins enable incorporation of recombinant proteins which are fusions of gpHoc or gpSoc with foreign elements, e.g., active oligopeptides, proteins, etc.[9-14]

T4 phage has been shown to be a good display platform, since it has been modified successfully with extraneous proteins several times. Active anti-lysozyme IgG, domains of the HIV1-CD4 receptor, multicomponent anthrax toxin, and a peptide from *Neisseria meningitidis* (PorA) were fused to decorative capsid proteins Soc or Hoc and as a result displayed on the T4 capsid surface. These foreign elements retained their activity and/or immunogenicity required for anticipated biological applications.[9-14]

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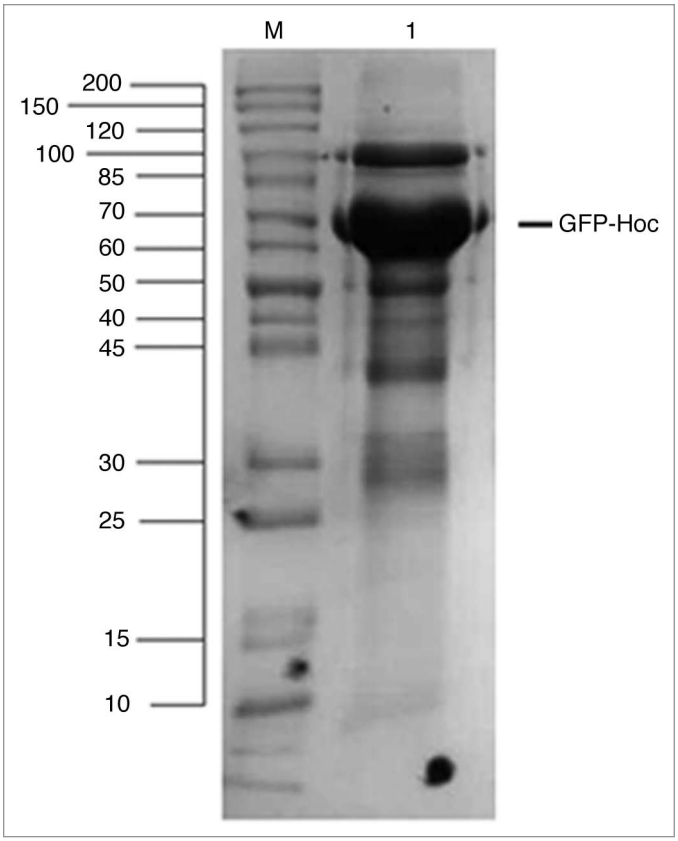

Figure 1. Expression of recombinant GFP-Hoc fusion in *E. coli*. Expression plasmids containing *GFP-hoc* fusion were tested for their effectiveness in production of GFP-Hoc proteins as showed by SDS-PAGE. M, marker; 1, induced GFP-Hoc expressing bacteria.

Bacteriophages decorated with GFP by modification of the phage genome have been previously proposed for detection of *E. coli* in the environment, i.e., in sewage water as well for the assessment of *E. coli* viability in the aquatic environment.[15,16] However, they have never been applied in studies of phage circulation or pharmacokinetics in mammals. Although many other biological objects have been successfully visualized in cells and tissues by GFP-labeling (e.g., proteins, bacteria, cancer cells), the utility of this method for phage detection in mammals has never been determined. Here we propose a new GFP-based method for T4 phage molecular imaging in living systems. The method employs decoration of a phage capsid with GFP fused to the N-terminus of Hoc protein by in vivo phage display. Fluorescent phages were assessed as regards their applicability for detection inside living mammalian cells (by phagocytosis) and tissues (filtering and retention by spleen and lymph nodes).

Results and Discussion

Previous studies of phage display on T4-like phages have shown that effective presentation of large foreign proteins can be done successfully by their fusion to the N-terminal part of decorative protein Hoc.[8,10] Thus, to ensure good exposition of GFP on the capsid, N-terminal fusion of GFP to Hoc was exploited

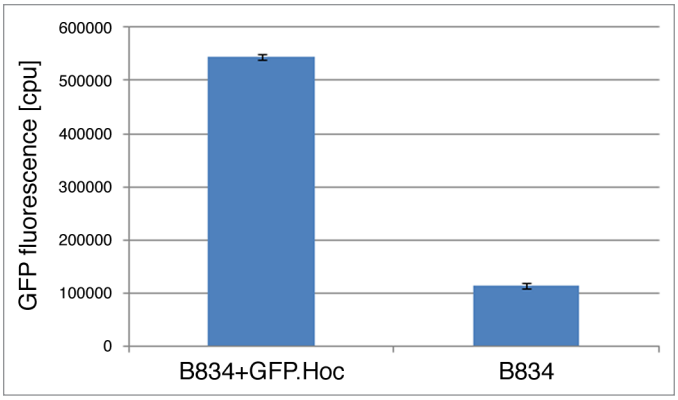

Figure 2. Fluorescence of GFP-Hoc fusion in *E. coli.* Effective fluorescence of the GFP-Hoc fusion was acuction and control *E. coli* (expressing protein Hoc without GFP).

in this study. In order to obtain functional *E. coli* clones expressing GFP-Hoc fusion, a new expression vector based on pDEST17 containing the *gfp-hoc* sequence was created. Expression was verified in *E. coli B834* strain; production of expected fusion was confirmed by SDS-page of the complete protein profile of induced expression bacteria (**Fig. 1**). Fluorescence of the produced GFP-Hoc fusion was further assessed by fluorescence measurement of induced expression bacteria; fluorescence of GFP-Hoc fusion expressing bacteria was significantly higher in comparison to the control ($P = 0.0253$) (**Fig. 2**).

E. coli B834 strain effectively expressing fluorescent GFP-Hoc fusion was used for propagation of HAP1 phage (T4 phage without protein Hoc, display platform).[17] The phage display was thus completed in vivo, in bacterial cells infected by the phage after induction for recombinant protein expression. Then, phages were separated from bacterial debris, also from GFP-Hoc fusions not incorporated into the phage capsid by size exclusion chromatography (**Fig. 3**), and dialyzed against PBS. The final phage titer of purified GFP-labeled phages was 1×10^{11} pfu per ml. Fluorescence of this preparation was significantly higher ($P = 0.0495$) than that of the control, which was an identically purified but non-modified phage (**Fig. 4**).

According to previous reports, macrophages are not crucial for the clearance of phages from blood, however they have been clearly shown as capable of phage phagocytosis.[18,19] In order to determine how GFP-labeled bacteriophages can be visualized in mammalian cells, fluorescent bacteriophages (GFP.Hoc.HAP1 phages) were incubated with murine macrophages (J774A.1). After the incubation, labeled phages were visualized inside the cells by fluorescent microscopy. Since 10 min of incubation did not result in visible uptake of the phages, while 20 and 40 min revealed an increasing fluorescent signal in the cells (**Fig. 5**), the amount of uptake was dependant on incubation time. This is in line with studies of the uptake of phages by rabbit macrophages performed by Aronow et al.[20] who used electron microscopy. These authors showed an increasing uptake of a phage by macrophages from approximately 7 min to 30 min. Intracellular destruction of ingested phages occurred some 2 h after their uptake.

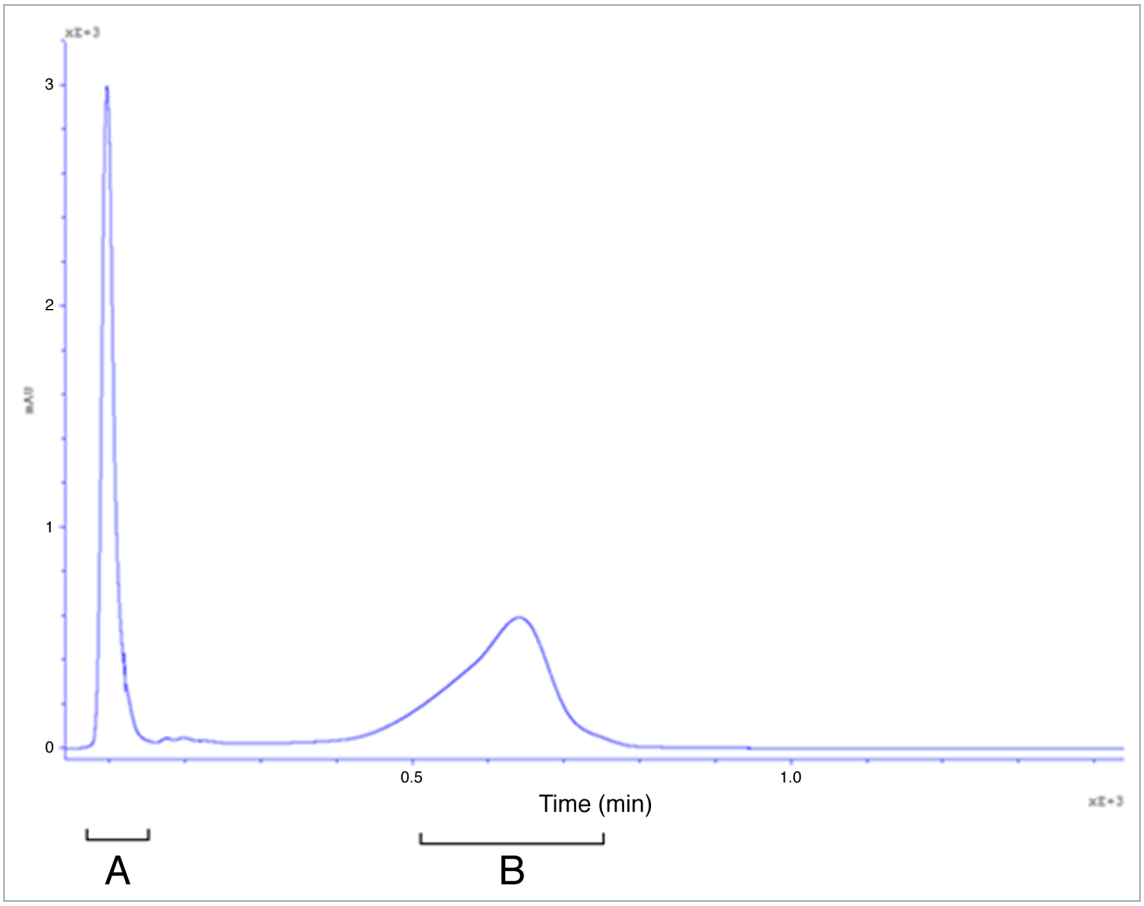

Figure 3. Separation of modified bacteriophages by *fast protein liquid chromatography.* (**A**) Fraction containing bacteriophages. (**B**) Fraction containing non-incorporated GFP-Hoc fusions and other proteins.

In order to determine if GFP-labeled bacteriophages can also be visualized in murine tissues, fluorescent phages were injected i.v. into the lateral tail vein. After 1.5 h, fluorescence of particular organs was detected in imaging hood, revealing a visible signal of GFP.Hoc.HAP1 phages in the spleen (**Fig. 6B**) and in the lymph node (**Fig. 6D**). In control mice injected with non-modified phages, only background signal from the tissues was visible (**Fig. 6A and C**), however much lower than that of the GFP labeled phage.

These results show that molecular imaging can be utilized for investigation of bacteriophages in living systems. Visualization can be done both in living mammalian cells and in tissues/organs, including quantitation of the results. This offers a good method for studies of time-dependent effects (like presented phagocytosis of bacteriophages) or multiple comparison of phage circulation aspects e.g., in tissues. T4-like phages can be effectively furnished with the popular biofluorescent marker GFP by phage display technique, engaging simple fusion of GFP to one of the decorative capsid proteins. Since this technique does not employ complex molecular or chemical modifications of the phage, we propose it as a useful tool for further studies of phage biology.

In previous studies of Mullaney et al.,[21] GFP was targeted into T4 heads as a probe of the internal environment. That approach offered an idea for the method of phage visualization (in vitro). Internal localization of a fluorescent marker makes Hoc protein available for other fusions, including fusions with targeting molecules or active compounds, which demonstrates a potential for developing visualization methods in the field of bacteriophage biology and applications.

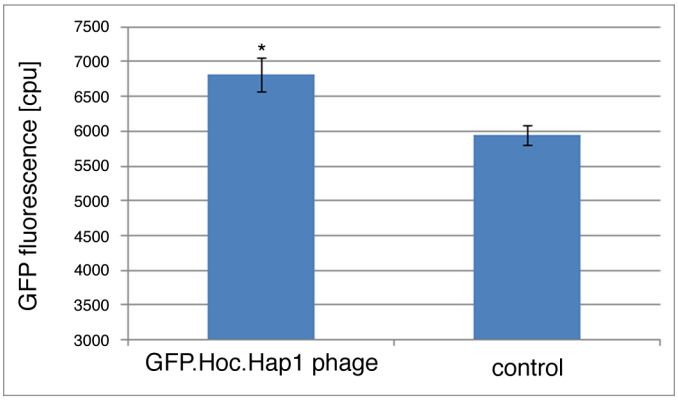

Figure 4. Comparison of fluorescence in GFP-modified and control phage. Effective fluorescence of the purified GFP.Hoc.HAP1 phages was compared with the fluorescence of HAP1 phage (without GFP, purified identically by FPLC).

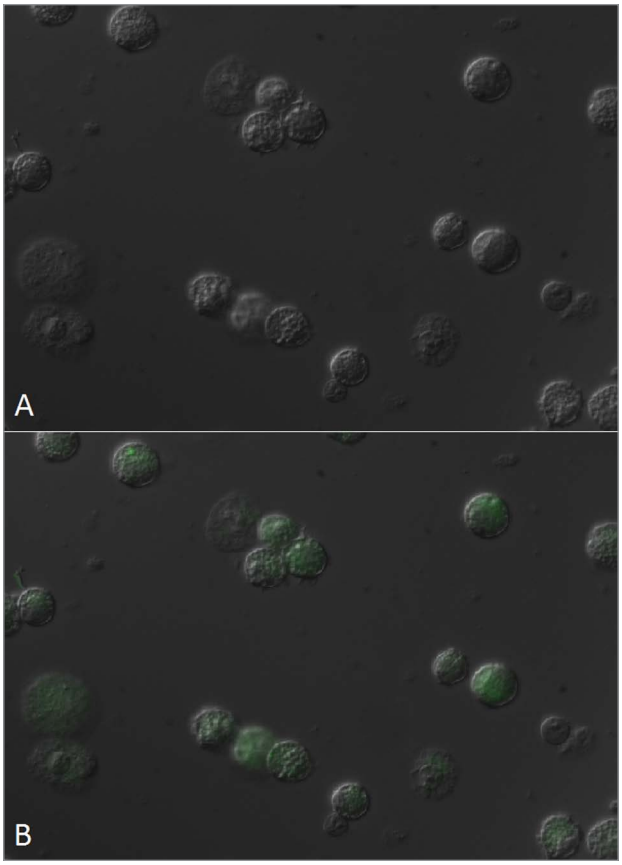

Figure 5. Visualization of GFP-modified bacteriophages in phagocytic cells J774A.1 (murine macrophages). Fluorescence of murine macrophage cell line was visualized in fluorescent microscopy. (**A**) control, phagocytes before incubation with GFP.Hoc.HAP1 phage, (**B**) phagocytes after 40 min incubation with GFP.Hoc.HAP1 phage.

Visualization of bacteriophages in living systems can be a potent tool for progress in bacteriophage therapy, in the time of the insufficient antibacterial drugs arsenal. Easy observation and quantification of phage deposition in particular tissues and organs may induce progress in determination of phage dosage, timing regiments and other aspects of phage pharmacokinetics, which are critical for efficacy and safety of phage therapy. Thus, this novel technique may contribute to the development of new alternatives in antibacterial treatment.

Materials and Methods

Construction of *gfp-hoc* fusion in the expression vector

For gene cloning, a combination of Gateway recombination cloning technology (Invitrogen, Life Technologies Corporation) and a standard restriction/ligation approach was applied. Three steps allowed construction of expression plasmids: i) construction of an entry clone in a non-expression vector, pDONR™221 (a pUC origin and universal M13 sequencing sites, kanamycin resistance) (Invitrogen, Life Technologies Corporation, http://tools.invitrogen.com/content/sfs/vectors/pdonr221_pdonrzeo_map.pdf) by introduction of the *hoc* gene and restriction sites at the 5′-terminus of the gene, then ii) introduction of the *gfp* gene into an entry clone using of restriction sites, and iii) transfer of the coding fragment (*gfp-hoc* fusion) to a destination expression vector, pDEST17 (Invitrogen, Life Technologies Corporation, http://tools.invitrogen.com/content/sfs/vectors/pdest15_map.pdf) for production of the recombinant proteins. Recombinant engineering (i and iii) was done according to the Gateway technology manual. Both the *hoc* gene and the *gfp* gene were cloned into the vector as PCR products. Recombination sites (*hoc*) or restriction sites (*gfp*) that allowed incorporation of the products into the vector were introduced with PCR primers (hoc forward primer: GGCAAAGTTT GTACAAAAAA GCAGGCTCCC GGGAAAGAA TTCATGACTT TTACAGTTGA TATAACTC, hoc reverse primer: GGGGACCACT TTGTACAAGA AAGCTGGGTC CTATGGATAG GTATAGATGA TACC gfp forward primer: ACCCGGGAAA ACCTGTATTT TCAGGGCAGC AGCAGCATGA GTAAAGGAGA AGAACTTTTC, gfp reverse primer: AAAGAATTC**G CTTGGGGCGC TTGGGGC**TTT GTATAGTTCA TCCATGCCAT GTGTAATCC). The high solubility motif APSAPS was introduced between GFP and Hoc by a relevant sequence in the *gfp* reverse primer (marked in bold). Polymerase chain reaction (PCR) was performed on a template of T4 total DNA (*hoc*) or pcDNA-DEST53

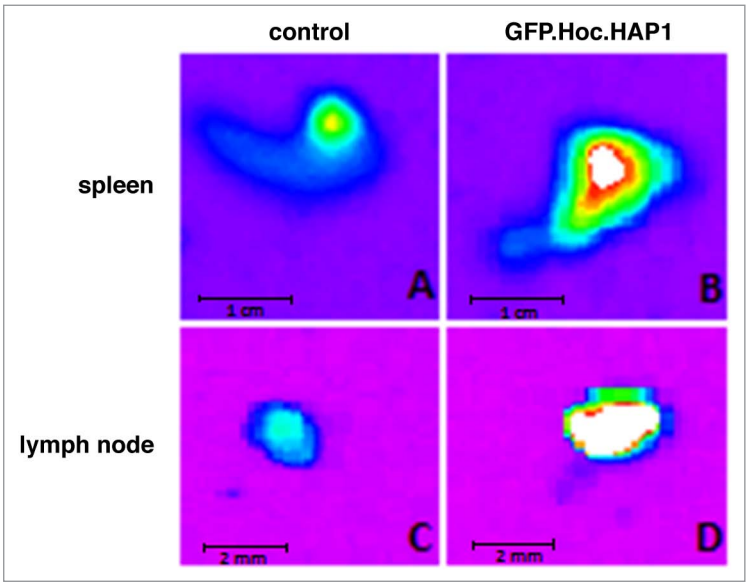

Figure 6. Visualization of GFP-modified bacteriophages in murine tissues. GFP.Hoc.HAP1 phages and non-modified HAP1 phages (control) were injected into murine lateral tail vein (10^{11} pfu/mouse). Fluorescence of spleen and lymph nodes was visualized in an imaging hood. (**A**) spleen of mice injected with non-modified HAP1 phage, (**B**) spleen of mice injected with GFP.Hoc.HAP1 phage, (**C**) lymph node of mice injected with non-modified HAP1 phage, (**D**) lymph node of mice injected with GFP.Hoc.HAP1 phage.

(Invitrogen, Life Technologies Corporation) (*gfp*). PCR products of the *hoc* gene were introduced into the pDONR221 using BP Clonase™ II Enzyme Mix (Invitrogen, Life Technologies Corporation) according to the manufacturer's instructions. Constructions were verified by automated Sanger sequencing with standard M13 primers using a 3730 DNA Analyzer, Applied Biosystems, Hitachi, DNA Sequencing KitBig Dye™ Terminator Cycle Sequencing version 1.1 (Oligo, Institute of Biochemistry and Biophysics, Warsaw, Poland). The apropriate clones were used for restriction/ligation with *gfp* PCR products; restriction enzymes: EcoRI and SmaI (FastDigest, Fermentas) and ligase (LigaFast, Promega) were used. Constructions were verified by automated Sanger sequencing with standard M13 primers using a 3730 DNA Analyzer, Applied Biosystems, Hitachi, DNA Sequencing KitBig Dye™ Terminator Cycle Sequencing version 1.1 (Oligo, Institute of Biochemistry and Biophysics, Warsaw, Poland). Proper fusions of the genes (*gfp-hoc*) were transferred by recombination to the expression vector pDEST17 (Invitrogen, Life Technologies Corporation) in the LR reaction, according to the manufacturer's instructions.

Expression clones were tested in *E. coli* B834(DE3) F^- ompT $hsdS_B(r_B^- m_B^-)$ gal dcm met (DE3) (EMD, Europe); the strains were checked for their ability to express the recombinant proteins *gfp-hoc* as follows: they were cultured with intensive aeration in LB high salts (10 g/l of NaCl) culture medium (Sigma-Aldrich, Europe or AppliChem, Europe) with appropriate selection antibiotics (ampicillin) at 37 °C and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.2 mM) in the exponential growth phase as determined by OD_{600} measurements (usually at OD_{600} = 0.8). Further expression was conducted overnight at 25 °C. Bacteria were harvested by centrifugation (6000 rpm, 5 min) and analyzed by SDS-page and fluorescence measurement in a multilabel plate reader (EnSpire, Perkin Elmer).

Phage display

As a display platform, HAP1 phage from the IIET Microorganisms Collection was used; HAP1 is a mutant of T4 phage (American Type Culture Collection, USA) with a nonsense mutation in the *hoc* gene with no functional Hoc.[22]

Bacterial cells were transformed with an expression vector containing *gfp-hoc* fusion. Transformed bacteria were grown at 37 °C in Luria broth (LB) with ampicillin as a selection antibiotic until optical density (OD_{600}) 0.7 was reached. Next they were transferred to fresh LB medium containing 0.2 mM IPTG; IPTG was an expression inductor. After induction of protein expression in bacteria, 10^6 pfu HAP1 (in 1 ml volume) was added. This infected culture was incubated at 37 °C for 8 h. Next the culture was incubated for 3 d at 4 °C. Lysates were filtered by 0.22 μm sterile syringe filters. Phage titer was determined by Routine Test Dilution (RTD).[23] Phage modified with GFP by phage display was denominated: "GFP.Hoc.HAP1 phage".

Purification of the GFP-modified phages (Fast protein liquid chromatography, FPLC)

GFP.Hoc.HAP1 phages were purified by size-exclusion chromatography as described by Boratyński et al.[24]; briefly, the suspension was chromatographed on a sepharose 4B column (2.5 × 95 cm, eluent 0.063 M phosphate buffer, pH 7.2, flow 0.3 ml min^{-1}). Bacteriophages were eluted in the highest molecular weight fraction, as confirmed by fraction titration. Phages were filtered by 0.22 μm sterile syringe filters. Phage titer was determined by RTD.[23]

Additionally, fluorescence of subsequent fractions was assessed in an imaging hood: In-Vivo MS FX PRO (Carestream Molecular Imaging). The GFP-displaying phage (GFP.Hoc.HAP1) fraction, other fractions resulting from FPLC, and a control phage (HAP1) were dropped into the detection area and analyzed (examined volume: 10 μl, excitation: 470 nm, detection: 535 nm). intensity of fluorescence was normalized as numerical data by Carestream Imaging software.

Phage imaging in living cells and tissues

J774A.1 cells (cell line, murine macrophages, ATCC collection) were incubated at 37 °C with GFP.Hoc.HAP1 phage for 10, 20, or 40 min (10^6 pfu per 10^6 cells), washed with PBS three times, and analyzed. Detection of the signal was performed in a fluorescent Fully Automated Inverted Research Microscope for Biomedical Research: Leica DMI6000B.

Female C57/Bl6 (8 wk) mice were purchased from the Center of Experimental Medicine, Medical University of Bialystok, and kept in specific pathogen free (SPF) conditions in the Animal Breeding Centre of the Institute of Immunology and Experimental Therapy (IIET). All experiments were approved by the 1st Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland (no. 64/2009).

Mice were injected intravenously (i.v.) with GFP.Hoc.HAP1 phage 2×10^{11} pfu per mouse, control mice were injected i.v. with the phage without GFP protein (HAP1) 2×10^{11} pfu per mouse. 90 min later mice were sacrificed and lymph nodes were excised. Imaging was performed in In-Vivo MS FX PRO (Carestream Molecular Imaging).

Statistical methods

ANOVA, Mann-Whitney U test, and the Statistica 6.0 software packages were applied (www.statsoft.pl).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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