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ScienceDirect
Microbes and Infection 8 (2006) 2539–2546
Microbes and Infection
www.elsevier.com/locate/micinf

Original article

Prophylactic anti-tumor immunity against a murine fibrosarcoma triggered by the *Salmonella* type III secretion system

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Received 9 May 2006; accepted 10 July 2006
Available online 1 August 2006

Abstract

The potential of an attenuated *Salmonella enterica* serovar Typhimurium strain as a prophylactic anti-tumor vaccine against the murine fibrosarcoma WEHI 164 was evaluated. Tumor cells were transfected with the DNA sequence encoding the MHC class I-restricted peptide $p60_{217-225}$ from *Listeria monocytogenes*. BALB/c mice received a single orogastric immunization with *Salmonella* that translocates a chimeric p60 protein via its type III secretion system. Mice were subsequently challenged subcutaneously with $p60_{217-225}$-expressing WEHI cells. In vivo protection studies revealed that 80% of these mice remained free of the fibrosarcoma after challenge, whereas all animals of the non-vaccinated control group did develop tumor growth. In further experiments, the distribution of tetramer-positive $p60_{217-225}$-specific effector and memory CD8 T cells after *Salmonella*-based immunization and tumor application was analyzed. Costaining with CD62L and CD127 revealed a predominance of p60-specific central memory and effector memory CD8 T cells in spleens, whereas in blood samples the majority of p60-specific lymphocytes belonged to effector and effector memory CD8 T cell subsets. This is the first report demonstrating that a bacterial type III secretion system can be used for heterologous antigen delivery to induce cytotoxic effector and memory CD8 T cell responses resulting in an efficient prevention of tumor growth.

Keywords: *Salmonella enterica* serovar Typhimurium; Type III secretion system; Tumor vaccine; CD8 T cell; Memory T cell

1. Introduction

Attenuated recombinant *Salmonella enterica* serovar Typhimurium has emerged as a promising delivery system for foreign vaccine antigens [1]. Upon close contact with the eukaryotic cell, a type III secretion system (T3SS) encoded by the ''*Salmonella* pathogenicity island 1'' mediates *Salmonella* invasion of the host cell, where the bacterium resides within *Salmonella*-containing vacuoles. The T3SS is designed to translocate *Salmonella* type III effector proteins directly into the cytosol of target cells [2]. Our laboratory has focused its research on the genetic manipulation of attenuated *Salmonella* strains to endow them with the ability for efficient induction of major histocompatibility complex (MHC) class I-restricted immune responses [3,4]. We have developed a new vaccination strategy by using the *Salmonella*-T3SS to

Abbreviations: IFN-γ, interferon-gamma; MHC, major histocompatibility complex; T_E, effector CD8 T cells; T_{EM}, effector memory CD8 T cells; T_{CM}, central memory CD8 T cells; T3SS, type III secretion system; YopE, *Yersinia* outer protein E.

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doi:10.1016/j.micinf.2006.07.004

translocate antigens from microbial pathogens directly into the cytosol of antigen-presenting cells. In a first approach, an immunodominant CD8 epitope of the murine lymphocytic choriomeningitis virus was inserted between two functional domains of a translocated *Salmonella* type III effector protein [3]. Mice orally vaccinated with an attenuated *Salmonella* strain expressing this hybrid protein were protected against a lethal viral infection. In further studies, the immunodominant listerial p60 antigen was fused to the defined N-terminal translocation domain of the *Yersinia* outer protein E (YopE). Translocation and cytosolic delivery of the chimeric YopE/p60 protein into macrophages led to efficient MHC class I-restricted antigen presentation of the p60 nonamer peptide $p60_{217-225}$ [4]. As determined by enzyme-linked immunospot assay, mice orally vaccinated with a single dose of attenuated *Salmonella* expressing translocated YopE/p60 protein revealed high numbers of interferon-gamma (IFN-γ)-producing CD8 T cells reactive with $p60_{217-225}$. These T lymphocytes conferred protection against a challenge with the intracellular pathogen *Listeria monocytogenes*.

The use of *Salmonella*'s T3SS to induce antigen-specific cytotoxic T cells might be also an attractive strategy to develop vaccines for the immunoprophylaxis of tumors. CD8 T cells have been shown to contribute to the eradication of solid tumors [5,6]. Many experimental tumor systems have utilized tumors transfected with model antigens such as ovalbumin [7] or immunogenic viral components [8,9]. In the present study, we wanted to evaluate the potential of *Salmonella*'s T3SS to induce a CD8 T cell-mediated anti-tumor immunity against an aggressive fibrosarcoma transfected with $p60_{217-225}$.

2. Materials and methods

2.1. Plasmids, bacterial strains, and growth conditions

The construction of plasmid pHR241 has been outlined in detail [4]. This low-copy-number expression vector bears the genetic information for the translocated chimeric $YopE_{1-138}$/$p60_{130-477}$/M45 fusion protein under expression control of the *lac* promoter. M45 is derived from the E4-6/7 protein of adenovirus and its use for chimeric protein tagging has been described [4].

2.2. Stable transfection of fibrosarcoma cell line

To obtain WEHI-p60 cells, the H-2^d fibrosarcoma WEHI 164 (ATCC # CRL-1751), a methylcolanthrene-induced tumor of BALB/c origin, was transfected with a double-stranded synthetic oligonucleotide (oligonucleotide A, 5′-CCGGT GCCACCATGAAATACGGTGTTTCTGTTCAAGACATTTG AG-3′; oligonucleotide B, 5′-GATCCTCAAATGTCTT GA ACAGAAACACCGTATTTCATGGTGGCA-3′) encoding the $p60_{217-225}$-epitope which was inserted into the mammalian expression vector pIRESneo3 (BD Biosciences, Heidelberg, Germany). Cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, 2-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μg/ml), and G418 (400 μg/ml) (Sigma, Deisenhofen, Germany).

2.3. Generation and purification of H2-K^d tetramers

Tetrameric H2-K^d/$p60_{217-225}$ complexes were generated as previously described [10]. Briefly, recombinant H2-K^d heavy chain and β_2-microglobulin were expressed as insoluble inclusion bodies in *Escherichia coli* and were further purified. The H2-K^d heavy chain molecule was mutated to remove the transmembrane and cytosolic domain and to add a specific biotinylation site at the C-terminus. Purified proteins were refolded in vitro in the presence of high concentrations of synthetic peptides (Biosythan, Berlin, Germany) to form stable and soluble MHC/peptide complexes. Complexes were specifically biotinylated in vitro by adding the enzyme BirA, d-biotin, and ATP. After further purification, biotinylated MHC/peptide complexes were multimerized with streptavidin-PE (SA-PE; Molecular Probes, Eugene, USA). Tetrameric complexes were purified by gel filtration and stored at 2–5 mg/ml at 4 °C in phosphate-buffered saline (pH 8.0) containing 0.02% sodium azide, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 0.5 ml EDTA.

2.4. In vitro antigen presentation assay

A CD8 T cell line specific for $p60_{217-225}$ was derived from *L. monocytogenes*-infected BALB/c mice and propagated by repeated restimulation in the presence of 1×10^{-9} M $p60_{217-225}$ as described previously [11]. The detection limit of the CD8 T cell line was between 10^{-11} and 10^{-12} M peptide. T cell activation by WEHI-p60 cells was measured by the detection of IFN-γ in culture supernatants. Between 1×10^5 and 1×10^2 p60-expressing antigen-presenting cells were seeded in 96-well round-bottom microwell plates. For the peptide control 1×10^5 non-transfected WEHI 164 cells were loaded for 1 h with graded amounts of $p60_{217-225}$. Per well 3×10^4 T cells were added and after 12–18 h at 37 °C supernatants were harvested and the IFN-γ concentration was measured in an IFN-γ-specific enzyme-linked immunoassay that binds and detects IFN-γ with a pair of specific monoclonal antibodies. Results were corrected for dilution of the sample to yield the sample concentration in ng/ml.

2.5. Mice and orogastric immunization with recombinant Salmonella

Female BALB/c mice, 6–8 weeks old, were kept under specific-pathogen-free conditions. Groups of five mice were orogastrically immunized with a single dose of 5×10^8 colony-forming units of *Salmonella* strain SB824 or SB824 (pHR241) expressing YopE/p60. The respective bacterial suspensions were delivered by round bottomed feeding needles. Animal experiments were repeated at least twice with similar results.