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Generation of an immune microenvironment as a novel mechanism for myotoxins to potentiate genetic vaccines

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ABSTRACT

We recently reported that administration of low doses of myotoxins at vaccination sites potentiated antigen-specific T-cell immunity induced by genetic cancer vaccines in mice, an effect which was superior to TLR agonists. In the current study, we found unexpectedly that the mechanism of this potent adjuvant effect was immune-mediated. Myotoxins induced sterile inflammation at vaccination sites, associated with a predominant infiltration of dendritic cells (DC). Inhibition of DC recruitment abrogated the immune stimulation effect of myotoxins, suggesting the requirement for DC. Genetic profiling of myotoxin-treated tissues revealed characteristics of an immune microenvironment with up-regulation of chemokines, proinflammatory cytokines, Toll-like receptors (TLR) and their endogenous ligands, and activation of innate immunity. Mechanistic experiments in vivo also elucidated the requirement for genes triggering DC maturation including TLR signaling and CD40. These studies suggest that myotoxins-induced sterile inflammation generates a favorable microenvironment that promotes multiple stages in the development of adaptive immunity. This novel mechanism of immune potentiation may be exploited for development of adjuvants for genetic vaccines against infectious pathogens and cancer.

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1. Introduction

The idea of genetic vaccination originated from the observation that injection of DNA into living animals resulted in expression of gene products in vivo [1–3]. Preclinical studies revealed that genetic immunization induced both antigen-specific antibody and cytotoxic T lymphocyte [4,5]. Early human trials of DNA vaccines have been mainly focused on infectious disease, and vaccines against malignant disease have activated anti-tumor immunity in cancer patients and extended remission [6–9]. Compared with proteins or peptides, genetic vaccines are highly appealing. The antigen synthesized in host cellular machinery is naturally processed by antigen presenting cells (APC) to release all potential antigenic epitopes for priming antigen-specific immunity, avoiding HLA restriction that is absolutely required for peptide vaccine. Moreover, simplicity in large-scale production and purification makes DNA vaccines more attractive than protein. However, the goal of using DNA vaccines as a clinical therapy has not yet been achieved. The major obstacle preventing general use of DNA vaccines on patients is that the plasmid DNA, especially those encoding non-immunogenic, tumor-associated self-antigens, is relatively weak in inducing immunity. Developing an efficient strategy to enhance immunogenicity is therefore vital for translational vaccine development.

An immune adjuvant is a substance that is used to improve the efficiency of vaccine. It is usually designed to boost a critical step in the development of adaptive immunity. The downstream effects of adjuvant can be linked to enhancing the function of dendritic cells (DC) in antigen uptake and presentation. Examples include chemokines for DC recruitment [10], Toll-like receptor (TLR) agonists for DC maturation [11,12], and CD40 ligand and interferon-α which enhance T-cell activation [13,14]. Given that development of adaptive immunity includes a series of sequential events, it can be speculated that a strategy that targets multiple steps in activation of adaptive immunity would be more beneficial than an individual adjuvant in improving the potency of vaccine.

Many adjuvant candidates such as inflammatory chemokines, TLR ligands and interferon α, are the important elements of innate immune system. Based on this concept, we hypothesized that inducing an innate immune response at vaccination sites could

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create an immune microenvironment containing the required elements to facilitate vaccine-triggered adaptive immunity. For example, tissue damage may present as an alternative pathway leading to innate immunity. This idea is supported by the observation that endogenous ligands of toll-like receptors are released following tissue necrosis and activate various cellular elements of the innate immune system [15]. Hence, we reasoned that induction of controlled tissue necrosis prior to vaccination might lead to activation of innate immune system and potentiate the adaptive immune responses resulting from the vaccination. Myotoxins including cardiotoxin, especially at low doses, cause local tissue damage that is quickly resolved by muscular regeneration [16]. When combined with a lymphoma idiotype DNA vaccine as shown in our previous report, they considerably potentiated vaccine-induced T-cell immunity and tumor protection [17]. Lymphoma idiotype is a tumor-specific antigen. The vaccine derived from lymphoma idiotype protein elicited tumor-specific immune responses [7] and prolonged disease-free survival in patients with follicular lymphoma [9]. Our current mechanistic study showed that cardiotoxin produced a favorable immune microenvironment at vaccination sites, triggering the recruitment and activation of antigen-presenting cells for T-cell priming. Hence, we describe a novel, immune potentiating mechanism for myotoxins to improve the immunogenicity of genetic vaccines by generating an inflammatory microenvironment at vaccination sites.

2. Materials and methods

2.1. Mice

Balb/c and C57/BL mice were obtained from the National Cancer Institute. Interleukin 1β receptor-deficient and CD40-deficient mice were purchased from Jackson Laboratories. Myd88-deficient mice were bred in house. The mice were maintained in a pathogen-free mouse facility according to institutional guidelines. The protocol of vaccination studies on mice was approved by the Institutional Animal Care and Use Committee at The University of Texas M.D. Anderson Cancer Center.

2.2. Vaccination

Plasmid constructs encoding A20 idiotype single chain (sFv) fused with monocyte chemotactic protein-3 (MCP3) was previously generated in the lab [18–20]. Chicken ovalbumin (Ova) DNA vaccine was made by genetic fusion of MCP3 gene with the full-length Ova cDNA that was cloned from the total RNA of Ova-expressing B16 melanoma cells using RT-PCR approach. All the plasmid DNA used in this study was scaled up by Aldevron, LLC (Fargo, ND). For vaccinating mice, a dose of 50 μg plasmid DNA dissolved in 50 μl PBS was injected in quadriceps. A total of three rounds of vaccination were given with a two-week interval. During the first two rounds quadriceps was injected with 6.8 μg cardiotoxin in an injection volume of 100 μl (Celtic Biotec) 5 days before vaccination. Two weeks after final vaccination, a lethal dose of 2×10^5 A20 lymphoma cells was given by i.p. Tumor development was closed monitored, and data were statistically analyzed by using the Kaplan-Meier method with a log-rank *P* value.

2.3. Immunohistochemistry

Quadriceps treated with 6.8 μg Cardiotoxin for 1, 3, 5 and 7 days respectively, were isolated for either paraffin fixation or cryo-fixation. The paraffin-fixed tissue sections were stained for hematoxylin and eosin to identify cellular infiltration. The cryo-sections were immunostained for antibodies for neutrophils (Gr-1), monocytes or macrophages (F4/80), dendritic cells (CD11c), B cells (B220), and T cells (CD3). The tissue samples were examined under a 20×/0.40 PH1 objective of a Leica DMLB microscope (Meyer Instruments Inc., Houston, TX) equipped with a SPOT RT color camera 2.2.1 (Diagnostic Instruments Inc. Sterling Heights, MI). Images were captured using SPOT Advanced software 4.7.

2.4. Ova-tetramer staining

The peripheral blood samples were collected from tail veins 10 days after vaccination of mice with 50 μg MCP3-Ova plasmid DNA by i.m. on cardiotoxin-treated or untreated quadriceps. In some cases, mice were given i.m. with 100ng Diphtheria toxin (Sigma, St. Louis, MO) on cardiotoxin-treated muscles the day before vaccination for depleting cardiotoxin-induced DC infiltration at vaccination sites. The blood samples were treated with ACK lysis buffer to remove red blood cells, and stained with antibodies including CD3-allophycocyanin, CD8-fluorescein isothiocyanate (BD Biosciences, San Jose, CA) and $Ova_{(257–264)}$ H-$2K^b$ tetramer-phycoerythrin (Beckman Coulter, Miami Fl). The data was obtained on BD FACSCalibur flow cytometer and analyzed using FlowJo 7.2.5 software.

2.5. Microarray

A Qiangen RNeasyMicroKit was used to isolate the total RNA from cryo-preserved muscular tissues. The RNA pooled from groups of 10 quadriceps treated with cardiotoxin for 1, 3 and 5 days, respectively as well as untreated muscles were used for synthesis of double strand cDNA. The quality control of RNA samples and the microarray experiment using Mouse 430 2.2 Affymetrix GeneChips were conducted by Expression Analysis (Durham, NC). Targets were prepared as per Affymetrix Technical Manual. The data were normalized to untreated muscular tissues. Differential expression was determined by Affymetrix Pairwise comparison via GeneChip Operating system (GCOS). The microarray data of selected genes was presented as a Java Treeview file.

3. Results

3.1. Pre-treatment of vaccination sites with myotoxins potentiated adaptive T-cell responses to two model antigens

We previously described a DNA vaccine which encodes lymphoma idiotype antigen (sFv) fused with monocyte chemotactic protein-3 (MCP3-sFv). This clinically relevant vaccine elicited $CD8^+$ T cell-dependent protective and therapeutic anti-lymphoma immunity in mice [18,19]. In our recently published study, we observed that particularly MCP3-sFv fusion DNA vaccine-induced tumor protection was significantly improved by pre-treating the vaccination sites with low dose cardiotoxin [17]. Cardiotoxin pre-treatment was also an effective strategy to covert a less immunogenic tumor antigen into a protective tumor vaccine, as even vaccination of mice with plasmid DNA encoding a unfused sFv with cardiotoxin, but not without, protected mice from lethal tumor challenge (log-rank $P = 0.04$), although the level of protection was less than that induced by the fusion vaccine (Fig. 1A).

Given the technical challenge of quantifying idiotype-specific $CD8^+$ T-cell immunity, we used Ova model antigen to explore the mechanism that cardiotoxin acts as an immune adjuvant. Similarly, antigen-specific T-cell responses to Ova were significantly enhanced by combination of DNA vaccine encoding MCP3-Ova with cardiotoxin, compared MCP3-Ova DNA vaccine alone (Fig. 1B). Overall, compared with $1.39 \pm 0.28\%$ $Ova_{(257–264)}$ H-$2K^b$ tetramer-positive $CD8^+$ peripheral blood T cells elicited by MCP3-Ova DNA vaccination, the cardiotoxin-combined strategy elicited an average