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Vaccine draining lymph nodes are a source of antigen-specific B cells

Stephanie C. Pero [a], Yu-Jing Sun [a], Girja S. Shukla [a], Chelsea L. Carman [a], Christopher C. Krag [a], Cory Teuscher [b], Dimitry N. Krementsov [b], David N. Krag [a,*]

[a] Department of Surgery, Vermont Cancer Center, University of Vermont College of Medicine, 89 Beaumont Avenue, Given Building E310, Burlington, VT 05405, USA
[b] Department of Medicine, University of Vermont College of Medicine, 89 Beaumont Avenue, Given Building C329, Burlington, VT 05405, USA

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ABSTRACT

Purpose: Our research is focused on using vaccine draining lymph nodes as a source of immune cells to better understand the immune response and to attempt to generate new anti-cancer reagents. Following a vaccine, harvesting the lymph node can only be done once. We endeavored to determine the range of times that B cells secreting anti-KLH antibodies were present in the node of KLH-vaccinated mice.
Results: Following vaccination the total number of mononuclear cells (MNCs) increased in the vaccine-draining lymph node (VDN). The percentage of MNCs that were B cells nearly doubled. B cells recovered from the node that secreted anti-KLH antibodies were evident by day 7. The number continued to increase and then slowly decreased over the observed time range to 28 days after vaccination. The VDN, compared to the spleen, the bone marrow and the nonVDN, contained a higher percentage of B cells that secreted anti-KLH antibodies.
Conclusions: After a vaccine, there is a multi-week window of time when an increasing number of B cells are present in a VDN that secrete anti-KLH antibodies. These results support using the VDN as a source for B cells that secrete anti-vaccine antibodies.

1. Introduction

Our research is focused on using vaccine or tumor draining lymph nodes as a source of immune cells to better understand the immune response and to attempt to generate new anti-cancer reagents. Interstitial injection of antigen or antigen released from a tumor is absorbed into lymphatic ducts and transported to regional lymph nodes. It is in the lymph node that antigen is processed and the initial immune response occurs. The development of lymphatic mapping with radioactive tracers allows precise localization of the draining lymph nodes from almost any tissue in the body [1,2]. Lymphatic mapping can also be used to identify the precise lymph node receiving drainage from a vaccine and guide removal of the draining node [3]. The ability to reliably identify the vaccine draining node (VDN) with precision and remove it with minimal morbidity creates opportunities to investigate the properties of immune cells within the lymph node that have presented and processed the antigen.

Systemic amplification of the immune response is an important component of multiple immunotherapy strategies including checkpoint inhibitors and vaccines. An alternative to systemic amplification of components of the immune system is ex vivo amplification followed by reinfusion. Indeed, the development of adoptive T cell therapy was enabled when it became possible to significantly expand autologous T cells outside of the human body [4,5]. The strategy of ex vivo amplification of autologous T cells has shown some of the most dramatic responses in human cancer patients [6]. B cells do not grow in culture as well as T cells and consequently has been less well studied as a potential therapeutic modality. However, B cells, unlike T cells offer the opportunity of reinfusion of antibodies rather than reinfusion of expanded cells.

The rationale for using autologous antibodies is based on experimental evidence demonstrating the capacity of autologous antibodies to inhibit or eradicate tumors. Vaccine studies in animals using selected tumor antigens [7,8] or tumor cell lysates [9] have demonstrated the contribution of vaccine-stimulated anticancer antibodies. These animal studies demonstrated that antibodies stimulated by a vaccine could be recovered from the serum and when transferred to a next animal had antitumor activity. A study in humans used an innovative approach to evaluate the antitumor effect of elevated levels of autologous antibodies [10]. Patients were first vaccinated with autologous tumor and Freund's adjuvant. In patients with elevated antitumor serum antibodies, blood was collected and the antibody fraction concentrated. The concentrated autologous antibodies were injected into and around

* Corresponding author.
E-mail address: David.Krag@uvm.edu (D.N. Krag).

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subcutaneous metastatic nodules. All nodules injected with the concentrated autologous antibodies decreased in size. More than half of the nodules became nonpalpable within 10 days. Biopsy demonstrated central tumor necrosis, a rim of nonviable tumor cells and no histologic evidence of normal tissue destruction. The authors acknowledged the inability to increase autologous antitumor antibodies systemically to high levels and designed their experiment to determine the effect of increased antibody concentration on a tumor. Although this is an older study it was well controlled and saline or nonspecific immunoglobulin injected into subcutaneous nodules had no effect.

Generating antibodies from B cells ex vivo will benefit from having a good starting set of B cells that have responded to a vaccine. The VDN may provide this source of B cells. Using viral-induced immortalization, phage display or hybridoma methods, anti-tumor antibodies have been generated from tumor draining nodes [11–13] and vaccine draining nodes [14]. The focus of these types of studies has mostly been to derive an antibody that can be used to treat many patients or to identify targetable antigens. Although antibodies have been generated from cells recovered from lymph nodes using a variety of methods, there is a paucity of information on the presence and timing of antibody secreting cells in a lymph node following a vaccine.

Following a vaccine, harvesting the VDN can only be done once. We endeavored to demonstrate the presence of B cells in VDNs that secrete anti-vaccine antibodies and determine the range of times following KLH vaccine that B cells secreting anti-KLH antibodies were present in the VDN. As a robust and well-studied experimental antigen, KLH was chosen for the vaccine. Preliminary work with KLH and the potent Freund's adjuvant indicated that a single vaccine resulted in elevated serum titers to KLH. This indicated that a response was likely present in the VDN. Our goal here was not to maximize the serum antibody response but to use experimental conditions that would reliably and relatively simply induce a response in the VDN.

We describe here the time course of B cells in VDNs to respond to KLH vaccine. A multi-week window of time was identified where B cells were recovered that secreted anti-KLH antibodies. The response in the VDN was compared to other tissues containing B cells. The percent of B cells in the VDN that secrete anti-KLH antibodies was higher during all time points compared to B cells recovered from other tissue sources. These data support the strategy of using the VDN as a source of B cells for ex vivo generation of anti-vaccine antibodies.

2. Materials and methods

2.1. Animals

Female 7–8 week old BALB/c mice (Strain 028, Charles River, Wilmington, MA) were allowed to acclimate to our animal facility before starting the vaccinations. Mice were maintained on a 12-h light/12-h dark cycle and received food and water ad libitum. The University of Vermont Institutional Animal Care and Use Committee approved all animal procedures.

2.2. Vaccination

The animals were immunized with KLH (Sigma, St. Louis, MO) in Freunds Complete Adjuvant (Thermo Fisher Scientific, Waltham, MA) through one subcutaneous injection consisting of 50 μL KLH (1.2 mg) + 50 μl adjuvant delivered through a 25G needle. The location of injection was on the top of the right flank because this location reliably drains to a single lymph node located in the ipsilateral inguinal lymph node [15]. Mice were euthanized by CO_2 inhalation. Tissues and blood were obtained after 3, 5, 7, 14, 21, and 28 days after vaccination. Each time point consisted of four mice per group.

2.3. Tissue processing

Tissues were harvested in a sterile manner immediately after sacrificing the mouse. Harvested tissues included the VDN, a nonVDN on the contralateral side of the mouse, bone marrow, and spleen. The tissues were placed between two pieces of nylon mesh (150 μm) and gently mashed with the rubber end of a syringe plunger. The disaggregated cells were rinsed in RPMI-1640 (Hyclone, Thermo Fisher Scientific) containing 10%FBS (Sigma)/1X Penicillin-Streptomycin (Cellgro, Thermo Fisher Scientific)/50 mM 2-mercaptoethanol (Invitrogen, Carlsbad, CA), referred to as R10 media. Blood was collected from the sacrificed mouse by cardiac puncture and transferred to a 1.5 mL centrifuge tube containing EDTA to prevent clotting. Blood was centrifuged at 1000*g* for 10 min to separate the plasma and cells. The plasma was stored in aliquots at −20 °C. Bone marrow was extracted from the femoral bone by cutting both ends and flushing the marrow twice using 3 mL PBS containing 0.5% bovine serum albumin and 2 mM EDTA. Bone marrow was filtered through a 40 μm cell strainer (BD Biosciences, San Jose, CA) to remove debris. Bone marrow cells were rinsed in PBS containing 0.5% bovine serum albumin and 2 mM EDTA. All cells were counted, viability determine using trypan blue exclusion (MP Biomedicals, Santa Ana, CA), and cryopreserved in 90% FBS/10% dimethyl sulfoxide (Sigma).

2.4. ELISA

Flat-bottom MaxiSorp 96-well plates (Nunc, Rochester, New York) were coated overnight at 4 °C with 0.5 μg KLH in carbonate buffer, pH 9.6, or buffer alone for negative control wells. Wells were thoroughly washed with TBST-0.1% and nonspecific binding sites were blocked for 2 h with casein-TBS (Thermo Fisher Scientific). Diluted mouse plasma (1:10) was added to antigen coated wells for 2 h at room temperature. After washing the plates, the KLH reactive antibodies were detected with goat anti-mouse IgG (H+L)-horseradish peroxidase (1:1000 dilution in Casein-TBS Blocker + 0.1% Tween) incubation with gentle shaking for 1 h. After washing 4 times (3 minutes each) with TBST-0.1% and once with PBS, the bound antibody was detected with 3,3′,5,5′-tetramethyl benzidine soluble substrate (Millipore, Jaffrey, NH). The formation of blue-colored horseradish peroxidase product was read immediately at 650 nm for 15 min at 3-min intervals using a Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT). Data were expressed as the average ± SD of the 4 mice in each experimental group.

2.5. B Cell ELISPOT

Secretion of IgG by B cells was quantitated using B cell ELISPOT assay [16]. Multiscreen IP-PVDF filter microplates (Millipore) were pre-rinsed with ethanol and rinsed twice in PBS following coating with either 2 μg KLH (Sigma) or 1 μg goat anti-mouse IgG antibody (Sigma) as capture reagent. Plates were washed 3 times with media and then blocked for 2–4 h at 37 °C in R10 media before cells were added. Mononuclear cells from each tissue source were stimulated with 2 μg/ml lipopolysaccharide (Sigma) for 5 days [17]. Stimulated cells were washed once with media. Following cell and viability counts estimation, 100,000 cells per well were added to each well. For wells coated with anti-IgG, 5000–25,000 cells were incubated per well. Multiple combinations of stimulants were tested, including R848, mouse Interleukin-2, *S. aureus* Cowan, pokeweed mitogen extract, fully phosphothioiated CpG

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