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Neem leaf glycoprotein promotes dual generation of central and effector memory CD8$^+$ T cells against sarcoma antigen vaccine to induce protective anti-tumor immunity

Sarbari Ghosh, Madhurima Sarkar, Tithi Ghosh, Ipsita Guha, Avishek Bhuniya, Akata Saha, Shayani Dasgupta, Subhasis Barik, Anamika Bose[1], Rathindranath Baral[*,1]

Department of Immunoregulation and Immunodiagnostics, Chittaranjan National Cancer Institute (CNCI), 37, S. P. Mukherjee Road, Kolkata 700026, India

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ABSTRACT

We have previously shown that Neem Leaf Glycoprotein (NLGP) mediates sustained tumor protection by activating host immune response. Now we report that adjuvant help from NLGP predominantly generates CD44$^+$CD62L^{high}CCR7^{high} central memory (TCM; in lymph node) and CD44$^+$CD62L^{low}CCR7^{low} effector memory (TEM; in spleen) CD8$^+$ T cells of Swiss mice after vaccination with sarcoma antigen (SarAg). Generated TCM and TEM participated either to replenish memory cell pool for sustained disease free states or in rapid tumor eradication respectively. TCM generated after SarAg + NLGP vaccination underwent significant proliferation and IL-2 secretion following SarAg re-stimulation. Furthermore, SarAg + NLGP vaccination helps in greater survival of the memory precursor effector cells at the peak of the effector response and their maintenance as mature memory cells, in comparison to single modality treatment. Such response is corroborated with the reduced phosphorylation of FOXO in the cytosol and increased KLF2 in the nucleus associated with enhanced CD62L, CCR7 expression of lymph node-resident CD8$^+$ T cells. However, spleen-resident CD8$^+$ T memory cells show superior efficacy for immediate memory-to-effector cell conversion. The data support in all aspects that SarAg + NLGP demonstrate superiority than SarAg vaccination alone that benefits the host by rapid effector functions whenever required, whereas, central-memory cells are thought to replenish the memory cell pool for ultimate sustained disease free survival till 60 days following post-vaccination tumor inoculation.

1. Introduction

Cancer vaccinology deals with the awakening of the immune system to cancer by presenting antigens. These antigens are associated with tumor cells and participating in either prevention of the cancer development (prophylactic cancer vaccines) or existing cancer treatment (therapeutic cancer vaccine). In use for cancer prevention, vaccines must elicit long term memory without the potential of causing autoimmunity (Finn, 2003). The major problem in developing an efficient cancer vaccine is the lack of TSAs (antigens present only on tumor cells) and the weakness of immune responses against TAAs (antigens present mostly on tumor cells but also on some normal cells), usually recognized by the immune system as self-antigens (Buonaguro et al., 2011; Cunto-Amesty et al., 2003; Kaech et al., 2002). Nevertheless, in the last 20 years, several different vaccines from whole tumor cells or tumor–cell lysates have been evaluated in preclinical models and clinical trials. Vaccine adjuvants trigger the activation and maturation of dendritic cells (DCs), so that DCs loaded with antigen, migrate to the proximal lymph nodes and acquire the ability to optimally present antigens for initiation of *de novo* T cell responses (Dubensky and Reed, 2010; Mohan et al., 2013).

A productive encounter of naïve CD8$^+$ T cells to antigen stimulation follows a prototypical, tri-phasic response consisting of: (i) activation phase; (ii) death phase and iii. immunologic memory phase (Klebanoff et al., 2006). According to Sallusto et al. (2004); Sallusto et al. (1999), memory T cells are divided broadly into central (TCM) and effector (TEM) memory T cells. TCMs are antigen-experienced cells that constitutively express CD62L and CCR7, two surface molecules necessary for cellular extravasation in high endothelial venules and migration to T-cell zones of peripheral lymph nodes. Whereas TEMs are antigen-experienced T cells that have these markers significantly downregulated and popu-

* Corresponding author. Fax: +91 033 2475 7606.
E-mail address: baralrathin@hotmail.com (R. Baral).
[1] Both authors contributed equally to this work.

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late to the peripheral tissues, such as, the liver and lung (Masopust et al., 2001). Adoptively transferred self/tumor-reactive $CD8^+$ TCMs are observed to be superior mediator of antitumor immunity to an established cancer compared to TEM cells, when given in combination with a systemically administered tumor antigen vaccine (Klebanoff et al., 2005). TCM cells have greater proliferative capacity upon antigen re-encounter compared with TEM cells (Bachmann et al., 2005), thereby, leading to generation of a larger absolute number of terminally differentiated effector T cells that can infiltrate the peripheral sites to mediate antigen clearance. These data suggest that TCM may be more potent on a per cell basis in mediating antigen clearance compared with TEM cells. Therefore, generation of TCM should be an important immunologic end-point to consider in future preventive and therapeutic vaccine trials.

In the present study, we have reported that vaccination with sarcoma antigen (SarAg), along with neem leaf glycoprotein (NLGP), a global immunomodulator, generates antigen-specific central memory $CD8^+$ T cells (TCM) in lymph nodes and effector memory $CD8^+$ T cells (TEM) in spleen during vaccination-post vaccination window. Furthermore, this vaccination schedule shows significant protection against growth of sarcoma tumor expressing SarAg by inducing effective conversion of memory-to-effector cells. Superior protection from tumor growth, along with tumor free survival till experiment termination, provides evidence in favor of the adjuvant efficacy of NLGP.

2. Materials and methods

2.1. Antibodies and reagents

RPMI-1640 and Fetal Bovine Serum (FBS) were purchased from Life Technologies (NY, USA). Lymphocyte separation media (LSM) was procured from MP Biomedicals, Irvine, CA, USA and HiMedia, Mumbai, India. Fluorescence conjugated different anti-mouse antibodies (CD44, CD127, CD69, Ly6C-FITC conjugated and CD8, CD62L, CCR7, Granzyme B-PE conjugated), purified anti-mouse antibodies (CD8, Ki67, KLF2, FOXO3, pFOXO3, *p*AKT (ser), *p*AKT (thr), *p*mTOR) were procured from either BD-Pharmingen or Biolegend (San Diego, CA, USA) or Santa Cruz (Dallas, Texas, USA). TMB substrate solutions (for ELISA), CytoFix/CytoPerm solutions (for intracellular staining) were procured from BD-Pharmingen, San Diego, CA, USA. LDH cytotoxicity detection kit was purchased from Roche Diagnostics, Mannheim, Germany. RT-PCR primers were designed and procured from MWG Biotech AG, Bangalore, India.

2.2. Neem leaf glycoprotein (NLGP)

Extract from neem (*Azadirachta indica*) leaves was prepared by the method as described previously (Chakraborty et al., 2010). Mature leaves of same size and color (indicative of same age), taken from a standard source, were shed-dried and pulverized. Leaf powder was soaked overnight in phosphate-buffered saline (PBS), pH 7.4. Supernatant was collected by centrifugation at 1500 rpm, extensively dialyzed against PBS, pH 7.4 and concentrated by Centricon membrane filter (Millipore Corporation, MA, USA) with 10 kDa molecular weight cut-off. Purified NLGP was checked for its quality by electrophoresis and HPLC using routine laboratory methods. Biological activity of purified NLGP was checked by tumor growth restriction assay before use. The protein concentration was measured by Folin–Lowry method (Lowry et al., 1951).

2.3. Sarcoma antigen preparation

Peritoneally grown sarcoma cells were collected, washed in PBS and then lysed by five freeze (in liquid nitrogen)-thaw (at room temperature) cycles (Mallick et al., 2014). Total cell disruption was microscopically validated. Disrupted cells were sonicated and lysates were centrifuged at $15{,}000 \times g$ (30 min, 4 °C). The supernatant was recovered as SarAg. The protein concentration was measured using Folin's phenol reagent (Lowry et al., 1951) and stored at −20 °C. Similarly, melanoma antigen (MelAg) was prepared from B16 melanoma cells grown in culture.

2.4. Mice and immunization

Female Swiss mice (Age: 4–6 weeks; Body weight: 24–27 g) were obtained from the Institutional Animal Care and Maintenance Department of Chittaranjan National Cancer Institute, Kolkata, and maintained under standard laboratory conditions. Autoclaved dry pellet diet and water were given *ad libitum*. Mice ($n = 6$) were vaccinated sub-cutaneously with the SarAg and SarAg + NLGP in the lower right flank. Vaccination was done at an interval of seven days for 4 weeks' time period (Kalli et al., 2013; Hariharan et al., 1995). One group of mice was kept as vaccine free control (PBS treated). Animal experiments were performed according to the guidelines established by the Institutional Animal Care and Ethics Committee of CNCI, Kolkata, India, following their approval.

2.5. Tumor inoculation in vaccinated mice

Three groups of Swiss mice ($n = 6$ in each group) that were vaccinated with PBS, SarAg and SarAg + NLGP, were inoculated with sarcoma cells (1×106 cells/mice) in the lower right flank (the site of immunization) 30 days after the completion of the vaccination schedule. Growth of solid tumor (in mm^3) and survivability was monitored bi-weekly in these three cohorts of mice (PBS, SarAg and SarAg + NLGP) by caliper measurement using the formula: $(\text{width}^2 \times \text{length})/2$. Tumors were monitored till day 60 from the day of tumor inoculation. Tumors were checked macroscopically by caliper measurement regularly and tumor size was recorded as a tumor area (in mm^3) and mice were sacrificed if tumors became ulcerated or reached a size >250 mm^2 within 120 days (Saha et al., 2006). At experiment termination tumors were checked microscopically after histological preparation.

2.6. Generation and culture of bone marrow derived DCs and antigen pulsing

Primary bone marrow-derived DCs (BmDCs) were obtained from mouse bone marrow (from tibia and femurs) precursor according to the protocol described (Mallick et al., 2014). Tissue pieces were minced through a nylon mesh into a single-cell suspension. Next, erythrocytes were lysed by resuspending the cell pellet in a hypotonic buffer (9.84 g/l NH_4Cl, 1 g/l $KHCO_3$, 0.1 mM EDTA) and incubating the cell suspension for 10 min on ice. The cells were washed and cultured in a six-well plate at 2×10^6 cells/well with RPMI-1640 containing rmGM-CSF (10 ng/ml) and rmIL-4 (5 ng/ml). On day 6 of culture, non-adherent cells obtained from these cultures were considered to be immature bone marrow-derived DCs. Immature BmDCs (1×10^6 cells/ml) on day 8 were incubated with SarAg/MelAg (5 μg/ml of culture) for overnight in same culture condition. MelAg was used as an unrelated, non-specific control to see the antigen specificity of the generated TCM phenotype cells for SarAg.

2.7. $CD8^+CD62L^{high}$ T cell (central memory phenotype) purification

$CD8^+CD62L^{high}$ T cells were purified from the single cell suspension of TDLN and spleen using Magnetic Assisted Cell Sorter (MACS) according to the manufacturer's instruction (Barik et al., 2013a). In brief, $CD8^+$ T cells were purified by magnetic bead