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Neem leaf glycoprotein generates superior tumor specific central memory CD8$^+$ T cells than cyclophosphamide that averts post-surgery solid sarcoma recurrence

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ABSTRACT

The success of cancer vaccines is limited as most of them induce corrupted CD8$^+$ T cell memory populations. We reported earlier that a natural immunomodulator, neem leaf glycoprotein (NLGP), therapeutically restricts tumor growth in a CD8$^+$ T cell-dependent manner. Here, our objective is to study whether memory CD8$^+$ T cell population is generated in sarcoma hosts after therapeutic NLGP treatment and their role in prevention of post-surgery tumor recurrence, in comparison to the immunostimulatory metronomic cyclophosphamide (CTX) treatment. We found that therapeutic NLGP and CTX treatment generates central memory CD8$^+$ T (TCM) cells with characteristic CD44$^+$CD62L^{high}CCR7^{high}IL-2^{high} phenotypes. But these TCM cells are functionally impaired to prevent re-appearance of tumors along with compromised proliferative, IL-2 secretive and cytotoxic status. This might be due to the presence of tumor load, even a small one in the host, which serves as a persistent source of tumor antigens thereby corrupting the TCM cells so generated. Surgical removal of the persisting tumors from the host restored the functional characteristics of memory CD8$^+$ T cells, preventing tumor recurrence after surgery till end of the experiment. Moreover, we observed that generation of superior TCM cells in NLGP treated surgically removed tumor hosts is related to the activation of Wnt signalling in memory CD8$^+$ T cells with concomitant inhibition of GSK-3β and stabilisation of β-catenin, which ultimately activates transcription of Wnt target genes, like, eomesodermin, a signature molecule of CD8$^+$ TCM cells.

1. Introduction

Cancer immunotherapy is associated with generation of a memory response that prevents tumor recurrence. Memory CD8$^+$ T cells constitute a population of rare naïve CD8$^+$ T cells, programmed to expand clonally upon tumor antigen encounter during recurrence [1–3]. They respond more rapidly and for an extended duration, thereby killing tumor cells effectively [4,5].

Memory CD8$^+$ T cells are heterogeneous with respect to phenotypic markers, effector functions and tissue-homing capabilities and are classified as central (TCM) and effector (TEM) memory T cells [6]. TCM are antigen-experienced cells characterized as CD62L^{high}CCR7^{high} that migrate to peripheral lymph nodes (LNs), whereas TEM are antigen-experienced T cells characterized as CD62L^{low}CCR7^{low}, which migrate to peripheral tissues and inflammatory sites [7,8]. Klebanoff et al. [9] showed that adoptively transferred self/tumor reactive CD8$^+$ TCM cells are superior to TEM cells in therapeutic anti-tumor immunity and possess greater proliferative capacity upon antigen re-exposure.

However, persistent tumor derived antigen stimulation hampers CD8$^+$ memory T cell functions [10–13], leading to impaired proliferation and IL-2 secretion [14,15]. One possible way to reduce such antigen stimulation is the surgical removal of the residual tumor mass, which weakens tumor's immune suppressive effects [16,17]. Moreover, recent literature suggests that Wnt/β-catenin signalling pathway has a pivotal role in the generation and maintenance of the CD8$^+$ T cell memory [18–20].

We report that neem leaf glycoprotein (NLGP), an immunomodulator, therapeutically restricts murine tumor growth in CD8$^+$ T cell-dependent manner [21–23]. Despite good TCM response during NLGP vaccination, complete tumor restriction was observed in 37.5% mice, while 62.5% mice exhibited significantly small tumor volume (Table 1). We recently reported that adjuvant help

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Table 1
Number of tumor free, tumor bearing and dead mice on mentioned days after tumor inoculation on day 0.

Total number of mice/group	Number of mice tumor free			Number of mice tumor					
				Bearing			Died		
	D30	D60	D90	D30	D60	D90	D30	D60	D90
PBS (n = 32)	0 (0%)	0 (0%)	0 (0%)	29 (90.6%)	15 (46.8%)	3 (9.3%)	3 (9.3%)	17 (53.1%)	29 (90.6%)
NLGP (n = 32)	4 (12.5%)	8 (25.0%)	12 (37.5%)	28 (87.5%)	22 (68.7%)	12 (37.5%)	0 (0%)	2 (6.2%)	8 (25.0%)
CTX (n = 8)	0 (0%)	0 (0%)	0 (0%)	8 (100%)	4 (50.0%)	2 (25.0%)	0 (0%)	4 (50.0%)	6 (75.0%)

from NLGP for sarcoma antigen prophylactic vaccine generates TCM and TEM responses, which checks tumor incidence in immunized hosts [24]. Therefore, the potential of NLGP generated TCM response to eradicate established solid mice sarcoma is examined here in parallel comparison to cyclophosphamide (CTX), acting as an immunomodulator in metronomic doses [25,26].

2. Materials and methods

2.1. Antibodies and reagents

RPMI-1640 and Fetal Bovine Serum were purchased from Life Technologies, NY, USA. Lymphocyte separation media was procured from HiMedia, Mumbai, India. Fluorescence conjugated (FITC conjugated CD44, KLRG1 and PE conjugated CD8, CD127, CD62L, CCR7), purified (CD8 and Ki67) anti-mouse antibodies were procured from BD-Pharmingen or Biolegend, San Diego, CA, USA. TMB substrate and CytoFix/CytoPerm solutions were procured from BD-Pharmingen, San Diego, CA, USA. The cytotoxicity detection kit was purchased from Roche Diagnostics, Mannheim, Germany. Trizol reagent was purchased from Life Technologies, NY, USA. RT-PCR primers were procured from MWG Biotech AG, Bangalore, India. SYBR green PCR mix was obtained from Roche, Germany. Cyclophosphamide (Endoxan-N) was purchased from Zydus Oncosciences, Ahmedabad, India. The chromatin immunoprecipitation kit was purchased from Millipore, Darmstadt, Germany. Thiopentone sodium [Pentothal Sodium] was procured from Abbott Laboratories, India.

2.2. Neem leaf glycoprotein (NLGP)

The extract from mature neem (*Azadirachta indica*) leaves was prepared and purified by the method described [27]. The bioactivity of purified NLGP was checked by tumor growth restriction assay after determining protein concentration by Bradford's method.

2.3. Mice and tumors

Female Swiss mice (Age: 4–6 weeks; Body weight: 24–27 g) were obtained from the Institutional Animal Care and Maintenance Department. Autoclaved dry pellet diet and water were given *ad libitum*. For solid tumors, mice were inoculated subcutaneously (s.c.) in right hind leg quarters with sarcoma cells (1×10^6 cells/mice). Animal experiments were performed after approval by the Institutional Animal Care and Ethics Committee of the CNCI, Kolkata, India (Animal Ethical Proposal No. IAEC-1774/RB-5/2015/7). Tumor area (length x width) and tumor volume ($width^2$ - $\times$ length)/2 were calculated. Tumor hosts were euthanized by intra-peritoneal injection of thiopentone sodium (@100 mg/kg body weight), when tumors reached 20 mm in either direction.

2.4. Tumor growth restriction assay

After seven days of tumor inoculation, mice with palpable tumors were injected with NLGP (s.c.; 25 μg/mice/injection; weekly) and metronomic CTX (20 mg/kg mice body weight/day; every 4 days in drinking water) along with a control PBS group for four weeks. Solid tumor growth (in mm^2) was monitored by caliper measurement using the formula: (length $\times$ width). Mice survival was monitored till day 60 or 90.

2.5. Surgical excision of the primary tumors

After therapy, tumor surgeries of the mice were carried out aseptically after anesthetization with thiopentone sodium. Once unconscious, an incision was made on tumor site using a sterile blade and after tumor visualization, it was removed surgically. The surgery site was sutured using a surgical needle and nylon threads. The area was dabbed with alcohol soaked cotton and covered with an antibiotic powder for a few days. The mice were kept on a cotton bed under frequent monitoring until the wounds healed.

2.6. Generation and culture of bone marrow derived dendritic cells (DCs) and antigen pulsing

Primary bone marrow-derived DCs (BmDCs) were obtained from mouse bone marrow (from tibia and femurs) precursors according to the protocol described [30]. The immature BmDCs (1×10^6 cells/ml) were incubated with SarAg/MelAg (5 μg/ml of culture) overnight.

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

$CD8^+CD62L^{high}$ T cells were purified from single cell suspension of TDLN using BD IMag cell separation system as mentioned [22]. Cells were labeled with biotinylated anti-CD62L antibody followed by incubation with streptavidin microbeads and loaded on the BD IMag to pass through. Next, $CD8^+$ T cells were purified by a magnetic bead attached anti-CD8 antibody. A cellular fraction that stuck to the tube walls is the desired fraction. Cell purity was checked by flow-cytometry and cell preparation with >90% purity was taken for experiment.

2.8. Flow-cytometric analysis of LN cells

LN cells were labeled (1×10^6) with mouse fluorescence tagged antibodies (CD8, CD44, CD62L, CCR7, KLRG1 and CD127). Cells were washed in FACS buffer (PBS with 1% FBS), fixed with 1% paraformaldehyde and analysed with FACSCaliber (Becton Dickinson, Mountainview, CA), Cell Quest and FlowJo software (Treestar, Ashland, OR). Suitable negative isotype controls were used.

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