Vaccine 31 (2013) 2449–2456

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Expression of membrane anchored cytokines and B7-1 alters tumor microenvironment and induces protective antitumor immunity in a murine breast cancer model

Erica N. Bozeman[a,1], Ashley Cimino-Mathews[a,1], Deepa K. Machiah[b], Jaina M. Patel[a], Arun Krishnamoorthy[a], Linda Tien[a], Rangaiah Shashidharamurthy[a], Periasamy Selvaraj[a,*]

[a] Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia 30322, United States
[b] Yerkes National Primate Research Center, Emory University, Atlanta, Georgia, 30329, United States

ARTICLE INFO

Article history:
Received 1 November 2012
Received in revised form 25 February 2013
Accepted 15 March 2013
Available online 28 March 2013

Keywords:
Breast cancer
Membrane anchored cytokines
Antitumor immunity
Tumor microenvironment

ABSTRACT

Many studies have shown that the systemic administration of cytokines or vaccination with cytokine-secreting tumors augments an antitumor immune response that can result in eradication of tumors. However, these approaches are hampered by the risk of systemic toxicity induced by soluble cytokines. In this study, we have evaluated the efficacy of 4TO7, a highly tumorigenic murine mammary tumor cell line, expressing glycosyl phosphatidylinositol (GPI)-anchored form of cytokine molecules alone or in combination with the costimulatory molecule B7-1 as a model for potential cell or membrane-based breast cancer vaccines. We observed that the GPI-anchored cytokines expressed on the surface of tumor cells greatly reduced the overall tumorigenicity of the 4TO7 tumor cells following direct live cell challenge as evidenced by transient tumor growth and complete regression within 30 days post challenge. Tumors co-expressing B7-1 and GPI-IL-12 grew the least and for the shortest duration, suggesting that this combination of immunostimulatory molecules is most potent. Protective immune responses were also observed following secondary tumor challenge. Further, the 4TO7-B7-1/GPI-IL-2 and 4TO7-B7-1/GPI-IL-12 transfectants were capable of inducing regression of a wild-type tumor growing at a distant site in a concomitant tumor challenge model, suggesting the tumor immunity elicited by the transfectants can act systemically and inhibit the tumor growth at a distant site. Additionally, when used as irradiated whole cell vaccines, 4TO7-B7-1/GPI-IL-12 led to a significant inhibition in tumor growth of day 7 established tumors. Lastly, we observed a significant decrease in the prevalence of myeloid-derived suppressor cells and regulatory T-cells in the tumor microenvironment on day 7 post challenge with 4TO7-B7-1/GPI-IL-12 cells, which provides mechanistic insight into antitumor efficacy of the tumor-cell membrane expressed IL-12. These studies have implications in designing membrane-based therapeutic vaccines with GPI-anchored cytokines for breast cancer.

1. Introduction

Breast cancer is among the leading types of cancer among women in the United States, with an estimated 229,060 new cases in 2012 alone [1] and devising new strategies of breast cancer therapy remains a priority in medical research. While there have been numerous preclinical studies that have evaluated different methods and approaches to enhance the overall immunogenicity of tumor cells, few have been capable of inducing clinically relevant responses. One such approach includes the genetic modification of tumor cells to secrete cytokines including IL-12 and GM-CSF [2–4], or using GM-CSF secreting tumor vaccines with or without concomitant chemotherapy [5,6].

In order for a cancer immunotherapy to be effective in a clinical therapeutic setting, the immune suppressive nature of the tumor microenvironment must be overcome [7,8]. Tumor cells have been shown to up-regulate the expression of the inhibitory molecules PD-L1 and CTLA-4 [9–11]. Moreover, tumors also produce inhibitory cytokines and factors such as IL-10, vascular endothelial growth factor (VEGF), prostaglandins and transforming growth factor beta (TGF-β) that can induce immune tolerance by

* Corresponding author at: Department of Pathology and Laboratory Medicine, Emory University School of Medicine 7309 Woodruff Memorial Research Building 101, Woodruff Circle Atlanta, GA 30322, United States. Tel.: +1 404 727 5929; fax: +1 404 727 5764.
E-mail addresses: ebozema@emory.edu (E.N. Bozeman), acimino1@jhmi.edu (A. Cimino-Mathews), dkodand@emory.edu (D.K. Machiah), jmpate2@emory.edu (J.M. Patel), arun.krishnamoorthy@duke.edu (A. Krishnamoorthy), ltien@uchicago.edu (L. Tien), rangaiahsh@pcom.edu (R. Shashidharamurthy), pselvar@emory.edu (P. Selvaraj).
[1] These two authors contributed equally.

0264-410X/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2013.03.028

preventing dendritic cell (DC) maturation [12,13] and promoting the differentiation and maturation of regulatory T cells (Tregs) [14,15] and myeloid derived suppressor cells (MDSCs) [16].

To potentially address this issue, we have established glycosyl phosphatidylinositol (GPI)- anchored forms of the cytokines IL-2 and IL-12 for stable surface expression onto tumor cells along with the costimulatory molecule B7-1. The expression of these molecules allows for immune activation to take place locally at the vaccination site rather than systemic activation, which could potentially be toxic to patients [17–19]. This localized immune activation can effectively manipulate or skew the tumor microenvironment towards being less immune suppressive. Additionally, direct targeting of tumor antigens to antigen presenting cells is more likely to occur following engagement of the modified tumor cells expressing IL-2 and IL-12 with their cognate receptors found on DCs.

Herein, we report for the first time the direct effects of membrane-anchored cytokines such as IL-2 and IL-12 on the tumorigenicity of a highly tumorigenic mouse mammary cancer model. Additionally, our studies provide insight into the potential mechanisms underlying the reduced tumorigenicity of these genetically modified cells as evidenced by a significant reduction in the local and peripheral immune suppressive microenvironment of tumor-bearing hosts.

2. Materials and methods

2.1. Cell culture and animals

4TO7 tumor cells, a kind gift of Fred Miller (Wayne State University), were cultured in DMEM media (Cellgro) with 10% FBS at 37 °C. cDNA encoding GPI-anchored forms of murine IL-2, and IL-12 were constructed in our laboratory by attaching a GPI-anchor signal sequence as previously described [20,21]. cDNA encoding murine B7-1 was kindly provided by Gordan Freeman (Boston, MA). The cDNAs were subcloned into the pUB6A expression vector (Invitrogen Corp). Cells were transfected using FuGene6 transfection reagent (Roche Molecular Biochemicals) and selected with blasticidin (10 μg/ml). In order to select the population of cells expressing the GPI-molecules, the cells were subjected sequentially to a) magnetic activated cell sorting (MACS) (Dynal Biotech Dynabeads, Invitrogen), b) panning [22] and c) fluorescence activated cell sorting (FACS). The wild-type 4TO7 (4TO7-WT) cell population was later subjected to four rounds of *in vivo* passage following subcutaneous (s.c.) injection into BALB/c mice to yield more reproducible, aggressive tumor growth with palpable tumor development within 6 days (4TO7RG).

Female BALB/C mice 6–8 weeks of age were purchased from Jackson Laboratories and were maintained in accordance with IACUC approved institutional guidelines and protocols.

2.2. Characterization of tumor cells

2.2.1. Flow cytometry

Surface expression of B7-1, IL-2 and IL-12 was determined by flow cytometry analysis. Briefly, cells were incubated for 30 min at 4 °C with directly-conjugated antibodies as follows: IL-2-PE (clone S4B6), IL-12-PE (clone 17.8), and B7-1-FITC (clone 1G10) (BD Biosciences). The cells were then washed, formalin-fixed and analyzed using a FACSCaliber cytometer and analyzed with FlowJo software.

2.2.2. ELISA and Western blot analysis

Cell transfectants (2×10^5/well) were seeded in 24-well plates for 48 h. After which, culture supernatant was collected, cells were washed and lysed using 2% octyl-β-glucoside, 50 mM Tris–HCl pH8, 2 mM PMSF, 5 mM EDTA and protease inhibitor cocktail (1:100, Sigma). IL-2 and IL-12 in the cell lysate and culture supernatant was detected by sandwich ELISA according to the manufacturer's instructions (eBioscience) and western blotting techniques as previously described [23].

2.2.3. PIPLC (phosphatidylinositol phospholipase-C) treatment

Cell transfectants were treated with a 1:1000 dilution of the PIPLC enzyme (Glyko Prozyme, San Leandro, CA) in PBS/0.1% Ovalbumin and incubated for 45 min in a 37 °C water bath with slight agitation every 10 min. At the end of the incubation, the cells were centrifuged and washed with FACS buffer (PBS/1%CCS/1%EDTA) and stained for FACS analysis.

2.2.4. CFSE dilution

CFSE staining was used to determine the growth rate of tumor cells using adapted methods as previously described [24]. Cells were then washed with FACS buffer and either analyzed immediately to verify CFSE incorporation or cultured for FACS analysis at the specified time points.

2.3. Tumor challenge studies

2.3.1. Direct challenge

Mice ($n = 5$/group) were challenged subcutaneously (s.c.) in the rear hind flank with 4TO7-WT or transfected 4TO7 cells (2×10^5). Tumor size (mm^2) was measured using Vernier calipers every 2–3 days with 2×2 perpendicular measurements. Tumor-free mice were subjected to a secondary challenge with 4TO7-WT cells (2×10^5) 30–33 days later on the opposite hind flank. Mice were monitored weekly for tumor growth. Mice were euthanized when the tumor size reached >2 cm^2.

2.3.2. Concomitant immunity

Mice ($n = 5$/group) were challenged with 4TO7RG cells (2×10^5) on the right hind flank and simultaneously challenged with each of the 4TO7 transfectants (2×10^5) on the opposite hind flank (s.c.). Mice were monitored as mentioned previously.

2.3.3. Therapeutic whole cell vaccination studies

Prior to vaccination, tumor cells were exposed to 80 Gy of gamma irradiation. Mice were challenged subcutaneously with 5×10^4 4TO7RG cells on the right hind flank and vaccinated with 2×10^5 irradiated cells on the opposite hind flank seven days later. Mice were monitored as previously mentioned.

2.4. Cellular phenotyping of immune infiltrates and immunohistochemistry staining (IHC)

Tumor cells (2×10^5) were mixed in a 1:1 ratio with 250 μL of Matrigel™ (BD Falcon) and injected into the hind flank of BALB/c mice (s.c.). Seven days post inoculation, the spleens, tumor-draining lymph nodes (TDLNs) and Matrigel were harvested. The Matrigels were either digested using collagenase type III (Sigma) for 1 h or formalin-fixed for IHC staining. Single cell suspensions were prepared from the digested Matrigels and red blood cells were lysed. Cells were then washed, Fc blocked (clone 2.4G2) and stained with directly conjugated antibodies (eBioscience) for 25 min at 4 °C to detect T cells ($CD4^+$ and $CD8^+$), B cells ($B220^+$), DCs ($CD11b^+CD11c^+$), Tregs ($CD4^+CD25^+FoxP3^+$) and MDSCs ($CD11b^+Gr1^+$). Samples were analyzed as described previously. Formalin-fixed Matrigels were embedded in paraffin blocks, sectioned and stained with hematoxylin and eosin (H&E). Blood vessels were visualized by staining endothelial cells with a CD31 primary antibody at 1:200 dilution (Abcam).