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Enhancement of T cell localization in mammary tumors through transient inhibition of T cell myosin function

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Abstract

Adoptive immunotherapy is hampered by poor lymphocyte localization in tumors. The polarized, adhesive phenotype of activated lymphocytes may contribute to this problem by making the cells prone to trapping and damage in pulmonary microvasculature. We found that transient inhibition of T cell polarization prior to i.v. infusion reduces trapping and improves tumor localization. Activated T cells were rendered nonpolar and nonadhesive by treatment with myosin light-chain kinase inhibitor ML-7. Polarity, adhesiveness, and motility recovered by 6 h after treatment, cytotoxicity, and proliferation by 24 h. ErbB2-specific T cells were infused i.v. into mice bearing ErbB2-expressing mammary tumors. ML-7 pre-treatment reduced T cell arrest in lungs by a factor of eight, improved tumor localization by 4-fold, and increased lymph node homing. Although this improvement alone proved insufficient to alter outcome in an immunotherapy experiment, this study indicates that cytoskeletal modification is a promising strategy for altering the trafficking of infused lymphocytes.

Keywords: T cell; Immunotherapy; Breast cancer; Traffic; Migration; Cytoskeleton; Actin; Myosin

1. Introduction

Immunotherapy by adoptive transfer of T cells is potentially powerful because it allows a T cell response against tumor antigens to be generated under optimal conditions, removed from tumor-derived suppressive and tolerogenic influences. This is a particular advantage during periods of immunosuppression following radiation and chemotherapy [1]. Adoptive transfer therapy also permits the use of promising new genetic manipulations of the T cells and NK cells during ex vivo culture, such as the expression of specific Ag receptors and of cytokines which enhance anti-tumor response and T cell survival [2,3]. To further improve adoptive immunotherapy we tested a strategy intended to increase the ability of cultured lymphocytes to traffic and survive when returned to circulation. Activated T cells, after i.v. infusion, show massive short-term lung localization, which mainly reflects adhesion and embolization in lung microvasculature. The trapped cells soon leave the lung but then localize heavily in liver, suggesting that they have been damaged or altered in ways which limit their ability to circulate [4,5]. Even though some transferred cells certainly do show long-term engraftment and growth [2,3,6] the effectiveness of adoptive therapy still reflects the activity of a small percentage of infusate reaches tumor sites or lymph nodes [7–9].

We hypothesize that pulmonary arrest stems in large part from the polarized, adhesive phenotype of the cultured activated T cells. T cells normally circulate in a state of rest or early activation, in which they are round, smooth, and nonadhesive. Activation in vitro converts them into a polarized, spiky, adhesive, and highly motile form which they never normally achieve until after extravasation [10] and which, we propose, contributes

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to their embolization and trapping in pulmonary microvasculature after infusion, when cell density is highest. We also hypothesize that transient inhibition of this polarized phenotype, induced shortly before infusion, will allow the cells to escape trapping and its attendant damage, and to show more normal patterns of traffic. The polarized form of activated T cells is not reversed by removal from culture flasks, for it is maintained independent of adhesion to substrate and persists when cells are kept in suspension. We have also found it impractical to return activated T cells to the nonpolarized state by withdrawing stimulation, for this type of depolarization is accompanied by apoptosis for all but a minority of cells (not shown).

In activated T cells, actin–myosin contractility is programmed to produce appendages and to promote the formation of activated integrin-containing adhesive foci [11]. We now report that that activated T cells can be rendered temporarily nonpolar, devoid of appendages, and nonadhesive in vitro by treatment with a pharmacological inhibitor of myosin light chain kinase (MLCK), in protocols that allow return of full polarization, motility, and cytotoxic function. We demonstrate, in a murine mammary tumor model, that transient inhibition of myosin function prior to infusion greatly reduces the initial trapping of the cells in lung microvasculature and increases their later localization in tumor and peripheral lymph nodes. We also determined whether transient inhibition of myosin function improves outcome in an immunotherapy model.

2. Methods

2.1. Cell lines and reagents

D2F2/E2 was a mammary adenocarcinoma which arose from a spontaneous mammary hyperplasia of a BALB/C mouse and was stably transfected to express full-length human ErbB2 [12]. The antigen presenting cell line 3T3/EKB was a syngeneic fibroblastoid cell line engineered to stably express human ErbB2 as well as K(d), and the co-stimulator B7.1 [13].

The MLCK inhibitor ML-7 (Calbiochem, La Jolla, CA) was stored as a sterile 100 mM stock solution in DMSO and diluted just before use. The nonspecific green fluorescent tracking dye CFSE (“Cell Tracker,” Molecular Probes, Eugene OR) was stored as a 10 mM solution in DMSO.

2.2. T cell culture

BALB/c mice received bilateral s.c. flank injections of 5×10^6 irradiated D2F2/E2 cells. This was repeated 4 weeks later, and after an additional 4 weeks draining lymph nodes were excised. ErbB2-specific lymphocytes from these nodes were specifically expanded by culture on irradiated 3T3/E2 cells. The T cells were thinned and fed every 2 days with DME containing 10%FCS, 5U IL-2/ml, and 10 ng/ml IL-7, and restimulated with irradiated 3T3/E2 every 14–20 days. After two rounds of stimulation, the lymphocyte population reached a stable phenotype of >98% $CD3^+$, of which typically 55–60% were $CD4^+$, and 45–50% $CD8^+$. They were used in experiments from 7 to 10 days after their last stimulation. At this point the cells typically exerted approximately 50% specific cytotoxicity against D2F2E2 at a an E:T ratio of 50:1, 35% at 25:1, and 10% at ratio of 10:1, as determined by chromium release assay, whereas cytotoxicity vs. the unrelated syngeneic mammary tumor 410.4 was <5% [12].

2.3. Lymphocyte localization experiments

D2F2/E2 tumors were allowed to grow s.c. in the flanks of syngeneic mice to approximately 10 mm diameter (0.4–0.6 g), a process which took 3–4 weeks. ErbB2-specific T cells were labeled by 30 min incubation with 1 μM CFSE, a concentration found in pilot experiments to confer sufficient fluorescent brightness to track the cells for at least 48 h while not altering their specific cytotoxicity or ability to migrate into collagen matrix in vitro (not shown). After labeling, T cells were treated with ML-7 or DMSO vehicle control and washed as described above, and then infused into D2F2/E2-bearing mice via tail vein, 2×10^7 cells/0.5 ml HBSS per mouse. At 30 min, 2, 24, and 48 h, tissues were harvested, and weighed, to the nearest mg. Single-cell suspensions were prepared as follows. Lymph nodes (pooled inguinal, axillary, cervical, and mesenteric) were pressed through wire mesh. Spleens were pressed through wire mesh and the resulting suspension was enriched for viable lymphoid cells by centrifugation over Ficoll–Hypaque cushions. Livers were minced with a scalpel and incubated at 37 °C with constant shaking in an enzyme cocktail until a uniform suspension was obtained (usually 2–3 h). The cocktail consisted of Liberase Blendzyme two collagenase mixture (Roche), 1 U/ml; elastase II (Roche), 2 U/ml, DNAse (Sigma), 3 KU/ml, and 2% CS, all in phosphate-buffered saline (PBS). The resulting cell suspension was filtered through a 60 μM mesh Nitex screen. Tumors were treated like livers but in addition were centrifuged over Ficoll–Hypaque to deplete nonviable cells. It was recognized that these dispersal steps caused the loss of an unknown percentage of labeled cells, but there was no reason to believe that the losses were not random. It was therefore assumed that the losses did not bias the comparisons of localization of treated vs. control T cells within each tissue type.

The single-cell suspensions were fixed in 3% paraformaldehyde, washed, and resuspended in a known volume of PBS. A measured aliquot, usually 600 μl, was