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Research paper

Functional analysis of acquired CD28 mutations identified in cutaneous T cell lymphoma

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ABSTRACT

CD28 is the major costimulatory receptor on T cells regulating proliferation, survival and effector function. Acquired mutations in the extracellular domain of CD28 have been identified in patients with cutaneous T cell lymphoma, angioimmunoblastic T cell lymphoma and other T cell neoplasms, suggesting it may contribute to disease pathogenesis. We used a heterologous system in which mutant human CD28 was expressed on primary murine T cells deficient in CD28 to ascertain how specific mutations identified in a genetic screen of patients with cutaneous T cell lymphoma affected normal T cell function. All three mutant CD28 proteins examined enhanced CD28-dependent T cell proliferation and effector function. These data suggest that the mutant CD28 isoforms could accelerate tumor cell growth and increase tumor burden in affected patients. Interruption of CD28:ligand interactions may be an effective, targeted therapy for a subset of patients whose tumors bear the mutant CD28 receptor.

1. Introduction

Binding of CD28 to either of its ligands, CD80 (B7-1) or CD86 (B7-2), initiates signaling that synergizes with TCR engagement to augment T cell proliferation, cytokine secretion and cell survival [1]. Despite its critical role in normal T cell function, acquired mutations of CD28 have not previously been shown to cause human disease although some polymorphisms have been associated with susceptibility to autoimmune conditions [2–4].

Recurrent acquired mutations in the extracellular domain of CD28, as well as fusions between CTLA-4 (CD152), ICOS (CD278) and CD28 have been identified in cells from up to 10% of patients with cutaneous T cell lymphoma (CTCL), angioimmunoblastic T cell lymphoma (AITL), peripheral T cell lymphoma (PTCL) and adult T cell lymphoma-leukemia (ATL) [5–11]. However, whether and how these mutations contribute to the disease phenotype remains unknown. We expressed human CD28 receptors with either of 2 point mutations that had been identified as occurring with the greatest frequency in CTCL as well as a fusion protein between CD28 and CTLA-4 also identified in patients with CTCL in primary T cells isolated from CD28-deficient mice and assessed for changes in T cell proliferation and effector function [5,7,9,10]. Our studies show that T cells expressing the mutant CD28 isoforms have significantly higher proliferative responses and IL-2 secretion as compared to wild type CD28. Our data suggests that this is due to a higher binding affinity of the mutant proteins to CD86 and support a mechanism by which the transformed T cells in CTCL might receive an augmented CD86:CD28 mediated signal, which could drive further clonal expansion of the malignant cells. These data provide new insights into how mutations in CD28 may contribute to the pathogenesis of CTCL and possibly to other T cell neoplasms. Furthermore as approved therapies exist, such as abatacept or belatacept that interfere with engagement of CD28 by ligand [12], suggest a novel, targeted therapeutic strategy for treatment of a subset of patients.

2. Methods

2.1. Mice

CD28-deficient mice were bred into the DO11.10 strain mice in the Balb/c background, which recognize the $OVA_{(323–339)}$ peptide as previously described [13,14]. All mice were bred and housed in a specific pathogen free environment at Washington University School of Medicine. All protocols were reviewed and approved by the Washington University School of Medicine Animal Studies Committee.

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2.2. Cell lines, plasmids, antibodies

Full length human CD28 cDNA (originally provided by C. Thompson MD, Memorial Sloan-Kettering Cancer Center, New York, New York) was cloned into the GFP-RV vector (provided by K. Murphy, Washington University School of Medicine, St Louis, MO) and single point mutations made using the Q5 Site Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA) A CTLA-4-CD28 fusion protein was constructed consisting of the extracellular domain of murine CTLA-4 (amino acids 1–162) and the transmembrane and cytoplasmic domain of human CD28 (amino acids 152–219). All constructs were verified by direct sequencing. Chinese Hamster Ovary cells (CHO) expressing IA^d (gift from Dr. K. Murphy, Washington University School of Medicine, St Louis, MO) were stably transfected with plasmids expressing either human CD80 or CD86 (Sino Biologicals, China). PlatinumE (PlatE) packaging cell line and the retroviral packaging vector pCMV-10A1 were kindly provided by Dr. T. Egawa (Washington University School of Medicine, St Louis, MO).

Fluorescently labeled antibodies were purchased from Biolegend (San Diego, CA) unless otherwise stated. CTLA4Ig was purchased from BioXcell (West Lebanon, NH) and human CD80Ig and CD86Ig were purchased from ACRObiosystems (Newark, DE).

2.3. Retroviral transduction

PlatE cells were co-transfected with the pCMV-10A1 plasmid and GFP-RV encoding empty vector, wild-type or mutant CD28 as indicated. Culture supernatants were harvested 48 h later and used to transduce splenocytes from CD28-deficient DO11.10 mice that had been activated with OVA peptide (0.3 μM) for 48 h. The activated splenocytes were resuspended in the viral supernatant in the presence of polybrene (10 μg/ml, Sigma Chemical, St Louis, MO) and centrifuged at 400XG for 1.5 h at room temperature. The process was repeated on day 3 and the cells expanded in IL-2 for an additional 24 h. Infection efficiency was assessed by flow cytometry and ranged from 30% to 50% between experiments, but was similar for each construct within a given experiment (data not shown).

2.4. Proliferation assay

Splenocytes (1×10^5/well) from CD28-deficient DO11.10 mice retrovirally transduced with either empty vector, wild type human CD28 or human CD28-F51I or CD28-F51V mutant, or the CTLA-4-CD28 chimeric constructs were cocultured in 96 well plates with irradiated (1000 R) or mitomycin C treated splenocytes (2×10^5/well) from wild type Balb/CbyJ mice then treated with either media or $OVA_{(323–339)}$ peptide at the indicated doses alone or in the presence of CTLA4Ig, anti-human CD28 antibody (1.0 μg/ml, clone CD37.51, BD Biosciences, San Jose, CA) or antibodies against CD80 or CD86 (10 μg/ml, clones 16-10A1 and GL-1, BioLegend, San Diego, CA). Cultures were pulsed with 1 μCi/well of tritiated thymidine for the final 10 h of a 48 h culture period and proliferation assessed by tritiated thymidine incorporation. For some experiments, CD4 + cells were first enriched by magnetic bead selection (Miltenyi Biotec, San Diego, CA) followed by flow cytometric sorting on GFP (Sony iCyt Synergy BSC, Sony Biotechnology, San Jose, CA). Purified T cells expressing control, wild type or mutant CD28 constructs were incubated in a 1:1 ratio with CHO cells expressing either IA^d alone or co-expressing human CD80 or CD86. For stimulation with Ig fusion proteins, purified transduced T cells were stimulated with plate bound α-CD3 (0.01 μg/ml) along with graded doses plate bound Ig fusion protein. The cultures were pulsed with tritiated thymidine for the final 10 h of a 72 h culture. All experiments were performed a minimum of 3 times and representative data presented. Proliferation data is expressed as the mean ± standard deviation of triplicate wells.

2.5. Binding assays

Splenocytes from CD28-deficient DO11.10 mice were retrovirally transduced and incubated in PBS 1% BSA alone at 4 °C or in the presence of graded doses of CD80Ig- or CD86Ig fusion proteins followed by staining with a fluorescently conjugated anti-Ig secondary antibody. For analysis, the GFP positive cells were gated on and the mean fluorescence index (MFI) of the Ig fusion protein staining determined.

2.6. Cytokine assays

Splenocytes from CD28-deficient DO11.10 mice were retrovirally transduced with empty vector, wild type or mutant CD28. The transduced cells were plated into a 96 well plate (1×10^5 cells/well) and stimulated with OVA peptide (0.3 μM) for 48 h. Culture supernatants from triplicate wells were collected and cytokine concentrations determined using the Th1/Th2/Th17 Cytometric Bead Array (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Shown is the mean ± standard deviation of the triplicate samples. Data is representative of 3 independent experiments.

2.7. Statistics

All statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software Inc, La Jolla, CA). Unless otherwise indicated, the analysis performed was a 2 tailed unpaired T-test. Multiple comparison testing was performed when appropriate.

3. Results

3.1. CD28 mutations augment primary T cell proliferation and IL-2 secretion

Retroviral gene transfer was used to express wild type or mutant CD28 receptors in primary, antigen specific T cells isolated from CD28-deficient, TCR transgenic mice. Point mutations at position 51 as well as a chimeric construct encoding the extracellular domain of CTLA-4 and the transmembrane and cytoplasmic domains of CD28 were examined as these had been reported in patients with CTCL and other T cell neoplasms [5,7,8,10] (Fig. 1A). Transduction efficiency was high, ranging between 30 and 50% between experiments and within a given experiment efficiency differed between constructs by no more than 5%. Staining with anti-CD28 or anti-CTLA-4 confirmed similar levels of expression for each construct (Fig. 1B). As the level of expression of CD28 and CTLA-4 cannot be directly compared due to the different antibodies used, we compared the intensity of GFP expression. This was identical between all constructs further suggesting that expression levels of CD28 and CTLA-4 were similar between the CD28 and CTLA-4 encoding constructs (data not shown).

To test whether the mutations altered the T cell response to antigen stimulation, we co-cultured OVA specific T cells either lacking CD28 expression or expressing wild type CD28 or mutant forms of CD28 with autologous antigen presenting cells in the presence of cognate antigen (OVA peptide) and assayed proliferation, expression of activation markers and cytokine secretion. T cells expressing CD28- F51I, CD28-F51V or the CTLA-4-CD28 chimeric protein all proliferated significantly more than cells expressing wild type CD28 (Fig. 2A). The maximal proliferative response was increased as well as the sensitivity of the cells to antigen, with the mutants responding to between a 5 and 10-fold lower dose of antigen than wild type cells. Under these experimental conditions, costimulation is being delivered by endogenous murine CD80 or CD86 expressed on APC in the culture. When costimulation was provided by α-CD28 antibody, there was no difference in proliferation between wild type and mutant cells (Fig. 2B).

Under the OVA plus APC conditions both mutant isoforms proliferated more than the wild type (Fig. 2A), whereas addition of the