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Research paper

Development of a luciferase reporter Jurkat cell line under the control of endogenous interleukin-2 promoter

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ABSTRACT

During new drug development, it is critical to have a cell-based reporter bioassay to measure drug-mediated physiological changes. In a conventional reporter cell line, a reporter expression construct is randomly inserted into the host cell genome with the reporter gene under control of an engineered promoter. This design ensures high signal output but may not represent the true physiological cell signaling. Here we used the CRISPR/Cas9 technology to engineer a Jurkat cell line by replacing one interleukin 2 (*IL2*) allele with firefly luciferase gene while keeping the other *IL2* allele intact. The expression of luciferase is thus under control of endogenous *IL2* promoter. We demonstrated that, in this engineered cell line, the IL-2 secretion pathway remained intact and luciferase activity significantly increased upon stimulation with phorbol ester or CD3/CD28 antibodies. We next expressed glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) in this cell line and observed dose-dependent IL-2 and luciferase responses to GITR agonist antibody. Thus we have successfully constructed a reporter cell line by engineering a reporter gene under control of an endogenous target gene promoter. This novel strategy may provide a more physiologically relevant alternative to the traditional method of reporter cell line construction.

1. Introduction

The recent success in the development of antibodies against immune checkpoints cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) for cancer immunotherapy has highlighted key players in the immune system that can be harnessed to enhance anticancer immunity (Mellman et al., 2011). The CTLA-4 and PD-1 antagonists have exhibited remarkable response rates in clinic with largely unprecedented durability, providing compelling examples that immunotherapy is a viable approach for cancer treatment (Herbst et al., 2014; Robert et al., 2015a; Robert et al., 2015b). However, a significant portion of patients, as demonstrated by the clinical trial data to date, did not respond or only partially responded to PD-L1 or PD-1 antagonists (Rizvi et al., 2015), suggesting that other mechanisms of immunomodulation may work together or in parallel with PD-L1/PD-1-mediated immunosuppression. Drugs targeting many of these immune checkpoints are being evaluated in both preclinical and clinical studies. Some of the targets are inhibitory checkpoints such as TIM-3, LAG-3, TIGIT, and BTLA, whereas others are stimulatory checkpoints such as 4-1BB, OX40, and GITR (Vilgelm et al., 2016). Combination treatments are also being developed to augment immune responses to tumors, which are expected to further improve patient outcome.

In order to develop therapeutic drugs targeting immune modulators, a relevant cellular system is needed to screen, profile, and assess the functions of drug candidates. The human leukemia T-cell line Jurkat (Schneider et al., 1977) has been utilized extensively as an in vitro model system to understand activation and signaling of T cell receptor (TCR) (Abraham and Weiss, 2004). Jurkat cells are particularly robust producers of IL-2 after stimulation with phytohemagglutinin (Pawelec et al., 1982). IL-2 is one of the major cytokines secreted upon T cell activation and is an important factor in sustaining T cell-mediated immune responses. For Jurkat cells, two synergistic signals are required

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for maximal IL-2 production. The first signal comes from CD3 activation, whereas the second signal can be induced by the activation of co-stimulators (e.g. CD28) or phorbol esters (Weiss et al., 1984; Huse, 2009). This phenomenon is also observed in primary T cells (Weiss et al., 1984). IL-2 expression is controlled by many pathways. The essential part of the enhancer-promoter of the *IL2* gene is located within the 300-bp region immediately upstream of the transcription start site. A number of transcription factor binding sites in this region have been shown to be involved in the regulation of *IL2* transcriptional activity (Durand et al., 1988; Granelli-Piperno and Nolan, 1991; Parra et al., 1995). These sites include the distal binding site of the nuclear factor of activated T cells (NF-AT) (Shaw et al., 1988), the nuclear factor kB (NF-kB) binding site (Sen and Baltimore, 1986), the activation protein 1 (AP-1)-responsive element (Angel et al., 1987; Lee et al., 1987), the CD28-responsive element (CD28RE) (Fraser et al., 1991), and the octamer (OCT-1)-responsive element (Kamps et al., 1990). To study extracellular signals triggering intracellular signaling pathways in T cells modulated by these nuclear factors, an expression cassette is generally constructed by placing a reporter gene such as firefly luciferase under the control of these transcriptional control elements. This expression cassette can then be integrated into the genome of immortalized T cells (e.g. Jurkat). Upon external stimulation, the change of luciferase expression can be quantitatively measured by chemiluminescence. These reporter assays offer the potential to facilitate faster measurements of T cell activation, superior sensitivity, wider dynamic range and more standardized operation (Michelini et al., 2010). For example, Grailer et al. reported a collection of these assays for various immune modulators (Grailer et al., 2016).

However, these reporter assays may also suffer from their own limitations. First, since the reporter gene expression is engineered to be controlled by the enhancer of choice, its induction or inhibition is restricted to a specific signaling pathway; when physiological responses to external stimuli are mediated by multiple intracellular signaling pathways, only part of the signaling matrix can be assessed. Second, most of reporter cell lines are established by random integration of the expression cassette into the host cell genome. The impact of local chromatin structure on reporter expression cannot be controlled, and many clones need to be screened to obtain a robust and stable reporter cell line. To circumvent the limitation of only one signaling pathway readout, a recent effort was made by co-introducing NF-kB, AP-1, and NF-AT fluorescence reporters into the Jurkat cell (Jutz et al., 2016). This allows the measurement of simultaneous activation of all three major signaling pathways. However, the reporter gene expression cassettes were still randomly integrated into cellular genome, thus the interactions of these pathways with other regulatory elements and the precise interactions among these three transcription factors could still be very different from that of the endogenous signaling.

We have devised a novel approach to overcome these aforementioned limitations. In this approach, the endogenous target gene is replaced by a reporter gene. This will elicit a native response due to the preservation of the entire transcriptional machinery and the native chromatin environment. We first tested this strategy by replacing one of the bi-allelic *IL2* open reading frame (ORF) of the Jurkat cell line with a firefly luciferase gene using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology (Chu et al., 2015), while keeping the other *IL2* allele intact. As expected, the resulting Jurkat cell line (1F2) showed both increased luciferase expression and IL-2 secretion upon TCR stimulation. Studies were subsequently conducted with 1F2 cells engineered to express human glucocorticoid-Induced TNFR-related protein (hGITR) to evaluate the co-stimulatory effect of an agonistic GITR antibody. Our results suggest that inserting a reporter gene downstream of the endogenous target gene promoter may represent a more physiologically relevant method for constructing a reporter cell line.

2. Materials and methods

2.1. Cell lines and reagents

Parental Jurkat cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Phorbol 12-myristate 13-acetate (PMA) and ionomycin were obtained from Sigma-Aldrich (St. Louis, MO). Anti-CD3 and anti-CD28 antibodies were from eBioscience (San Diego, CA). Bright-Glo substrate was obtained from Promega (Madison, WI). Lentivirus vector for GITR expression was constructed at Merck and produced at System Biosciences (Palo Alto, CA). PE conjugated anti-human GITR was from R & D Systems (Minneapolis, MN). Monoclonal GITR agonist antibody was generated at Merck.

2.2. Single-guide RNA (sgRNA) design, preparation and Cel-1 assay

Five sgRNAs designed to cut DNA sequence close to the start codon of human IL-2 locus were prepared with in vitro transcription and their activity was tested in Cas9-expressing HEK293 cells using Cel-1 assay, as described in (Kouranova et al., 2016). sgRNA with the highest activity (sgRNA1) was used for IL-2 locus targeting experiments.

2.3. Generation of Jurkat cells with a luciferase gene inserted at endogenous IL-2 locus

CRIPSR/Cas9 technology is utilized to insert the luciferase ORF directly under ATG of one *IL2* allele while leaving the other *IL2* allele intact. Briefly, 2 μg Cas9 protein, 1 μg sgRNA and 0.5 μg circular donor plasmid in SE solution were nucleofected into 2×10^5 parental Jurkat cells with Lonza 96-well Shuttle Device using program CL-120. Nucleofected cells were cultured for 3 days before they were further plated at < 1 cell/well for selection of single cell clones. The insertion junctions of each clone were analyzed by junction PCRs and confirmed by sequencing. Approximately 300 clones were analyzed and a single clone (1F2) was identified to have luciferase replacement at one of the two alleles of *IL2* with unmodified copy at the other allele. PCR reactions that amplify the donor plasmid backbone confirmed that no extra copy of the donor plasmid exists in the genome as random integration.

2.4. Luciferase assay

The culture medium for the parental Jurkat cells or 1F2 cells was RPMI-1640 supplemented with 10% fetal calf serum (ThermoFisher, Waltham, MA), 4 mM L-glutamine (ThermoFisher, Waltham, MA) and 50 units/ml of penicillin and 50 units/ml of streptomycin (ThermoFisher, Waltham, MA). The cells were cultured in a 37 °C incubator with 5% CO_2. Cells were washed with phenol red-free RPMI media (ThermoFisher, Waltham, MA) prior to the luciferase assay. 200 ng/ml PMA plus various concentrations of ionomycin were utilized to stimulate the cells in phenol red-free RPMI media for 6 h at 37 °C followed by the addition of Bright-Glo substrate for incubation of additional 5 min. The plates were then analyzed by EnVision plate reader (PerkinElmer, Waltham, MA). Alternatively, cells were stimulated with immobilized 2 μg/ml (optimal) or 0.8 μg/ml (suboptimal) anti-human CD3 antibody combined with various concentrations of soluble anti-human CD28 antibody for 6 h before luciferase assay. For GITR over-expressing Jurkat-1F2 cells, 0.1–10 μg/ml anti-GITR antibody was added along with anti-CD3 antibody. The data were graphed and analyzed using GraphPad Prism software.

2.5. IL-2 secretion assay

Parental Jurkat cells or 1F2 cells were stimulated with immobilized 2 μg/ml (optimal) or 0.8 μg/ml (suboptimal) anti-CD3 antibody along with various concentrations of PMA for 48 h at 37 °C. Secreted IL-2 was measured using CisBio IL-2 assay kit (Bedford, MA) and EnVision plate