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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

reader. Alternatively, cells were stimulated with immobilized 0.8 μg/ml anti-CD3 antibody alone or combined with 0.5 μg/ml soluble anti-CD28 antibody for 48 h followed by IL-2 assay. For GITR overexpressing 1F2 cells, 0.1–10 μg/ml anti-GITR antibody was added along with anti-CD3 antibody.

2.6. Lentivirus transduction for the expression of human GITR

1F2 cells were re-suspended with fresh growth media at concentrations of 10^5–10^7 cells/ml. For transduction, cells were mixed with lentivirus expressing human GITR for 20 min at room temperature (RT). The multiplicity of infection (MOI) was 0.04. Cells were then centrifuged for 30 min at 800 × *g* at RT followed by removal of virus containing medium and re-suspension of cell pellet with fresh complete culture media. Transduced cells were cultured for 72 h prior to selection. For selection, the transduced 1F2 cell pool was diluted to 40,000 cells/ml in fresh culture medium containing 1 mg/ml Geneticin (Thermo Fisher, Waltham, MA). Cells were cultured for 4 days and the surface expression of human GITR was determined by staining with PE conjugated anti-human GITR followed by analysis using flow cytometer FACSCanto II (BD Biosciences, San Jose, CA).

2.7. Statistical analysis

Experiments were performed at least in triplicate. Data were plotted as the mean ± SEM. Statistical significance was calculated with Student's *t*-test and considered meaningful at $p < 0.05$.

3. Results and discussion

3.1. Development of Jurkat cells expressing luciferase reporter gene under the control of endogenous IL-2 promoter

Placing a reporter gene under the control of endogenous *IL2* promoter should preserve native chromatin structure and responsiveness to facilitate the identification of potent yet physiologically relevant immune modulator(s). To achieve this goal, we used the state of the art CRIPSR/Cas9 technology to insert the firefly luciferase reporter gene right at the translation start codon of endogenous *IL2* gene. Five different sgRNAs were designed within the 50 bp region around *IL2* ATG codon. Their activity of generating double strand DNA break were compared in Cas9-expressing HEK293 cells and sgRNA1 was chosen for Jurkat cell genome editing (Fig. S1). The donor plasmid contains a luciferase reporter gene flanked with sequences homologous to the sequences neighboring *IL2* ATG starting codon (Fig. 1A). The co-delivery of Cas9/sgRNA1 ribonucleoprotein complex and the donor plasmid into Jurkat cells produced a double strand DNA break close to *IL2* ATG codon and the *IL2* allele was replaced by luciferase reporter gene via homologous recombination (Fig. 1A and B). The cells were then undergone single-cell cloning process and clone 1F2 was selected and confirmed to have only one of the two *IL2* alleles replaced by luciferase gene and the other IL-2 allele remaining intact (Supplementary Fig. S1). The DNA sequence of the donor plasmid is shown in Supplementary File 2.

3.2. Evaluation of luciferase activities of 1F2 cell line upon stimulation

To evaluate luciferase activities in 1F2 cell line, 1F2 cells were stimulated under a range of stimulatory conditions. PMA can activate PKC in Jurkat cells to increase the transcription of IL-2 (Ohtsuka et al., 1996). Moreover, IL-2 mRNA can be stabilized by phorbol ester and Ca^{2+} ionophore, such as PMA and ionomycin, or via engagement of T cell receptor (TCR) via agonistic anti-CD3 along with anti-CD28 co-stimulation (Fraser et al., 1991; Ohtsuka et al., 1996). In this study, 1F2 cells were stimulated with 200 ng/ml PMA along with various concentrations of ionomycin. Increased luciferase activities were demonstrated in an ionomycin concentration-dependent manner (Fig. 2A). Dose-dependent enhancement of luciferase activities was also observed upon stimulation with anti-CD3 antibody along with various concentrations of anti-CD28 antibody (Fig. 2B). These data suggest that in 1F2 cells, IL-2 transcription is induced in a similar fashion to the parental Jurkat cells.

3.3. IL-2 secretion in 1F2 cell line upon stimulation

With intact TCR signaling and luciferase activities demonstrated in 1F2 cells, we next evaluated if 1F2 cells maintain the functional IL-2 secretion upon stimulation. In this study, for comparison purpose, both parental Jurkat cells and 1F2 cells were stimulated under the same conditions and secreted IL-2 was measured. Upon stimulation with anti-CD3 antibody along with various concentrations of PMA as a co-stimulator, increased IL-2 secretion was observed in both parental Jurkat cells and 1F2 cells (Fig. 3A). Consistent with only a single allele of *IL2* in 1F2 cells, the amount of IL-2 secreted from 1F2 cells is roughly half of parental Jurkat cells (Fig. 3A). Further stimulation was conducted with anti-CD3 antibody along with various concentrations of anti-CD28 as a co-stimulator. IL-2 secretion increased in an anti-CD28 dose-dependent manner (Fig. 3B). In agreement with the data from stimulation using anti-CD3 and PMA, the levels of IL-2 secretion from 1F2 cells were approximately half of the parental Jurkat cells (Fig. 3C). These results suggest that the IL-2 induction and secretion pathway remains intact in 1F2 cell line.

3.4. Evaluation of agonistic anti-human GITR in 1F2 cell line expressing human GITR (1F2-hGITR)

GITR, a member of the TNFR superfamily, is expressed in many components of the innate and adaptive immune system (Nocentini et al., 1997; Nocentini and Riccardi, 2005; Hanabuchi et al., 2006). GITR is an important costimulatory molecule for T cells (Shimizu et al.,

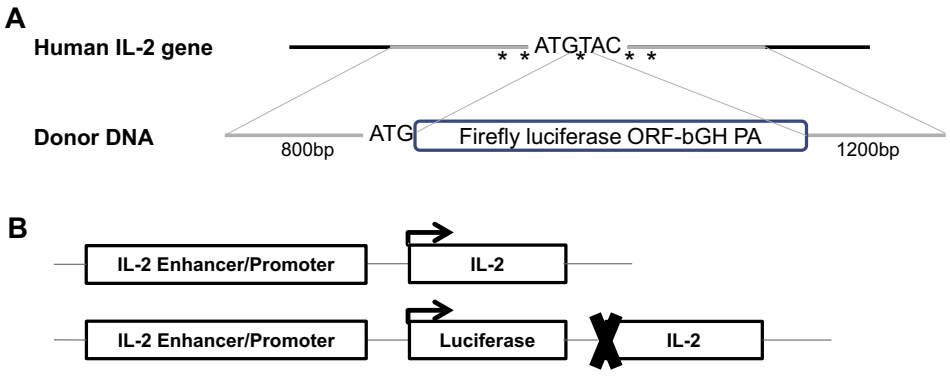

Fig. 1. Development of Jurkat cells expressing luciferase reporter under control of endogenous IL-2 promoter. (A) The design of CRISPR/Cas9 trageting strategy and donor template for homologous recombination. Five gRNA (indicated with "*") targeting sequences around the start codon of human IL-2 gene were tested. A donor template was designed to have sequences homologous to human IL-2 gene flanking the firefly luciferase open reading frame and bovine growth hormone poly A signal (bGH PA). (B). A schematic illustration of the replacement of only one IL-2 allele by firefly luciferase reporter gene.

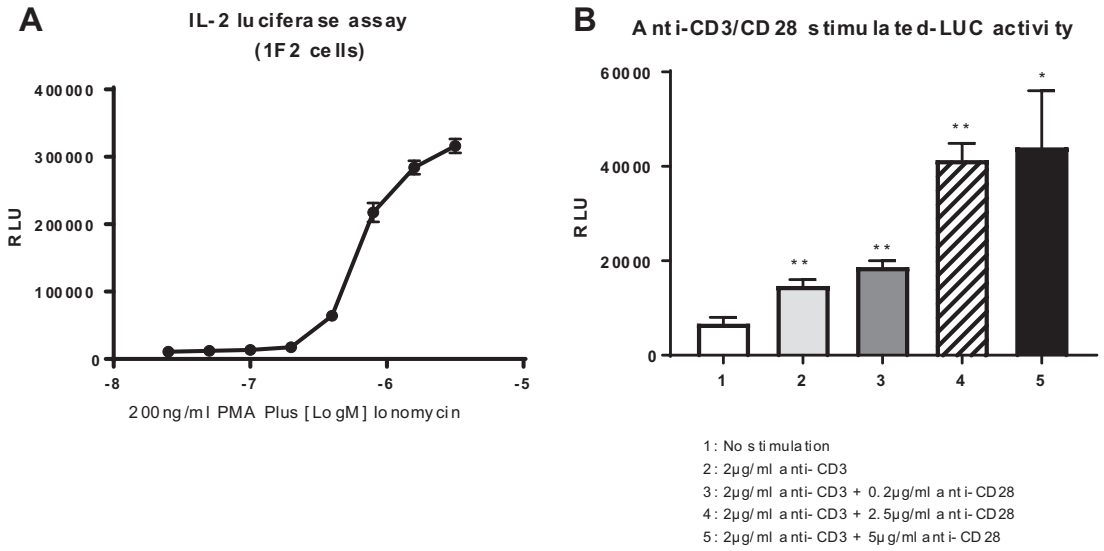

Fig. 2. Assessment of 1F2 cell line expressing firefly luciferase reporter under control of endogenous IL-2 promoter. (A). Luciferase reporter activities (shown as relative light units (RLU)) upon stimulation with 200 ng/ml PMA along with various concentrations of ionomycin. (B). Luciferase reporter activities in response to stimulation with optimal concentrations of agonistic anti-CD3 (2 μg/ml, immobilized) and 5 μg/ml soluble agonistic anti-CD28. The results are shown as the mean of triplicates with SEM. The experiment was repeated 3 times with the representative data shown here. *$p < 0.05$; **$p < 0.005$.

2002; Tone et al., 2003; Ronchetti et al., 2004). Agonistic anti-GITR antibody, but not the isotype-control antibody, in conjunction with anti-CD3 antibody, exerted a costimulatory effect on both CD4 + and CD8 + single-positive T cells from GITR + / + but not from GITR − / − mice (Ronchetti et al., 2004), suggesting that GITR functions as a co-stimulatory molecule in combination with the activation of the CD3–TCR complex. Indeed, numerous GITR stimulating agents demonstrated anti-tumor efficacy in preclinical models and several of them have entered clinical trials (Chrisann and Postow, 2016; Leyland et al., 2017). This warrants the need of a robust cell-based potency assay to screen GITR agonists and also control drug quality in clinical studies. A conventional way for the assessment of GITR agonistic activities is to insert a firefly luciferase reporter gene downstream to five copies of the NFκB response element in Jurkat cells (Leyland et al., 2017). The estimated agonist activities using this artificial promoter system may not truly reflect the physiological event considering the limitations of conventional reporter cell line described above. In our study, the co-stimulatory effect of a novel anti-GITR antibody was investigated in 1F2 cells transduced with lentivirus expressing human GITR. We intended to achieve approximately single copy of GITR per cell by using very low MOI (MOI = 0.04). After transduction, cell surface expression of GITR was confirmed by staining cells with fluorescence-labeled anti-GITR antibody followed by flow cytometery analysis (Fig. 4A). Luciferase activity of this cell line (1F2-hGITR) was subsequently assessed under various stimulation conditions, including combination of PMA and ionomycin, as well as immobilized anti-CD3 and soluble anti-CD28 at optimal concentrations (2 and 5 μg/ml respectively). Robust responses of luciferase activity were observed under these stimulatory conditions (Fig. 4B–C), suggesting an intact stimulatory response upon engaging TCR and the co-stimulatory molecule. This cell line also did not respond to GITR agonist antibody stimulation alone (Fig. 4D–E), consistent with the function of GITR as a co-stimulator of TCR. The co-stimulatory effect of GITR agonism was then evaluated with immobilized anti-CD3 antibody (0.8 μg/ml) in the presence of various concentrations of anti-GITR antibody. Luciferase activities were augmented in an anti-GITR antibody concentration-dependent manner (Fig. 4D), consistent with the co-stimulatory role of GITR. We further investigated IL-2 secretion in response to CD3 and GITR activation, with or without additional CD28 activation. Immobilized anti-CD3 antibody alone (0.8 μg/ml) or in combination with

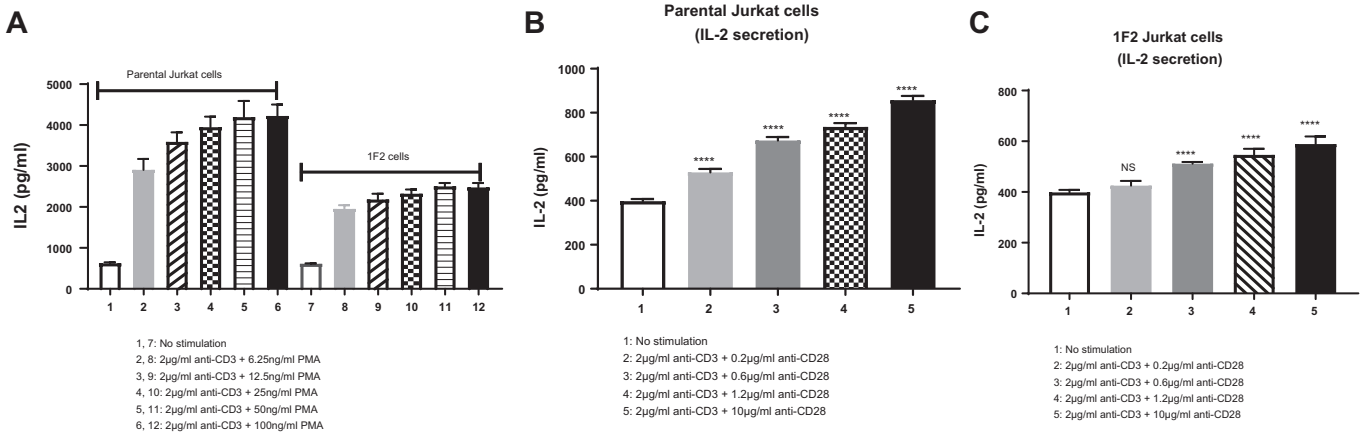

Fig. 3. Secretion of IL-2 by 1F2 cells in response to different stimulatory factors. (A). IL-2 secretion upon stimulation with 2 μg/ml anti-CD3 (immobilized) and various concentrations of PMA in parental Jurkat or 1F2 cells. (B and C). IL-2 secretion in (B) parental Jurkat and (C) 1F2 cells in response to stimulation of immobilized anti-CD3 (2 μg/ml) and various concentrations of anti-CD28. The results are shown as the mean of quadruplicates in (A) and octuplicates in (B) and (C) with SEM. The experiment was repeated 3 times with the representative data shown here. ****$p < 0.0001$.

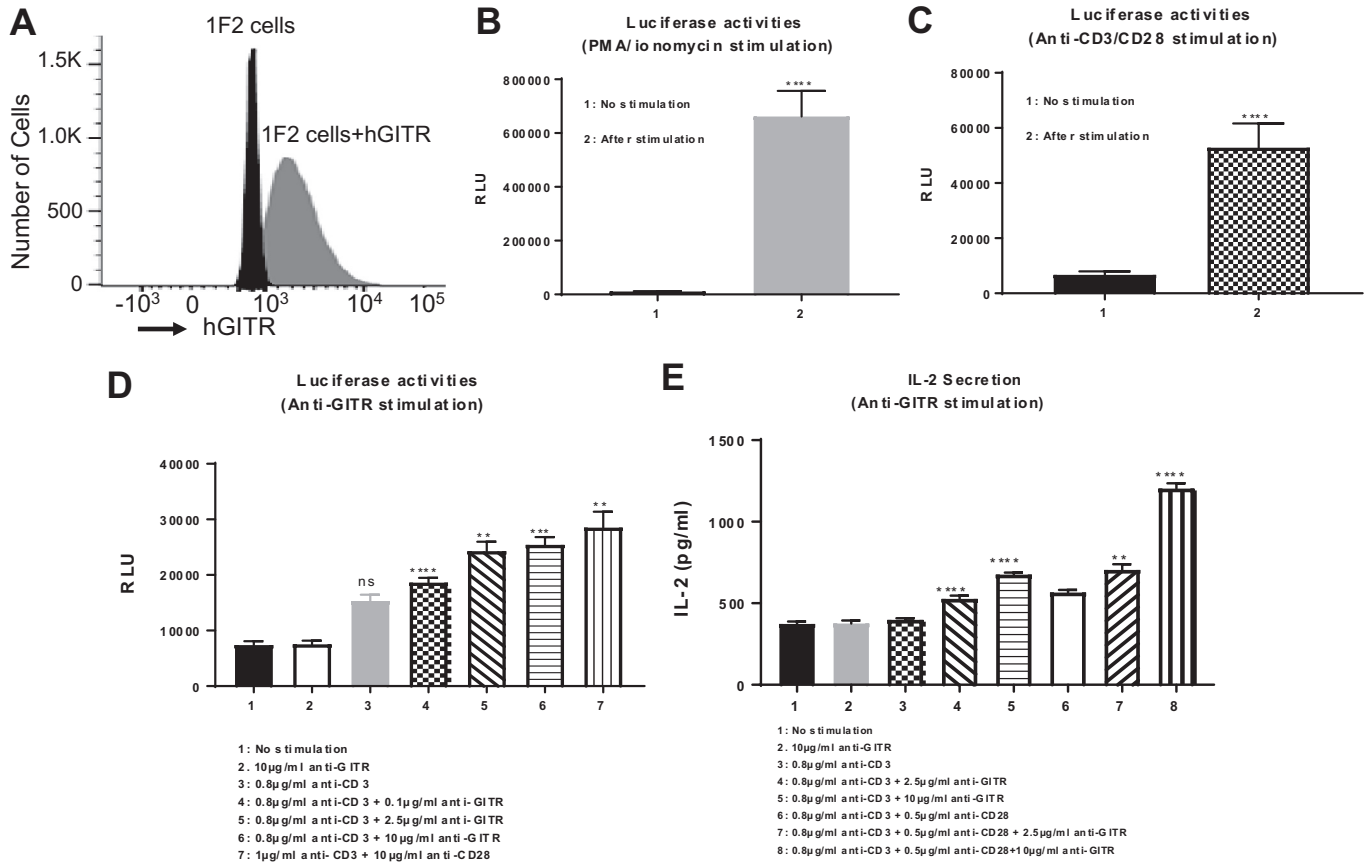

Fig. 4. Co-stimulatory activities of anti-hGITR in 1F2-hGITR cells. (A). 1F2 cells transduced by hGITR expressing lentivirus (1F2-hGITR) were analyzed by flow cytometry for surface expression of human GITR. (B). Luciferase activities of 1F2-hGITR cells in response to the stimulation of PMA (200 ng/ml) and ionomycin (1 μM). (C). Luciferase activities of 1F2-hGITR cells in response to immobilized anti-CD3 (2 μg/ml) and soluble anti-CD28 (5 μg/ml). The results are shown as the mean of 12 replicates with SEM. ****$p < 0.0001$. (D–E). (D) Luciferase reporter activities and (E) IL-2 secretion of 1F2-hGITR cells upon stimulation by various concentrations of agonistic anti-GITR antibody with or without suboptimal concentration of immobilized anti-CD3 (0.8 μg/ml), or with immobilized anti-CD3 (0.8 μg/ml) and soluble anti-CD28 (0.5 μg/ml). The experiments were repeated 3 times with the representative data shown here. In (D), the results are shown as the mean of replicates of six with SEM. **$p < 0.007$; ***$p < 0.0002$; ****$p < 0.0001$. In (E), the results are shown as the mean of replicates of eight with SEM. **$p < 0.002$; ****$p < 0.0001$.

soluble anti-CD28 antibody (0.5 μg/ml) were used in this study. A dose-dependent increase of IL-2 secretion upon GITR agonism in conjunction with TCR engagement was observed. Interestingly, this augmented IL-2 secretion increased further upon CD28 co-stimulation (Fig. 4E), suggesting a synergy of co-stimulatory role of CD28 and GITR. Our findings suggest that 1F2-hGITR cells could be utilized to interrogate agonistic anti-GITR antibodies in the context of native *IL2* "chromatin milieu". In comparison to the conventional reporter system under the control of artificial promoters/enhancers, this cell line represents a unique and potentially more physiologically relevant cell model to investigate and differentiate immune modulators. Our results also pave the way for using the same approach to construct reporter cell lines for evaluating other immuno modulators involved in different cell signaling pathways.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2017.08.006.

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Conflicts of interest

None.

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