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Glucocorticoids potentiate the inhibitory capacity of programmed cell death 1 by up-regulating its expression on T cells

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ABSTRACT

The inhibitory co-receptor programmed cell death 1 (PD-1, *Pdcd1*) plays critical roles in the regulation of autoimmunity, anti-cancer immunity, and immunity against infections. Immunotherapies targeting PD-1 have revolutionized cancer management and instigated various trials of improved cancer immunotherapies. Moreover, extensive trials are underway to potentiate PD-1 function in order to suppress harmful immune responses. Here, we found that both natural and synthetic glucocorticoids (GCs) up-regulate PD-1 on T cells without altering the expression levels of other co-receptors and cell-surface molecules. The GC-induced up-regulation of PD-1 depended on the transactivation of PD-1 transcription mediated through the glucocorticoid receptor (GR). We further found that a GC response element (GRE) 2525 bp upstream from the transcription start site of *Pdcd1* is responsible for GC-mediated transactivation. We also observed that *in vivo* administration of GCs significantly up-regulates PD-1 expression on tumor-infiltrating T cells. By analyzing T cells differing in PD-1 expression, we directly demonstrated that the amount of PD-1 on the cell surface correlates with its inhibitory effect. Accordingly, GCs potentiated the capacity of PD-1 to inhibit T cell activation, suggesting that this PD-1-mediated inhibition contributes, at least in part, to the anti-inflammatory and immunosuppressive effects of GCs. In light of the critical roles of PD-1 in the regulation of autoimmunity regulation, we expect that the potentiation of PD-1 activity may offer a promising therapeutic strategy for managing inflammatory and autoimmune diseases. Our current findings provide a rationale for strategies seeking to enhance the inhibitory effect of PD-1 by increasing its expression level.

The inhibitory co-receptor programmed cell death 1 (PD-1) is inducibly expressed on T cells upon activation and inhibits T cell receptor (TCR) signaling by recruiting SHP-2, a protein tyrosine phosphatase in a manner dependent on the engagement by either of its two ligands, PD-L1 and PD-L2 (1,2). PD-1-dependent regulation of TCR signal is required for the establishment and the maintenance of immune tolerance to self and the suppression of excess immune responses to pathogens. Mice deficient for PD-1 spontaneously develop tissue-specific autoimmune diseases and die of sever inflammatory tissue damage upon infection with pathogens that normally establish chronic infection in PD-1-sufficient mice (3,4). On the other hand, this immunoregulatory function of PD-1 is often hijacked by tumors to escape from the cancer immune surveillance. Cancer immunotherapies targeting PD-1 therefore successfully eradicates various types of tumors by restoring the tumoricidal activities of tumor-specific T cells (5,6). As anticipated from the autoimmune phenotypes of PD-1-deficient mice, targeted blockade of PD-1 activates not only tumor-specific T cells but also self-reactive T cells to provoke inflammatory tissue damages termed immune-related adverse events (irAEs)(7,8).

Various kinds of immunosuppressants have been developed and widely used for the treatment of autoimmune diseases, allergic diseases, transplant rejection and so on (9). However, most of these drugs have a low therapeutic index and can cause various side effects that are dependent on time and dose, requiring special caution in their use. Although the molecular mechanisms of action of immunosuppressants have been extensively analyzed, the actual effects of immunosuppressants on the expression and function of immune-related molecules including immune-checkpoint molecules have not been fully understood, which makes the rational design of regimen with higher efficacy and safety difficult.

Among various immunosuppressants, glucocorticoids (GCs) have been a mainstay drug in the treatment of numerous inflammatory diseases including irAEs of cancer immunotherapies. GCs, a class of steroid hormones playing critical roles in diverse physiological processes, have profound anti-inflammatory and immunosuppressive activities (10). Pharmacological effects of GCs are predominantly mediated through the glucocorticoid receptor (GR), which is a member of the nuclear receptor superfamily of ligand-dependent transcription factors (TFs). Upon interacting with GC in the cytoplasm, GR translocates into the nucleus where it functions either as a transcriptional activator or repressor (11). GCs attenuate the expression of pro-inflammatory cytokines, induce apoptotic cell death, or impede the recruitment of immune cells by inhibiting the expression of adhesion molecules and chemokines in the suppression of inflammation (10). In T cells, GCs suppress T cell activation by abrogating TCR-induced gene expression or inhibiting dendritic cell maturation (12-15). Despite its potent anti-inflammatory effects, recent studies revealed that GCs might also augment immune reposes by up-regulating genes involved in innate immunity (16,17). In addition to genomic effects, which involve the induction/suppression of genes, GCs have also been reported to function in a non-genomic manner (18). Thus, GCs have extremely diverse and complicated effects on immune and non-immune cells, which have not been fully understood.

In this study, we found that natural and synthetic GCs up-regulate PD-1 on T cells by augmenting PD-1 transcription without changing the expression levels of other co-receptors. We also observed that *in vivo* administration of GCs significantly up-regulated PD-1 expression on tumor-infiltrating T cells. We identified a GC response element (GRE) that is responsible for the GC-mediated transactivation in the promoter region of PD-1 gene. By analyzing T cells expressing PD-1 to a variable degree, we directly demonstrated that the amount of PD-1 on cell surface correlated with its inhibitory effect. Accordingly, GCs potentiated the inhibitory effect of PD-1 on the antigen-dependent functional T cell activation. These results provide new insights into the mechanisms underlying the immunosuppressive effects of GCs and provide a rationale for the strategy to enhance the inhibitory effect of PD-1 by augmenting its expression level.

Results

Dexamethasone strongly enhances PD-1 expression on T hybridoma cells

A variety of drugs have been developed as immunosuppressants with different mechanisms of action. First, we assessed the effects of immunosuppressants on PD-1 expression using DO11.10 T hybridoma cells that endogenously express PD-1 on their surface and up-regulate PD-1 expression upon TCR-dependent activation. Treatments with cyclophosphamide monohydrate (CPA, an alkylating agent inhibiting DNA synthesis) and mizoribine (MZB, an imidazole nucleoside inhibiting the de novo synthesis of guanosine) did not affect PD-1 expression levels at all (Fig. 1, *A* and *B*). The expression levels of PD-1 were slightly enhanced by everolimus (ERL, a selective inhibitor of the serine-threonine kinase mammalian target of rapamycin (mTOR)) (Fig. 1, *A* and *B*), consistent with the previous report that mTOR induces T-box transcription factor (T-bet) that represses the transcription of PD-1 mRNA (18,19). High-dose cyclosporin A (CPA, a selective inhibitor of calcineurin) reduced PD-1 expression (Fig. 1, *A* and *B*), which suggests that the endogenous expression of PD-1 on DO11.10 T hybridoma cells depends on Ca^{2+}-calcineurin-NFAT signaling, as is the case with PD-1 expression upon T cell activation (20). Notably, we found that PD-1 expression was strongly augmented by the treatment with dexamethasone (Dex, a synthetic GC with potent anti-inflammatory activities) in dose- and time-dependent manners (Fig. 1, *A–C*). Although Dex also induced apoptotic cell death of DO11.10 T hybridoma cells in dose- and time-dependent manners (Fig. 1, *D* and *E*; and Fig. S1), the augmentation of PD-1 expression was observed on live cells and preceded cell death, indicating that PD-1 up-regulation was not due to non-specific

effects caused by cell death. Consistently, cell death but not PD-1 up-regulation by Dex was canceled by Z-VAD-FMK, an inhibitor of apoptotic cell death (Fig. S1). In addition, no statistically significant correlation was detected between the changes in PD-1 expression level and viabilities of cells treated with immunosuppressants at various concentrations (Fig. S2). We also confirmed that apoptosis inducers do not necessarily up-regulate PD-1 expression (Fig. S3). Collectively, PD-1 up-regulation by Dex is likely mediated by its specific pharmacological action.

GCs selectively enhance PD-1 expression on T hybridoma cells

In addition to Dex, various synthetic analogues of GC have been developed for the treatment of inflammatory diseases. When we examined natural and synthetic GCs, all GCs tested enhanced PD-1 expression on DO11.10 T hybridoma cells to a similar extent as Dex. Intriguingly, expression levels of other cell surface molecules tested were not affected by Dex and hydrocortisone (HC) (Fig. 2, *A* and *B*; and Fig. S4, *A–C*).

DO11.10 T hybridoma cells recognize 323–339 segment of chicken ovalbumin ($pOVA_{323–339}$) in the context of $I\text{-}A^d$. When we stimulated DO11.10 T hybridoma cells by co-culturing with $pOVA_{323–339}$-pulsed IIA1.6 B lymphoma cells expressing $I\text{-}A^d$, DO11.10 T hybridoma cells were activated to express or up-regulate activation markers such as CD69, CD28, 4-1BB, and RANKL as well as PD-1 to a variable degree (Fig. 2*C*). The addition of Dex and HC in the co-culture attenuated the activation-induced expression/up-regulation of most activation markers, which likely reflects the reduced levels of T cell activation by GC-treatment. On the other hand, the expression level of PD-1 was further augmented by GCs, indicating that the magnitude of PD-1 up-regulation by GCs outweighs the magnitude of PD-1 down-regulation due to the reduced T cell activation by GCs (Fig. 2*C* and Fig. S4*D*). Therefore, the augmentation of the expression by the treatment with GCs was highly specific to PD-1.

GCs selectively enhance PD-1 expression on primary T cells

We next tested whether GCs also augment PD-1 expression on primary T cells. Because naïve T cells do not express PD-1, we induced PD-1 expression by stimulating naïve T cells with anti-CD3 and anti-CD28 Abs for 16 hours. When we added GCs during stimulation, the expression levels of PD-1 were augmented both on $CD4^+$ and $CD8^+$ T cells with a clear dependency on the dose of GCs (Fig. 3, *A* and *B*; and Fig. S5, *A* and *B*). In contrast, the induction of activation markers such as CD25, CD44, and 4-1BB was attenuated by GCs (Fig. 3*C*). The levels of their down-regulation by GCs were smaller compared to those in stimulated DO11.10 T hybridoma cells, probably because anti-CD3 and anti-CD28 Abs activated primary T cells so strongly that GCs could only partially inhibit T cell activation.

The addition of GCs for 24 hours after the stimulation also resulted in the augmentation of PD-1 expression both on $CD4^+$ and $CD8^+$ T cells (Fig. 3, *D* and *E*; and Fig. S5, *C* and *D*). As is the case with un-stimulated DO11.10 T hybridoma cells, Dex and HC did not substantially affect the expression levels of most cell surface molecules on pre-activated $CD8^+$ T cells (Fig. 3*F* and Fig. S5*E*). These results indicate that GCs selectively up-regulate PD-1 on primary T cells as well.

In vivo treatment with GC up-regulates PD-1 expression on tumor-infiltrating $CD8^+$ T cells

GCs are commonly used to mitigate irAEs in cancer patients receiving immunotherapies targeting PD-1 and CTLA-4 (8). To investigate the effect of GCs on PD-1 expression in the tumor setting, we analyzed PD-1 expression on T cells infiltrating into tumors of CT26 mouse colon carcinoma cells. As is well known, tumor-infiltrating $CD4^+$ and $CD8^+$ T cells expressed PD-1 at substantial levels (Fig. 4*A*). Notably, the administration of Dex significantly increased the expression levels of PD-1 as well as the proportion of PD-1 expressing cells among tumor-infiltrating $CD8^+$ T cells compared with control mice treated with the solvent, DMSO (Fig. 4, *A–C*). Although not statistically significant, tumor-infiltrating $CD4^+$ T cells also exhibited a tendency toward increased levels of PD-1 expression upon Dex treatment (Fig. 4, *A–C*). These results indicate that GCs have a potential to up-regulate PD-1 expression *in vivo*.

GC-GR complex up-regulates PD-1 mRNA by binding to a GRE in the promoter region

We tested the involvement of GR in the up-regulation of PD-1 and cell death by GCs using RU486, GR antagonist. The up-regulation of PD-1 as well as cell death by Dex was canceled by the addition of RU486, indicating that the up-regulation of PD-1 and cell death by GCs are mediated through GR (Fig. 5*A*). Then we examined the effects of GCs on PD-1 expression at the mRNA

level. The amount of PD-1 mRNA was increased about 2 folds by 1 hour after the addition of GCs and further augmented over time, which was canceled by RU486 (Fig. 5*B* and *C*). These results indicate that GCs augment PD-1 expression at the mRNA level through the binding to GR.

Then, we investigated whether the GR-GC complex directly increases the transcription of PD-1 gene. By using the TF binding site prediction database, we identified three putative GREs at 2,525, 2,740, and 2,790 bp upstream from the transcription start site of PD-1 mRNA and termed them as GRE1, 2, and 3, respectively. To examine whether the augmentation of PD-1 expression by GCs was mediated through these GREs, we generated a series of reporter constructs containing intact or mutated GREs. Dex-treatment strongly augmented the promoter activity in the presence of GRE1, suggesting that GRE1 is responsible for the augmentation of PD-1 expression by GCs (Fig. 5*D*).

GCs potentiate the inhibitory effect of PD-1 by increasing the cell surface amount of PD-1

Upon antigen stimulation, DO11.10 T hybridoma cells secrete IL-2 in a manner dependent on the amount of antigen. Thus, we can evaluate the magnitude of functional T cell activation based on the amount of secreted IL-2. As reported previously, IL-2 production from DO11.10 T hybridoma cells upon antigen stimulation was strongly inhibited when IIA1.6 cells overexpressing PD-L1 (IIA1.6-PD-L1 cells) were used as antigen presenting cells (APCs) and the inhibitory effect of PD-1 was completely canceled by anti-PD-L1 blocking Ab (21,22). The PD-L1-mediated inhibition was abolished by the targeted deletion of PD-1 gene in DO11.10 T hybridoma cells and restored by the retroviral reconstitution of PD-1 (Fig. 6, *A* and *B*). By using this system, we examined the correlation between the cell surface amount of PD-1 and its inhibitory effect. We overexpressed PD-1 at various levels by using five different promoters (EF1α, CMV, CAG, MC1, SV40) with or without a polyadenylation (pA) signal, which plays essential roles in the stabilization of mRNA (Fig. 6, *C* and *D*). These cells were stimulated with $pOVA_{323–339}$-pulsed IIA1.6-PD-L1 cells, and PD-1-mediated inhibitory effects were calculated by comparing the amount of secreted IL-2 in the presence or absence of anti-PD-L1 blocking Ab. We observed a strong positive correlation of the cell surface expression level of PD-1 with its inhibitory effect (Fig. 6*E*).

Then, we evaluated the functional consequence of PD-1 up-regulation by GCs. DO11.10 T hybridoma cells were treated with Dex and live cells were stimulated with $pOVA_{323–339}$. As anticipated from the immunosuppressive activity of GCs, the pre-treatment with Dex substantially reduced the amount of IL-2 secreted from DO11.10 T hybridoma cells upon antigen stimulation. PD-1 engagement further reduced the IL-2 production from activated DO11.10 T hybridoma cells. Intriguingly, when we compared the levels of PD-1-dependent inhibition with or without Dex pre-treatment, PD-1 inhibited IL-2 production more efficiently when DO11.10 T hybridoma cells were pre-treated with Dex (Fig. 6, *F* and *G*). Thus, GCs can potentiate the inhibitory effect of PD-1 by increasing the cell surface amount of PD-1.

No substantial effect of GCs on ectopic PD-1

In order to confirm the specificity of PD-1 up-regulation by GCs, we evaluated the effect of GCs on ectopic PD-1. We treated DO11.10 T hybridoma cells that express exogenous PD-1 under LTR promoter but lack endogenous PD-1 expression with GCs. As expected, GCs failed to augment the cell surface amount of ectopic PD-1 (Fig.S6*A*). Accordingly, GCs failed to potentiate the inhibitory effect of ectopic PD-1 (Fig. S6, *B* and *C*). These results strongly suggest that GC-GR complex transactivates PD-1 transcription by directly binding to GRE1 located in the promoter region of PD-1 gene.

Discussion

GCs have been widely used as anti-inflammatory and immunosuppressive agents for the treatment of a variety of inflammatory and autoimmune diseases. However, despite extensive clinical and experimental studies, the extremely diverse and complicated effects of GCs on immune and non-immune cells have not been fully understood. In the current study, we found that GCs augment the expression level of PD-1 on T cells but not other cell surface molecules including CD25, CD44, CD69, 4-1BB, RANKL, and LAG-3. Dex has been reported to enhance the expression of PD-1 and CTLA-4 on T cells, but the underlying mechanism and the functional consequence of the up-regulation were largely unknown (23-25). We revealed that the treatment with GCs leads to the transactivation of PD-1 expression in a manner dependent on GR and GRE in the promoter region of PD-1 gene. We directly demonstrated that the amount of PD-1 on cell surface strongly correlated with its inhibitory effect. Accordingly, augmented expression of PD-1 by GCs lead to the proportional

enhancement of the inhibitory effects of PD-1 against the antigen-dependent functional T cell activation. These findings suggest that PD-1-mediated inhibition contributes, at least in part, to the anti-inflammatory and immunosuppressive effects of GCs.

Naïve T cells do not express PD-1 but rapidly express PD-1 upon antigen stimulation. This initial induction of PD-1 is driven by multiple TFs including nuclear factor of activated T cells 1 (NFATc1) and c-Fos/activator protein-1 (AP-1), which are activated by signaling through TCR or Notch (20,26,27). In the setting of chronic viral infections and tumors, chronic antigen exposure induces high and sustained expression of PD-1 on $CD8^+$ T cells, resulting in their functional exhaustion (4,28). Forkhead box protein O1 (FoxO1), NFATc1, and nuclear receptor subfamily 4 group A (NR4A) have been reported to mediate the expression of PD-1 on exhausted $CD8^+$ T cells (29-32). While these TFs function as transcriptional activators in PD-1 expression, T-bet and Blimp-1 have been reported to function as transcriptional repressors in PD-1 expression (19,33). These TFs cooperatively or competitively regulate PD-1 expression by directly binding to the promoter region of PD-1 gene. GRs are known to exert a diverse function by binding to promoter sequences together with other TFs or by physically interacting with other TFs (11). In the present study, we identified a GRE in the promoter region of PD-1 gene that is responsible for the transactivation by GC-treatment. Therefore, it is likely that GR directly binds to the promoter region of PD-1 gene in response to GC-treatment and acts as a transcriptional activator to augment PD-1 transcription. Further studies are expected to reveal a possible positive or negative cooperation of GC-GR complex with other TFs in the regulation of PD-1 expression.

Endogenous GCs (cortisol in humans and corticosterone in mice) play critical roles in the regulation of various physiological and developmental processes. It was recently reported that endogenous GCs produced upon infection with mouse cytomegalovirus induced PD-1 expression on natural killer (NK) cells to restrain IFN-γ production from NK cells leading to the prevention of immunopathology (34). Another recent study showed that the deletion of GR in regulatory T cells (Tregs) resulted in the reduction of PD-1-expressing Tregs in spleen (35). These findings suggest that PD-1 expression is positively modulated by endogenous GCs. It is possible that the GRE found in the current study also mediates the expression of PD-1 on NK cells and Tregs by endogenous GCs under physiological and pathological conditions.

Because of the recent success of cancer immunotherapy targeting PD-1 and CTLA-4, many other inhibitory co-receptors are extensively investigated aiming to develop new cancer immunotherapies with higher efficacy. Revisiting the significance of inhibitory co-receptors in maintaining immunotolerance to self and preventing excess immune responses, the potentiation of their activities is expected to be a promising therapeutic strategy for inflammatory and autoimmune diseases. Our current findings clearly demonstrated that the expression level of PD-1 strongly correlated with its inhibitory function just like LAG-3, another inhibitory co-receptor (36). These results provide a rationale for the strategy to treat inflammatory and autoimmune diseases by augmenting the expression levels of inhibitory co-receptors.

Experimental procedures

Reagents

Immunosuppressants, natural and synthetic glucocorticoidsused, inhibitors of cell death, and apoptosis inducers used in this study are as follows: cyclosporin A (CyA, CAS No. 1202.635, Tokyo Chemical Industry), cyclophosphamide monohydrate (CPA, CAS No. 6055-19-2, Tokyo Chemical Industry), mizoribine (MZR, CAS No. 50924-49-7, Tokyo Chemical Industry), everolimus (ERL, CAS No. 159351-69-6, Selleck Chemicals), dexamethasone (Dex, CAS No. 50-02-2, Cayman Chemical), hydrocortisone (CAS No. 50-23-7, Sigma-Aldrich), prednisolone (CAS No. 50-24-8, Tokyo Chemical Industry), bethamethasone valerate (CAS No. 2152-44-5, LKT Laboratories), fluocinolone acetonide (CAS No. 67-73-2, Tokyo Chemical Industry), hydrocortisone 17-Butyrate (CAS No. 25122-46-7, Tokyo Chemical Industry), budesonide (CAS No. 51333-22-3, Tokyo Chemical Industry), Z-VAD-FMK (CAS No. 187389-52-2, AdooQ Bioscience), necrostatin-1 (CAS No. 4311-88-0, Selleck Chemicals), IM-54 (CAS No. 861891-50-1, Cayman Chemical), necrosulfonamide (CAS No. 1360614-48-7, Cayman Chemical), cisplatin (CDDP, CAS No. 15663-27-1, Tokyo Chemical Industry), camptothecin (CPT, CAS No. 7689-03-4, Tokyo Chemical Industry), etoposide (ETO, CAS No. 33419-42-0, Tokyo Chemical Industry), and nitidine chloride (NC, CAS No. 13063-04-2,

Sigma-Aldrich). Glucocorticoid receptor antagonist, RU486 (Mifepristone, CAS No. 84371-65-3) was purchased from Cayman Chemical. These reagents were dissolved in dimethyl sulfoxide (DMSO, Wako).

Cell culture

DO11.10 mouse hybridoma T cells and CT26 mouse colon carcinoma cells were kindly provided by Tasuku Honjo (Kyoto University). IIA1.6 mouse B lymphoma cells were kindly provided by Tomohiro Kurosaki (Osaka University). PD-1 knock-out DO11.10 T hybridoma cells were generated by using CRISPR/Cas9 system (37). These cell lines were maintained in RMPI 1640 medium (Gibco), supplemented with 10% (v/v) fetal bovine serum (FBS, Biowest), 0.5 mM Monothioglycerol (Wako), 2 mM L-alanyl-L-glutamine dipeptide (Gibco), 100 U/ml penicillin (Nacalai Tesque), and 100 μg/ml streptomycin (Nacalai Tesque). Plat-E cells, which were kindly provided by Toshio Kitamura (University of Tokyo), were maintained in Dulbecco's Modified Eagle Medium (D'MEM, Gibco), supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Plasmid and retroviral gene transduction

Fragments of cDNA were amplified by PCR and cloned into retroviral expression plasmid vectors modified from pFB-ires-neo (Agilent). For controlling the expression levels of PD-1, fragments of cDNA were cloned into retroviral expression plasmid vectors modified from pSUPER.retro.puro (Oligoengine), the promoter region of which was replaced with promoters of EF-1α (human elongation factor-1 alpha), CMV (cytomegalovirus), CAG (cytomegalovirus enhancer/chicken beta-actin), MC1 (polyoma virus enhancer/herpes simplex virus thymidine kinase), SV40 (simian virus 40) coupled with or without a poly(A) signal. Plasmids were transfected using the FuGENE HD (Promega) into Plat-E cells cultured in D'MEM, high glucose (Gibco) supplemented with 20% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, and supernatants containing viruses were used to transduce genes into target cells. Infected cells were selected with G418 (Wako), puromycin (Sigma-Aldrich), or blasticidin (InvivoGen).

Mice

C57BL/6J and BALB/c mice were obtained from Charles River Laboratories Japan and Japan SLC, respectively, and housed under specific pathogen-free conditions in environmentally controlled clean rooms. All experimental procedures were planed and conducted according to the institutional regulations complying with the Act on Welfare and Management of Animals and the related guidelines in Japan. All mouse protocols were approved by the Animal Experimentation Committee of Tokushima University.

Stimulation of DO11.10 T hybridoma cells and primary T cells

DO11.10 T hybridoma cells (5×10^4 cells/well) were stimulated with IIA1.6 cells (1×10^4 cells/well) in the presence of antigenic peptide (323–339 segment of chicken ovalbumin, $pOVA_{323-339}$, ISQAVHAAHAEINEAGR, >95% purity, eurofins) in 96-well round bottom plate (BD Bioscinences) for 24 hours. Where indicated, 1 μg/ml of anti-PD-L1 blocking Ab (1-111A) or rat IgG2a isotype control Ig (2A3, Bio X Cell) were added. Activated primary T cells were prepared by stimulating splenocytes from C57BL/6J mice with soluble anti-CD3ε (0.5–1 μg/ml, 145-2C-11, BioLegend) and anti-CD28 (0.5–1 μg/ml, 37.51, BioLegend) Abs. The concentration of IL-2 in the culture supernatant was determined by ELISA (BioLegend). The percent PD-1-mediated inhibition of IL-2 production was calculated as the ratio of IL-2 concentration in the presence to absence of anti-PD-L1 blocking Ab (1-111A). IL-2 concentrations with different amounts of antigens were summed up for the calculation of the percent PD-1-mediated inhibition.

Antibody and flowcytometric analysis

Cultured cell lines and primary cells were stained with the indicated Abs. Data were obtained with Gallios (Beckman Coulter) and analyzed using FlowJo (Tree Star). CF633-Dye-labeled anti-mouse LAG-3 Ab (TKB58) was prepared as described previously (38). All the other Abs used in this study were purchased from BioLegend: anti-mouse PD-1 (RMP1-30), TCR-β (H57-597), CD3ε (145-2C-11), CD44 (IM7), CD45 (30-F11), Thy1.2 (53-2.1), CD69 (H1.2F3), CD28 (37.51), 4-1BB (17B5), RANKL (IL22/5), CD25 (7D4), CD4 (RM4-5), and CD8a (53-6.7). Apoptotic cells were detected by using Annexin V (BioLegend).

Real-time quantitative PCR

Total RNA was extracted from cells using TRIzol reagent (Ambion), and then subjected to reverse transcription using High-Capacity cDNA

Reverse Transcription Kit (Applied Biosystems). Gene expression was analyzed by quantitative PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Values were normalized to the expression of *Gapdh*. The following specific primer sets were used: *Gapdh*, F (5′ - TTCACCACCATGGAGAAGGC - 3′) and R (5′ - GGCATGGACTGTGGTCATGA - 3′); *Pdcd1*, F (5′ - ACCCTGGTCATTCACTTGGG - 3′) and R (5′ - CATTTGCTCCCTCTGACACTG - 3′).

Reporter assay

Putative GREs in the promoter region of PD-1 gene were predicted by using the JASPAR database (39) and genomatix genome analyzer (Genomatix). A DNA fragment containing GRE1, GRE2, and GRE3 (2,790 to 2,339 bp upstream of the transcription start of PD-1 mRNA) was amplified from C57BL/6N Mouse BRC clone (B6Ng01-240G08, RIKEN BRC) by PCR and cloned into a retroviral expression plasmid vector modified from pSUPER.retro.puro. together with a synthetic minimal promoter sequence and EGFP cDNA. Where indicated, GRE sequences (AGAACAnnnTGTTCT) were mutated by over-hang PCR to AGGTCAnnnTGACCT. Reporter constructs were retrovirally introduced into DO11.10 T hybridoma cells overexpressing mouse GR. After selection with puromycin, cells were cultured in the presence of Dex (100 nM), phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma-Aldrich), and ionomycin (500 ng/ml, Sigma-Aldrich) for 24 hours and analyzed by flow cytometry.

Preparation of tumor-infiltrating T cells

Male and female BALB/c mice (8–10 weeks old) were inoculated subcutaneously with 1×10^6 CT26 murine colon carcinoma cells at shaved back. On days 10 and 12, Dex (400 μg) in 50% DMSO/PBS was subcutaneously injected near the site of tumor inoculation. On day 13, tumor tissues were dissected and mechanically dissociated by using gentleMACS Octo Dissociator with Heaters (Miltenyi Biotec) in RPMI 1640 containing Collagenase (1 mg/ml, Wako), Hyaluronidase (50 μg/ml, Sigma-Aldrich), and DNase I (10 μg/ml, Sigma-Aldrich). Single cell suspensions prepared from tumor tissues were used for the flow cytometric analysis.

Statistical analysis

Two-way ANOVA with Dunnett's and Bonferroni's multiple comparisons test, one-way ANOVA with Dunnett's multiple comparisons test, Pearson's correlation test, and two-tailed Student's *t*-test was used to evaluate statistical significance. $p < 0.05$ was considered statistically significant. These statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software).

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Author Contributions: NM, TM, and TO designed and analyzed the experiments. NM, TM, DS, KS, and IO established experimental systems and generated experimental materials. NM and TM performed the staining and functional experiments using cultured and mouse primary cells. NM, TM, and TO wrote the manuscript with all authors contributing to writing. TO supervised the project.

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FOOTNOTES
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The abbreviations used are: Dex, dexamethasone; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HC, hydrocortisone; irAE, immune-related adverse events; PD-1, programmed cell death 1; *Pdcd1*, mouse PD-1 gene; TCR, T cell receptor; TF, transcription factor

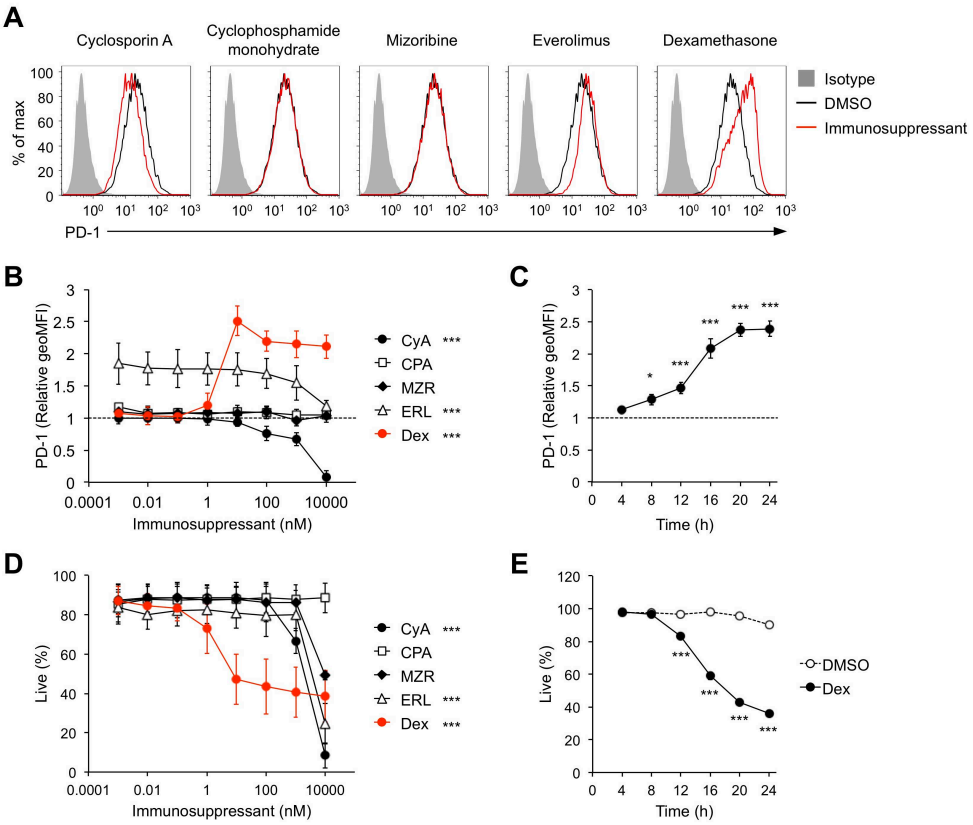

Figure 1. Effects of immunosuppressants on PD-1 expression. *A*, Surface PD-1 expression levels on DO11.10 T hybridoma cells after treatments with immunosuppressants. Representative histogram plots are shown for cells treated with indicated immunosuppressants (100 nM). Gray shaded histograms represent isotype control Ig staining. *B–E*, Dose- and time-dependent effects of immunosuppressants on PD-1 expression (B, C) and cell viability (D, E). DO11.10 T hybridoma cells were cultured in the presence of indicated immunosuppressants at indicated doses (B, D) for 24 hours or Dex (100 nM, C, E) for indicated hours and analyzed by flow cytometry. Fold changes in PD-1 expression relative to DMSO-treated cells (the relative geometric mean fluorescent intensity, geoMFI of PD-1) (B, C) and the percentages of live (propidium iodide (PI)-negative) cells are shown (D, E). Representative plots of three independent experiments (A) or the mean ± s.d. of three independent experiments (B–E) are shown. Two-way ANOVA with Dunnett's multiple comparisons test (compared to DMSO-treated cells) (B, D) or with Bonferroni's multiple comparisons test (compared to DMSO-treated cells) (E). One-way ANOVA with Dunnett's multiple comparisons test (compared to before treatment) (C). *$p < 0.05$, ***$p < 0.001$. CyA, cyclosporin A; CPA, cyclophosphamide monohydrate; MZR, mizoribine; ERL, everolimus; Dex, dexamethasone.

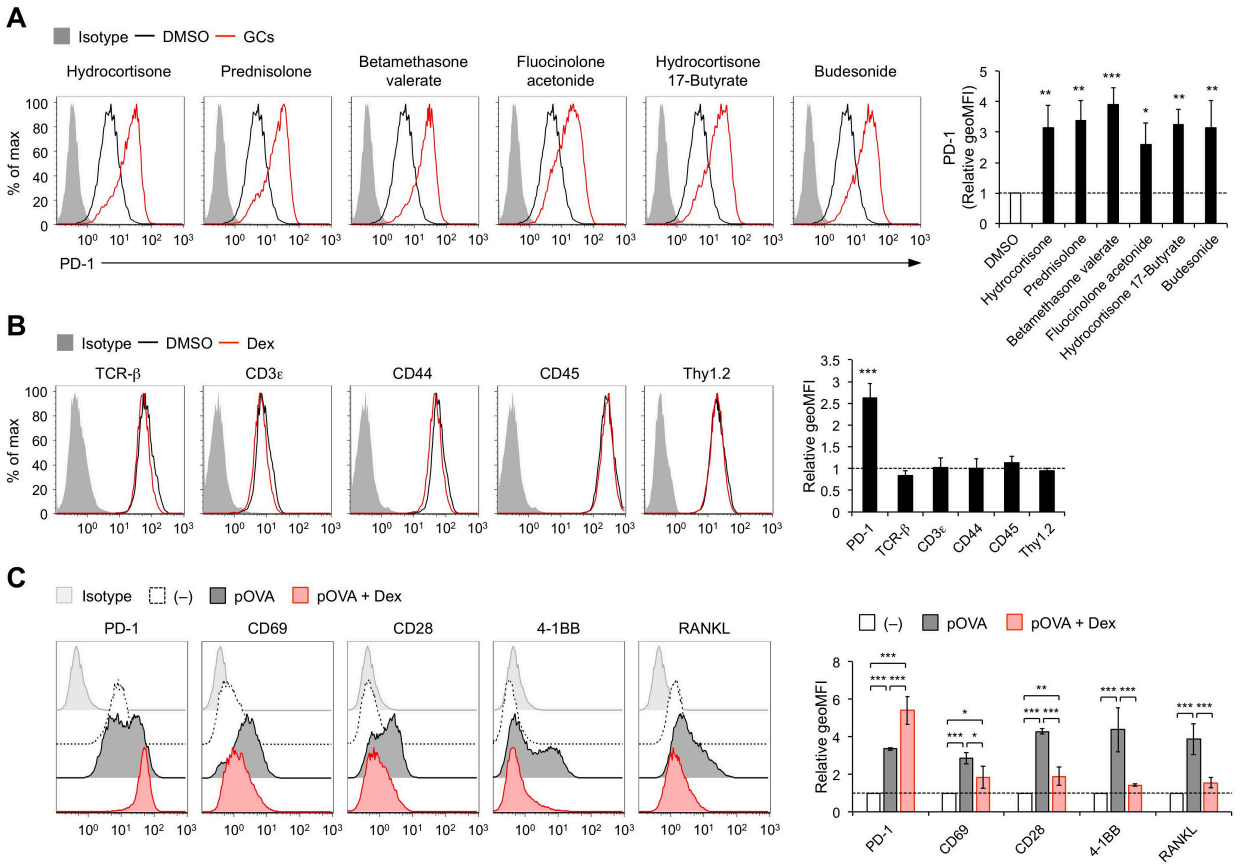

Figure 2. Selective up-regulation of PD-1 expression on T hybridoma cells by GCs. *A*, Surface PD-1 expression levels on DO11.10 T hybridoma cells after treatments with GCs. Cells were treated with indicated GCs (100 nM) for 24 hours and their PD-1 expression level was analyzed by flow cytometry. *B*, Expression levels of cell surface molecules on DO11.10 T hybridoma cells after Dex-treatment. Cells were treated with Dex (100 nM) for 24 hours and the expression levels of indicated molecules were analyzed by flow cytometry. *C*, Expression of cell surface molecules on DO11.10 T hybridoma cells after antigenic stimulation in the presence of Dex. DO11.10 T hybridoma cells were stimulated by co-culturing with $pOVA_{323–339}$-pulsed IIA1.6 cells for 24 hours in the presence Dex (100 nM) and the expression of indicated molecules on live DO11.10 T hybridoma cells ($PI^-B220^-TCR\text{-}\beta^+$) cells were analyzed by flow cytometry. Representative histogram plots and the mean ± s.d. of three independent experiments are shown (A–C). Gray shaded histograms represent isotype control Ig staining (A–C). One-way ANOVA with Dunnett's multiple comparisons test (compared to DMSO-treated cells) (A) and with Tukey's multiple comparisons test (B, C). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

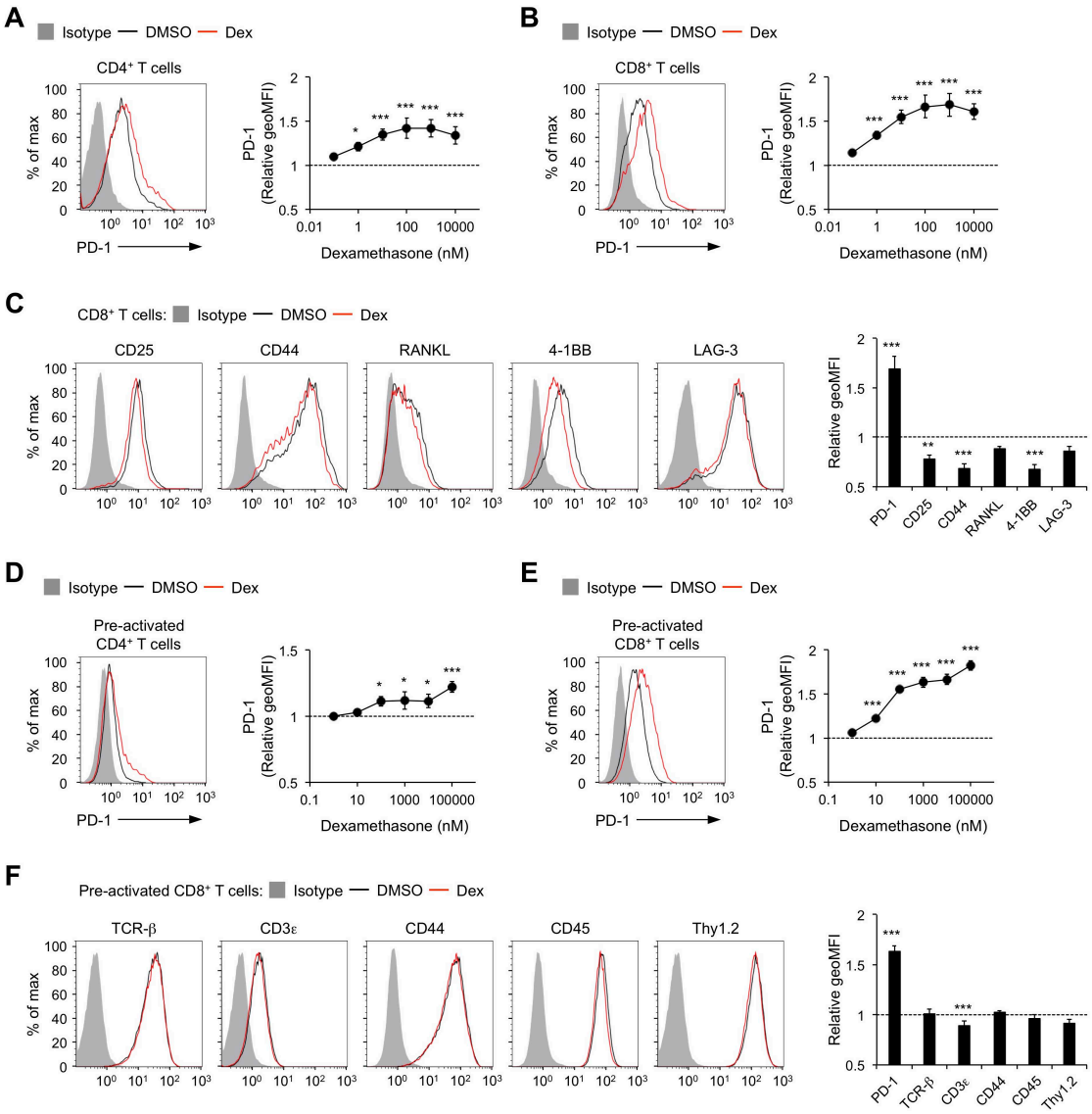

Figure 3. Selective up-regulation of PD-1 expression on primary T cells by GCs. *A–C*, Expression levels of PD-1 (A, B) and cell surface molecules (C) on primary T cells stimulated with anti-CD3ε/CD28 Abs in the presence of Dex. Splenocytes from C57BL/6J mice were cultured in the presence of soluble anti-CD3ε Ab, anti-CD28 Ab, and Dex for 16 hours and analyzed by flow cytometry. *D–F*, Effects of Dex on the expression of PD-1 (D, E) and cell surface molecules (F) of pre-activated primary T cells. Splenocytes from C57BL/6J mice were pre-activated with soluble anti-CD3ε and anti-CD28 Abs for 48 h. The pre-activated cells were cultured in the presence of Dex for 24 hours and analyzed by flow cytometry. Representative histogram plots of cells treated with Dex (1 μM) and changes in the expression levels of indicated molecules are shown for $CD4^+$ (gated on $PI^-TCR\text{-}\beta^+CD4^+$ cells) (A), $CD8^+$ (gated on $PI^-TCR\text{-}\beta^+CD8^+$ cells) (B, C), pre-activated $CD4^+$ (D), and pre-activated $CD8^+$ (E, F) T cells. Gray shaded histograms represent isotype control Ig staining (A–F). Data indicates the mean ± s.d. of biological triplicates in one representative experiment (A–F). Data are representative of at least two independent experiments (A–F). One-way ANOVA with Dunnett's multiple comparisons test (compared to DMSO-treated cells) (A–F). $*p < 0.05$, $***p < 0.001$.

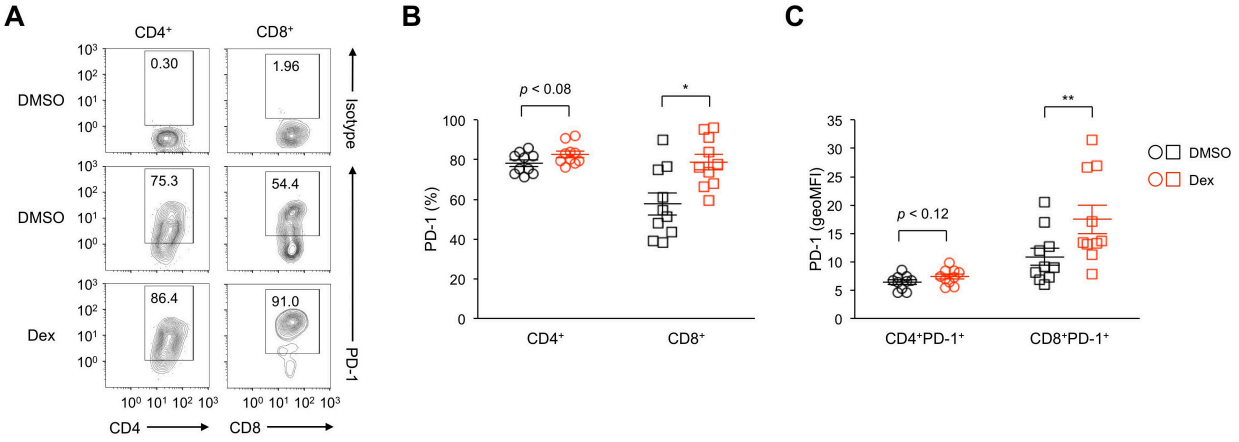

Figure 4. Up-regulation of PD-1 on tumor-infiltrating CD8$^+$ T cells by GCs. *A–C*, Surface PD-1 expression levels on T cells infiltrating in tumors of CT26 colon carcinoma cells. BALB/c mice were administered subcutaneously with DMSO or Dex (400 μg) at 10 and 12 days after the subcutaneous inoculation of CT26 cells. At day 13, the percentages of tumor-infiltrating CD4$^+$ and CD8$^+$ T cells expressing PD-1 (gated on PI$^-$CD45$^+$CD4$^+$ and PI$^-$CD45$^+$ CD8$^+$, respectively) (A, B) and the expression levels of PD-1 on PD-1-expressing CD4$^+$ and CD8$^+$ T cells (geoMFI, C) were analyzed by flow cytometry. Representative histogram plots of three independent experiments are shown (A). Each symbol represents an individual mouse (n = 10, each) and horizontal lines denote the mean ± s.e.m. of ten biological replicates pooled from three independent experiments. Two-tailed Student's t-test. *$p < 0.05$, **$p < 0.01$.

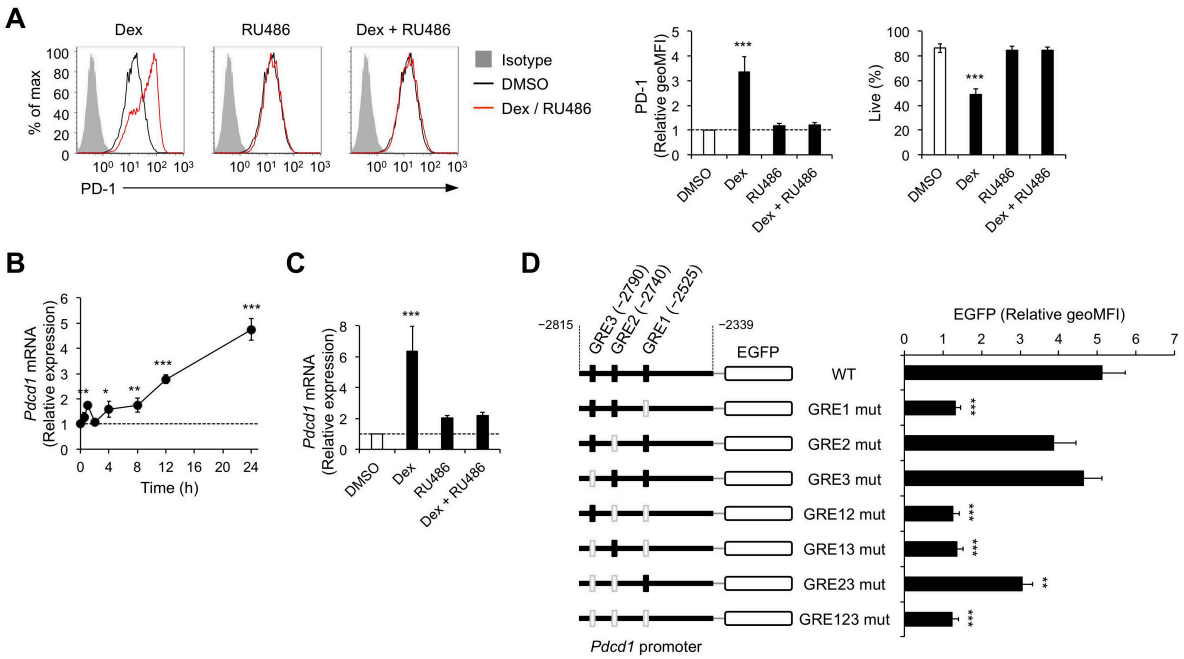

Figure 5. GR-dependent augmentation of PD-1 transcription by GC-treatment. *A*, Abrogation of GC-mediated PD-1 up-regulation and cell death by glucocorticoid receptor antagonist, RU486. DO11.10 T hybridoma cells were cultured in the presence of Dex (100 nM) and RU486 (10 μM) for 24 hours and analyzed by flow cytometry. Fold changes in PD-1 expression relative to DMSO-treated cells and the percentages of live cells are shown. Gray shaded histograms represent isotype control Ig staining. *B*, The time-course of PD-1 mRNA expression in DO11.10 T hybridoma cells after treatment with Dex. Expression levels of PD-1 mRNA were determined by real-time quantitative PCR and normalized to those of GAPDH mRNA. Fold changes relative to DMSO-treated cells are shown. *C*, Abrogation of GC-mediated PD-1 mRNA up-regulation by RU486. DO11.10 T hybridoma cells were cultured in the presence of Dex (100 nM) and RU486 (10 μM) for 24 hours and fold changes of PD-1 mRNA expression relative to DMSO-treated cells were determined as in (B). *D*, Promoter activity of DNA sequences containing three putative GREs in the promoter region of PD-1 upon GC-treatment. Schematic representations of reporter constructs encoding EGFP cDNA and the promoter region of PD-1 (2,790 to 2,339 bp upstream from the transcription start site) with or without amino acid mutations in the putative GREs (*left*). DO11.10 T hybridoma cells transduced with mouse GR and indicated reporter constructs were cultured in the presence of Dex (100 nM), PMA (50 ng/ml), and ionomycin (500 ng/ml) for 24 hours, and analyzed by flow cytometry. Relative geoMFIs of EGFP compared to DMSO-treated cells are shown. The mean ± s.d. (A, C) or s.e.m (D) of three independent experiments or the mean ± s.d. of technical triplicates in one out of three independent experiments (B) are shown. One-way ANOVA with Dunnett's multiple comparisons test compared to DMSO-treated cells (A–C) or cells with wild type (WT) reporter construct (D). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

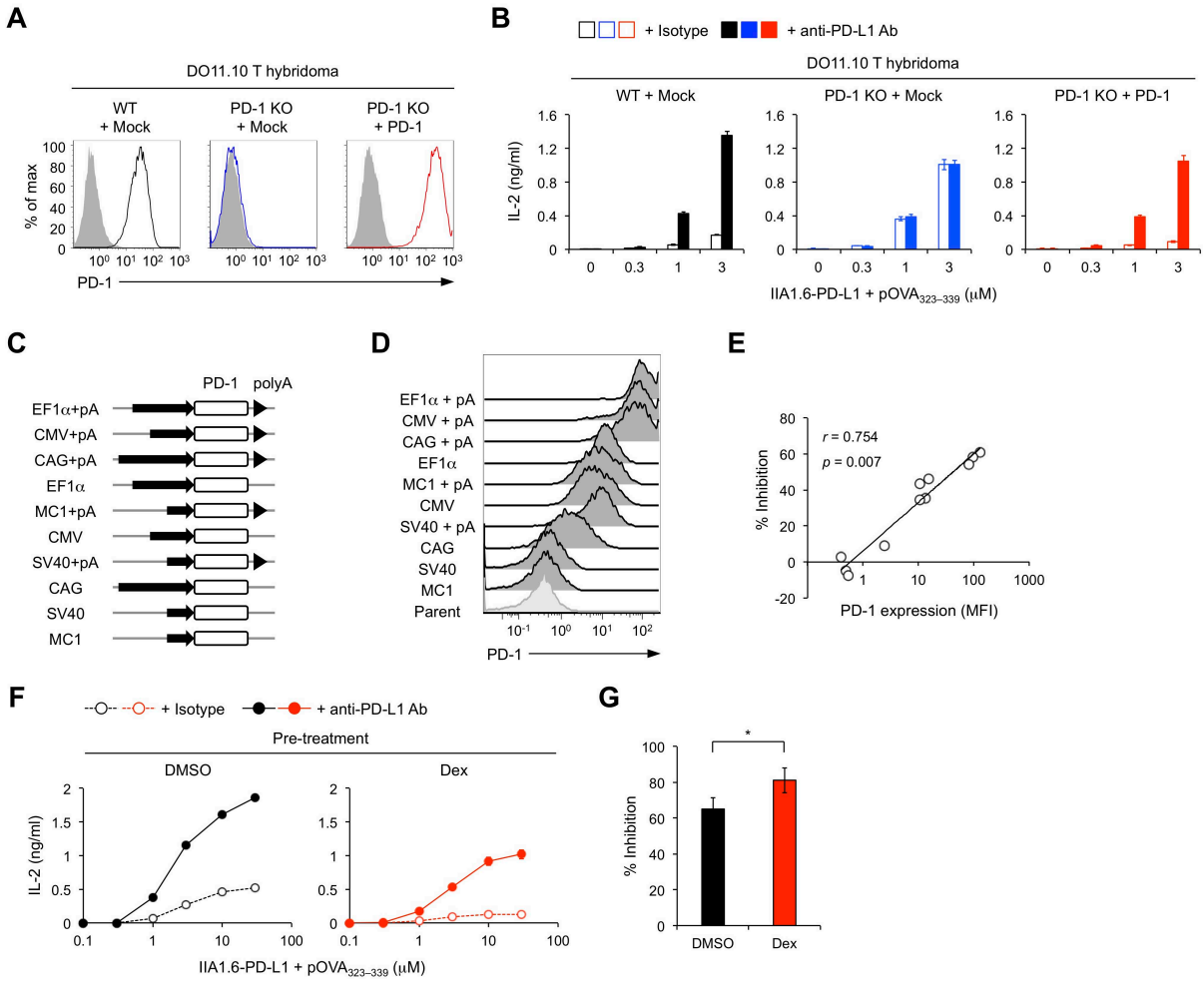

Figure 6. Potentiation of the inhibitory effect of PD-1 by GCs. *A*, Surface PD-1 expression levels on DO11.10 T hybridoma cells with or without deletion and overexpression of PD-1. *B*, Restoration of PD-1-mediaeted inhibitory effects by overexpression of PD-1 in DO11.10 T hybridoma cells with targeted deletion of PD-1 gene. IL-2 concentration in the culture supernatant is shown for indicated DO11.10 T hybridoma cells stimulated with pOVA$_{323–339}$-pulsed IIA1.6-PD-L1 cells in the presence or absence of anti-PD-L1 blocking Ab for 24 hours. *C*, Schematic representations of retroviral expression vectors. Mouse PD-1 cDNA was overexpressed in DO11.10 T hybridoma cells with targeted deletion of PD-1 gene using indicated promoters with or without polyA signal. *D*, Flow cytometric analysis of DO11.10 T hybridoma cells overexpressing PD-1 at various levels by using retroviral expression vectors shown in (C). *E*, The correlation between the percent inhibition of IL-2 production by PD-1 and the expression level of PD-1 (MFI). DO11.10 T hybridoma cells with different PD-1 expression levels were stimulated as in (B). *r*, Pearson's correlation coefficient. *F* and *G*, Augmentation of PD-1-mediated inhibitory effects by Dex. DO11.10 T hybridoma cells were treated with Dex (100 nM) or DMSO for 24 hours and rested for 6 hours. The same number of Dex- or DMSO-treated live cells was stimulated for 24 hours as in (B). IL-2 concentration in the culture supernatant (F) and the percent inhibition of IL-2 production by PD-1 (G) are shown. Data are the mean ± s.d. of technical duplicates in one representative experiment (B, F) or the mean ± s.d. of four independent experiments (G). Data are representative of at least three independent experiments (A, B, D–F). Two-tailed Student's *t*-test (G). *$p < 0.05$.

Glucocorticoids potentiate the inhibitory capacity of programmed cell death 1 by up-regulating its expression on T cells

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