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Potential role of inducible GPR3 expression under stimulated T cell conditions

Hiroko Shiraki [a], Shigeru Tanaka [a, *], Yun Guo [b], Kana Harada [a], Izumi Hide [a], Tomoharu Yasuda [b], Norio Sakai [a]

[a] Department of Molecular and Pharmacological Neuroscience, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
[b] Department of Immunology, Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

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ABSTRACT

G protein-coupled receptor 3 (GPR3) constitutively activates Gαs proteins without any ligands and is predominantly expressed in neurons. Since the expression and physiological role of GPR3 in immune cells is still unknown, we examined the possible role of GPR3 in T lymphocytes. The expression of GPR3 was upregulated 2 h after phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation and was sustained in Jurkat cells, a human T lymphocyte cell line. In addition, the expression of nuclear receptor 4 group A member 2 (*NR4A2*) was highly modulated by GPR3 expression. Additionally, GPR3 expression was linked with the transcriptional promoter activity of *NR4A* in Jurkat cells. In mouse $CD4^+$ T cells, transient GPR3 expression was induced immediately after the antigen receptor stimulation. However, the expression of *NR4A2* was not modulated in $CD4^+$ T cells from *GPR3*-knockout mice after stimulation, and the population of Treg cells in thymocytes and splenocytes was not affected by *GPR3* knockout. By contrast, spontaneous effector activation in both $CD4^+$ T cells and $CD8^+$ T cells was observed in *GPR3*-knockout mice. In summary, GPR3 is immediately induced by T cell stimulation and play an important role in the suppression of effector T cell activation.

1. Introduction

G protein-coupled receptor 3 (GPR3), along with GPR6 and GPR12, is a member of a subfamily that constitutively activates Gαs proteins, resulting in elevated basal levels of intracellular cyclic adenosine monophosphate (cAMP) in the absence of any ligands.[1] GPR3 is abundantly expressed in neurons and oocytes.[2–4] In oocytes, GPR3 expression produces high levels of cAMP, and plays a role in maintaining meiotic arrest.[3] The neuronal expression of GPR3 also leads to upregulated intracellular cAMP, which is associated with neurite outgrowth[4,5] and anti-apoptotic functions under various apoptotic stimuli.[6] Additionally, several reports have suggested a pathophysiological role for GPR3 in Alzheimer's disease[7] and multiple sclerosis.[8] However, the expression and physiological functions of GPR3 in immune cells have not been reported.

T cells are sensitized upon T cell receptor (TCR) stimulation, with diverse effects on T cell differentiation, survival, apoptosis, and cytokine production. Upon T cell stimulation, cAMP-mediated protein kinase A (PKA) activation is induced.[9] Interestingly, the duration of cAMP elevation was regulated differently depending on the strength of the TCR signal input, which affects different T cell functions.[10] Transient elevation in cAMP via segregation of stimulatory G-proteins contributed to T cell sensitization. By contrast, sustained cAMP elevation in T cells led to inhibition of effector T cell proliferation via Jak/Stat signaling and cell cycle arrest, thereby contributing to immunosuppression.[11–15] Effector T cell suppression by cAMP elevation is accomplished through adenosine via adenosine A2A receptor[16] and inhibition of phosphodiesterases (PDEs).[17] Under physiological conditions, adenosine is secreted from tumor cells and elevates the cAMP level in T cells, which leads to functional inactivation of T cells called T cell anergy.[18] Effector T

* Corresponding author. Department of Molecular and Pharmacological Neuroscience, Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan.
E-mail address: tanakamd@hiroshima-u.ac.jp (S. Tanaka).
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cell suppression is also determined by cell-to-cell contact, where a higher level of cAMP in regulatory T (Treg) cells is transferred into other T cells through gap junctions.[19] However, the upstream molecules that govern stimulated T cell-mediated cAMP activation still need to be elucidated.

The nuclear receptor 4 group A (NR4A) genes are orphan receptors that comprise a subfamily of nuclear receptors including NR4A1 (also called Nur77, NGFI-B), NR4A2 (also called Nurr1, NOT), and NR4A3 (also called Nor-1).[20] NR4A family genes are expressed early in response to various stimuli, and bind to the NGFI-B response element (NBRE) or Nur response element (NurRE) in the promoter of target genes, thereby playing a role in proliferation, differentiation, apoptosis, and remodeling in various tissue types.[20] In the NR4A family, NR4A2 is highly expressed in the central nervous system compared with the other receptors. It is involved in the development of dopaminergic neurons[21,22] and in the differentiation of regulatory T lymphocytes,[23,24] thereby affecting the pathogenesis of Parkinson's disease and multiple sclerosis.[24,25] NR4A family genes can be induced by parathyroid hormone (PTH) in a cAMP/PKA-dependent manner in osteoblasts.[26] They can also be induced by melanocortin-1 receptor, a ligand-dependent GPR coupled to Gαs, in melanocytes.[27] However, the upstream modulators of these factors remain largely unknown, especially in T lymphocytes.

In this study, we found for the first time that GPR3 sustainably induced upon Jurkat cell stimulation. We then examined the involvement of GPR3 in T cell functions using DNA gene microarray and revealed that NR4A2 as highly affected in terms of gene expression in Jurkat cells. We further explored the potential role of GPR3 in mouse primary T lymphocytes using *GPR3*-knockout mice.

2. Materials and methods

2.1. Animals

GPR3-knockout mice cryopreserved fertilized oocyte were obtained from the Mutant Mouse Resource & Research Centers (Bar Harbor, ME) and resuscitated at the Institute of Laboratory Animal Science, Hiroshima University under the license of Deltagen (San Mateo, CA), as previously described.[6] All experiments were approved by the Animal Care and Use Committee of Hiroshima University (approval numbers 25-193-3, 25-194-4, 30-153, 30-154).

2.2. Chemicals, inhibitors, siRNAs, and plasmid vectors

U0126 and ionomycin were from Calbiochem (La Jolla, CA). SB203580 was from Cayman Chemical (Ann Arbor, MI). GF109203X, H-89, and phorbol 12-myristate 13-acetate (PMA) were from Sigma–Aldrich (St. Louis, MO, USA). LY294002 was from Cell Signaling Technology (Denver, MA). The predesigned siRNA against human *GPR3* and the control siRNA were from Sigma–Aldrich. GPR3 in a FLAG-tagged-GFP fusion protein-expressing vector (pC-GPR3mAGFL) and the control vector (pC-mAGFL) have previously been described.[28]

2.3. Cell culture and transfection

Jurkat cells (clone E6-1) were from ATCC (Manassas, VA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum (Biological Industries, Cromwell, CT). The siRNA and expression plasmid vectors were transfected into Jurkat cells using a NEPA21 Super Electroporator (Napa Gene, Chiba, Japan) following the manufacturer's protocol.

2.4. RNA isolation and real-time RT-PCR analysis

Total RNA was isolated from 4×10^5 cells using an RNeasy® Mini Kit (Qiagen, Venlo, Netherlands) and then reverse transcribed using a QuantiTect® Reverse Transcription Kit (Qiagen). Primers and probes specific for the human and mouse genes encoding *GPR3*, *NR4A1*, *NR4A2*, *NR4A3*, and *β-actin* were from PrimeTime® Standard qPCR Assays (Integrated DNA Technologies, Coralville, IA). Real-time PCR analyses were performed using an ABI PRISM® 7700 sequence detection system (Applied Biosystems, Foster City, CA).

2.5. DNA microarray analysis

For RNA preparation for the DNA microarray analysis, isolated RNA was further purified using TURBO DNase free (Thermo Fisher Science), and the RNA quality was evaluated with an Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) using an Agilent 2100 Bioanalyzer system (Agilent Technologies). Each total RNA was used to prepare biotinylated, fragmented, single-stranded cDNA according to the manufacturer's instructions. Affymetrix GeneChip® expression arrays (Clariom S Assay, human) (Thermo Fisher Science) were hybridized with 2.3 μg of single-stranded DNA using a GeneChip™ WT PLUS Reagent Kit (Thermo Fisher Science) in a GeneChip® Hybridization Oven 645 (Affymeterix, Santa Clara, CA, USA). The arrays were then washed and stained using the GeneChip® Fluidics Station 450 (Affymetrix), and were finally scanned using the GeneChip® Scanner 3000 7G (Affymetrix). The comparison between control siRNA-treated cells and GPR3 siRNA-treated cells was performed using the Transcriptome Analysis Console (TAC) 3.0 onsite software using duplicated samples.

2.6. Dual luciferase reporter assay

Three copies of the oligonucleotides corresponding to the potential NR4A-binding site NBRE or NurRE[29,30] were cloned into the pNL3.2 NanoLuc plasmid (Promega, Madison, WI), designating pNL3.2-NBRE and pNL3.2-NurRE, respectively. The luciferase reporter assay was carried out using the Nano-Glo® Dual-Luciferase® Reporter Assay System (Promega) in accordance with the manufacturer's protocol. One day after the NanoLuc plasmid transfection, cells were seeded into 96-well white-colored plates at a density of 2×10^5 cells/well. After cell stimulation, the Nano-Glo® Dual-Luciferase Assay was measured using a TriStar LB941 Luminometer (Berthold, Bad Wildbad, Germany). Promoter activity was calculated as the light units relative to the light units of the control (pGL4.54-luc2).

2.7. $CD4^+$ T cells isolation and stimulation

Single-cell suspensions were prepared from the spleen of 7–8-week-old mice and red blood cells were removed using Gey's solution. Cells were first stained with biotin-conjugated antibodies for CD19 (1D3), CD8α (53–6.7), CD11b (M1/70), CD11c (HL3), Ter119, I-Ab (KH74), TCRγ/δ (GL3) (BD Biosciences, Franklin Lakes, NJ), B220 (RA3-6B2), and CD49b (HMα2) (Biolegend, San Diego, CA). Then, the cells were labeled with BD IMag™ Streptavidin Particles Plus-DM (#5578l12) and separated by BD IMagnet™ following the manufacturer's protocol. $CD4^+$ T cells were stimulated with plate-bounded anti-CD3ε (145-2C11) antibodies and anti-CD28 (37.51) antibodies in the medium (both 10 μg/mL, BD Biosciences) or with PMA (100 nM) and ionomycin (1 μg/mL) in the medium. Cells were harvested at the indicated time points.

2.8. Flow cytometry analysis

Single-cell suspensions were prepared from spleen and thymus. Cells were first reacted with monoclonal anti-mouse CD16/CD32 antibodies (BD Biosciences) to block Fc receptors. Then, cells were stained using the following antibodies: BV510-conjugated anti-CD4 (RM4-5), FITC-conjugated anti-CD62L (MEL-14), PE-Cy7-conjugated anti-CD44 (IM7), and APC-Cy7-conjugated anti-CD8α (53–6.8) (BD Biosciences). Flow cytometry was performed with FACSCanto II and analyzed using FlowJo software (BD Biosciences).

2.9. Statistical analysis

Statistical analysis was performed using Prism 9 software (GraphPad Software, San Diego, CA). Statistical significance was determined one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* tests. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Early and sustained GPR3 expression is induced upon human T lymphocytes stimulation

A recent report suggested that low GPR3 expression in peripheral blood mononuclear cells is a potential biomarker for poor prognosis of multiple sclerosis.[8] Based on these findings, we considered the possible role of GPR3 in T cell function. *GPR3* expression was relatively low under unstimulated conditions in Jurkat cells, a human T lymphocyte cell line (Fig. 1A–C). However, when Jurkat cells were stimulated by PMA, a potent protein kinase C (PKC) activator, *GPR3* expression was highly upregulated in a dose-dependent manner 3 h after stimulation and was further upregulated by combined stimulation with ionomycin, a calcium ionophore (Fig. 1A and B). Additionally, *GPR3* expression was induced as early as 2 h after PMA/ionomycin stimulation, and its expression level was sustained until 8 h (Fig. 1C). These results suggest that early and sustained *GPR3* expression is induced upon human T lymphocytes stimulation.

3.2. NR4A2 expression is regulated by sustained GPR3 expression in human T lymphocytes stimulation

To explore the role of sustained GPR3 expression by T cell stimulation, we performed a gene array analysis to search for modulated genes under *GPR3* suppression in Jurkat cells. When Jurkat cells were transfected with *GPR3* siRNA, *GPR3* expression was significantly decreased at 2, 3, 4, 6, and 12 h after PMA/ionomycin stimulation (Fig. 1D). Among the genes with suppressed expression at 6 h after PMA/ionomycin stimulation, the expression of NR4A receptor family members was highly suppressed (*NR4A2*: −3.1 $\log_2$fold change, $p = 0.016$ and *NR4A3*: −2.17 $\log_2$fold change, $p = 0.0045$; Suppl. 1A, B). *NR4A1* also appeared to be weakly suppressed among these family members, although this change was not significant (−1.4 $\log_2$fold change, $p = 0.0751$; data not shown). Among the various downregulated genes, NR4A2 was the second to highest modulated gene by GPR3 suppression and the NR4A receptor family was of most interest because this family plays a role in multiple functions in T lymphocytes.[24,31,32]

We further explored whether the expression of the NR4A receptor family is modulated by GPR3 expression using real-time RT-PCR. To address this question, we transfected control siRNA or *GPR3* siRNA into Jurkat cells and stimulated the cells with PMA and ionomycin 24 h after transfection. In control siRNA-treated cells, the expression of *NR4A1* and *NR4A3* was induced as early as 1–2 h after PMA/ionomycin stimulation and declined thereafter. However, the expression of *NR4A2* was increasingly upregulated until 6 h after stimulation and then declined. In *GPR3* siRNA-treated cells, *NR4A2* expression was significantly decreased at 6 and 12 h after stimulation. In contrast, significant changes in *NR4A1* and *NR4A3* were not observed under *GPR3* suppression (Fig. 1E–G).

We next investigated whether upregulated GPR3 expression affects the expression of the NR4A receptor family. To this end, we transfected mock or GPR3-expressing plasmid vectors into Jurkat cells, and then stimulated the cells with PMA and ionomycin 24 h after transfection. The expression levels of recombinant GPR3 were ~150-fold higher than those of endogenous GPR3 24 h after transfection (data not shown). *NR4A2* expression was markedly higher in the GPR3-transfected cells compared with the controls from 4 to 12 h after cell stimulation (Fig. 1I). Additionally, *NR4A1* and *NR4A3* expression was highly upregulated in the GPR3-transfected cells after cell stimulation (Fig. 1H, J).

These results suggest that sustained GPR3 expression by T cell stimulation induces *NR4A2* expression, but does not affect *NR4A1* or *NR4A3* expression. However, upregulated GPR3 expression has the potential to induce all NR4A receptors expression after T cell stimulation.

3.3. GPR3-mediated NR4A2 induction is modulated by the protein kinase A and mitogen-activated protein kinase signaling pathways

We further explored the signaling pathways involved in the GPR3-mediated induction of *NR4A2*. In mock-transfected Jurkat cells, the induction of *NR4A2* by PMA/ionomycin stimulation was significantly inhibited by PKC, mitogen-activated protein/extracellular signal-regulated kinase (MEK), and p38 mitogen-activated protein kinase (MAPK) inhibitors (Fig. 2). In GPR3-transfected cells, the induction of *NR4A2* was significantly increased 6 h after cell stimulation, and this effect was significantly inhibited by PKC, PKA, MEK, and p38 MAPK inhibitors, but not by PI3K inhibitor (Fig. 2). Because T lymphocytes are stimulated by a PKC activator, we could not evaluate the involvement of PKC in GPR3-mediated NR4A2 expression in current study. However, the GPR3-mediated *NR4A2* induction was regulated partly by the PKA and MAPK signaling pathways.

3.4. The transcriptional activity of NR4A is positively regulated by GPR3 expression

The NR4A subfamily members bind directly to the common NBRE promoter region as a monomer, and to the NurRE promoter region as a homodimer or heterodimer.[29,30] To further elucidate the effect of GPR3 expression on *NR4A2* in Jurkat cells, we created an *NR4A* reporter construct containing the NBRE or NurRE element upstream of the luciferase gene. *NR4A* transcriptional activity was increased in Jurkat cells until 12 h after stimulation by PMA and ionomycin (Fig. 3A and B). However, knock down of *GPR3* using siRNA led to significantly decreased *NR4A* transcriptional activity at 12 h after the stimulation of Jurkat cells (Fig. 3A and B). Conversely, GPR3 overexpression led to markedly increased *NR4A* transcriptional activity as early as 4 h after T cell stimulation compared with the control (Fig. 3C and D). These results suggest that the GPR3-mediated induction of *NR4A* correlated with its functional transcriptional activity in Jurkat cells.

3.5. Suppressive role of GPR3 in mouse primary T lymphocyte activation

We further explored the potential role of GPR3 using mouse primary splenocytes. As expected, *GPR3* expression in mouse $CD4^+$

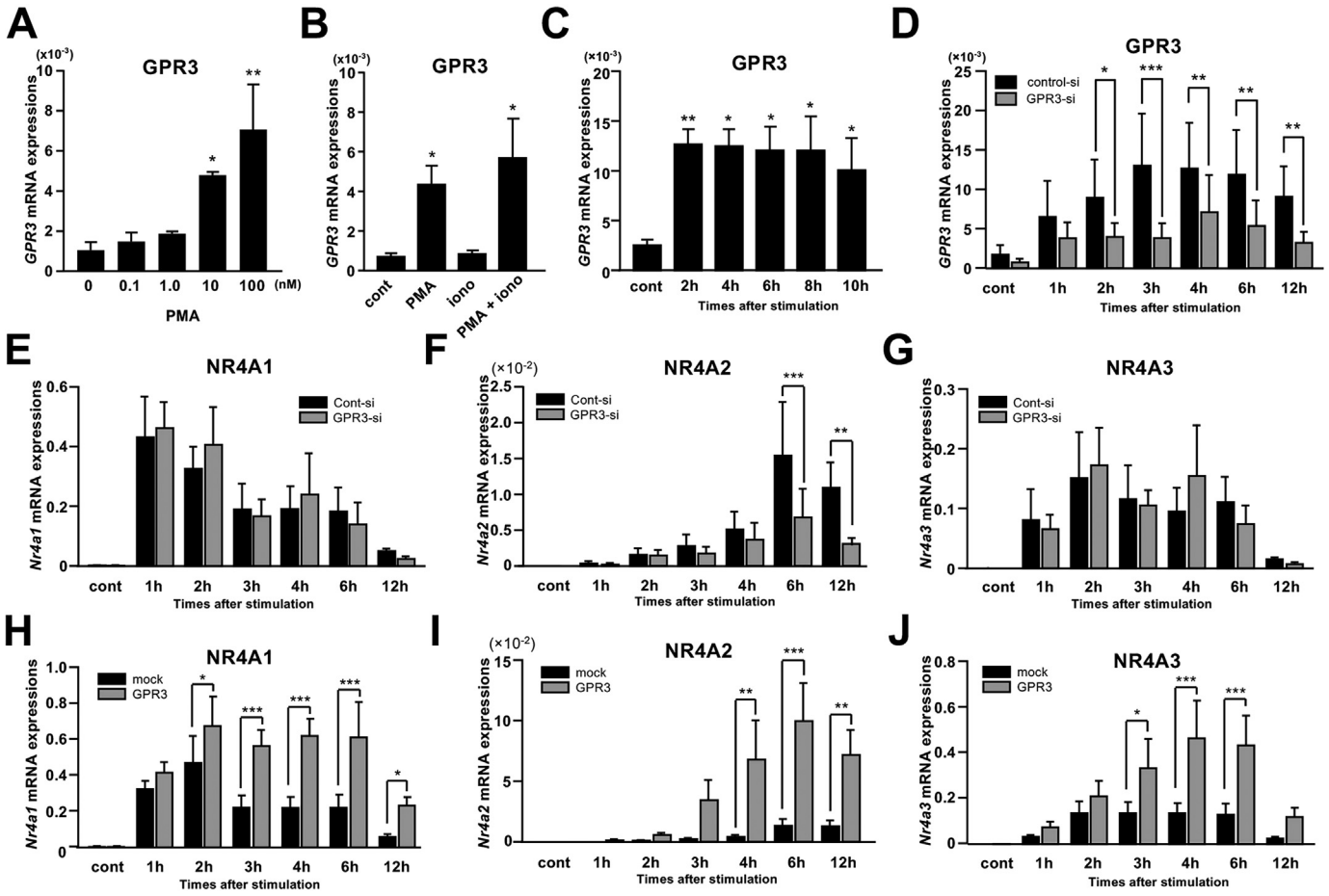

Fig. 1. GPR3 was induced upon T cell stimulation and modulated *NR4A2* expression in Jurkat cells. A) Jurkat cells were stimulated by PMA (0.1–100 nM) and the induction of *GPR3* mRNA was evaluated 3 h after stimulation. B) Jurkat cells were stimulated by PMA (100 nM), ionomycin (1 μg/mL), or PMA (100 nM) + ionomycin (1 μg/mL). Three hours after stimulation, the induction of *GPR3* mRNA was evaluated. C) Jurkat cells were stimulated by PMA (100 nM) + ionomycin (1 μg/mL), and the expression of *GPR3* mRNA was evaluated at 2, 4, 6, 8, and 10 h after stimulation. D–G) Jurkat cells were transfected with control siRNA or *GPR3* siRNA. Twenty-four hours after transfection, the cells were stimulated by PMA (100 nM) + ionomycin (1 μg/mL). The *GPR3* (D), *NR4A1* (E), *NR4A2* (F), and *NR4A3* (G) mRNA expression was evaluated 0, 1, 2, 3, 4, 6, and 12 h after stimulation. H–J) Jurkat cells were transfected with pC-mAGFL or pC-GPR3mAGFL. The *NR4A1* (H), *NR4A2* (I), and *NR4A3* (J) mRNA expression was evaluated 0, 1, 2, 3, 4, 6, and 12 h after stimulation. Data represent relative *GPR3* expression vs. control *GAPDH* (A, B) or *β-actin* (C–J). Results are presented as the mean ± SEM for each condition (three independent replicates). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. control.

splenocytes was induced as early as 1 h after stimulation by CD3/28 or PMA/ionomycin, however this expression was transient or sustained, respectively (Fig. 4A and B). Because the population of T cells, especially that of Treg cells, is modulated by *NR4A2* expression,[24] we examined the population of T cells in thymocytes and splenocytes from wild type or *GPR3*-knockout mice. The cell number and percentage of T cell subtypes in thymus and spleen was similar in wild type and *GPR3*-knockout mice (Fig. 4C–H). Additionally, the population of Treg cells in thymocytes and splenocytes was not affected by *GPR3*-knockout (Fig. 4I and J). By contrast, the percentage of effector T cells ($CD44^+CD62L^-$) was significantly greater in both the $CD4^+$ and $CD8^+$ T cell fractions from *GPR3*-knockout mice, compared with wild type mice (Fig. 5A and B). We further examined whether GPR3 modulates the expression of NR4A members under stimulated T cell conditions as observed in Jurkat cells. *GPR3* expression was immediately induced and sustained for 4 h after PMA/ionomycin stimulation in $CD4^+$ splenocytes from wild type mice, however significant changes were not observed in *NR4A1–3* expression between wild type and *GPR3*-knockout mice (Suppl. 2). These results suggest that *GPR3* is immediately induced by the TCR/CD28 stimulation in mouse $CD4^+$ T cells, and the duration of mRNA expression is differently regulated by the signal input. GPR3 plays a suppressive role in spontaneous T cell activation while the mechanism of suppression is remained as future issue.

4. Discussion

In the present study, we found that sustained GPR3 expression was induced after stimulation in Jurkat cells. Sustained GPR3 expression by T cell stimulation upregulated *NR4A2*, subsequently enhancing *NR4A* transcriptional activity in Jurkat cells. In mouse primary T lymphocytes, *GPR3* was also induced by T cell stimulation, but the duration of expression was regulated differently depending on the signal input. GPR3 expression did not affect the population of Treg cells; however, GPR3 had a suppressive role in spontaneous effector T cell activation.

The early induction of GPR3 upon T cell stimulation was observed in both Jurkat cells and mouse $CD4^+$ T cells, however the duration of *GPR3* expression differed depending on the type of cells and kind of T cell stimulation. Sustained GPR3 expression was achieved by PMA/ionomycin stimulation in $CD4^+$ T cells, but it lasted longer in Jurkat cells. Additionally, GPR3-mediated *NR4A2* modulation observed in Jurkat cells did not occur in mouse $CD4^+$ T cells upon PMA/ionomycin stimulation. Because Jurkat cells are a leukemia-derived cell line, they differ from normal physiological T

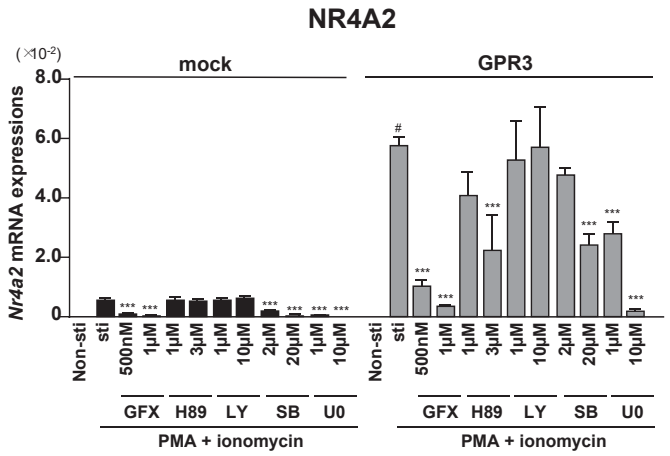

Fig. 2. GPR3-induced *NR4A2* expression was mediated by the PKA and MAPK signaling pathways. Jurkat cells were transfected with pC-mAGFL or pC-GPR3mAGFL vectors. Twenty-four hours after transfection, the cells were treated with the inhibitors GF109203X (PKC) (500 nM-1 μM), H89 (PKA) (1–3 μM), LY294002 (PI3K) (1–10 μM), SB203580 (p38 MAPK) (2–20 μM), or U0126 (MEK1/2) (1–10 μM) for 1 h, and then stimulated with PMA (100 nM) + ionomycin (1 μg/mL). The *NR4A2* mRNA expression was evaluated 6 h after stimulation. Data represent relative *GPR3* expression vs. control *β-actin*. Results are presented as the mean ± SEM of each condition (3–6 independent replicates). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs. no inhibitor in each stimulated group; #, $p < 0.001$ vs. no inhibitor in the unstimulated group.

cells. *In vivo*, in addition to TCR stimulation, costimulatory signals are required for the induction of NR4A1 expression in thymocytes.[33] Costimulatory molecules may be induced by T cell activation in Jurkat cells, resulting in sustained GPR3 expression that induces *NR4A2* expression. In human blood T cells, GPR3 expression is low in naïve T cells and resting Treg cells, but its expression increases in effector T cells and activated Treg cells (http://immunecellatlas.net) (Suppl. 3). Therefore, the difference in the duration and the stimulation of GPR3 expression may account for the discrepancy in NR4A2 modulation.

NR4A2 can be induced by PTH in osteoblasts[34] and by melanocortin-1 receptor in melanocytes.[35] Additionally, antimalaria drugs such as chloroquine have agonistic action on NR4A2, thereby modulating the immune response in T cells.[36] However, modulating agonists and agents for NR4A2 have rarely been reported. We revealed that GPR3 modulated *NR4A2* expression in a PKA- and MAPK-dependent manner in Jurkat cells. The human *NR4A2* gene has three transcriptional regulatory elements: cAMP response element (CRE), CArG-like element, and Sp-1 element.[37] Furthermore, the NR4A receptor subfamily induction can be mimicked by a cAMP activator.[38] Because GPR3 has the potential to increase the level of intracellular cAMP without ligands, GPR3-mediated *NR4A2* expression may partially depend on the cAMP-PKA signaling pathway, which is downstream of GPR3. Furthermore, GPR3-mediated *NR4A2* induction was mediated by MAPK

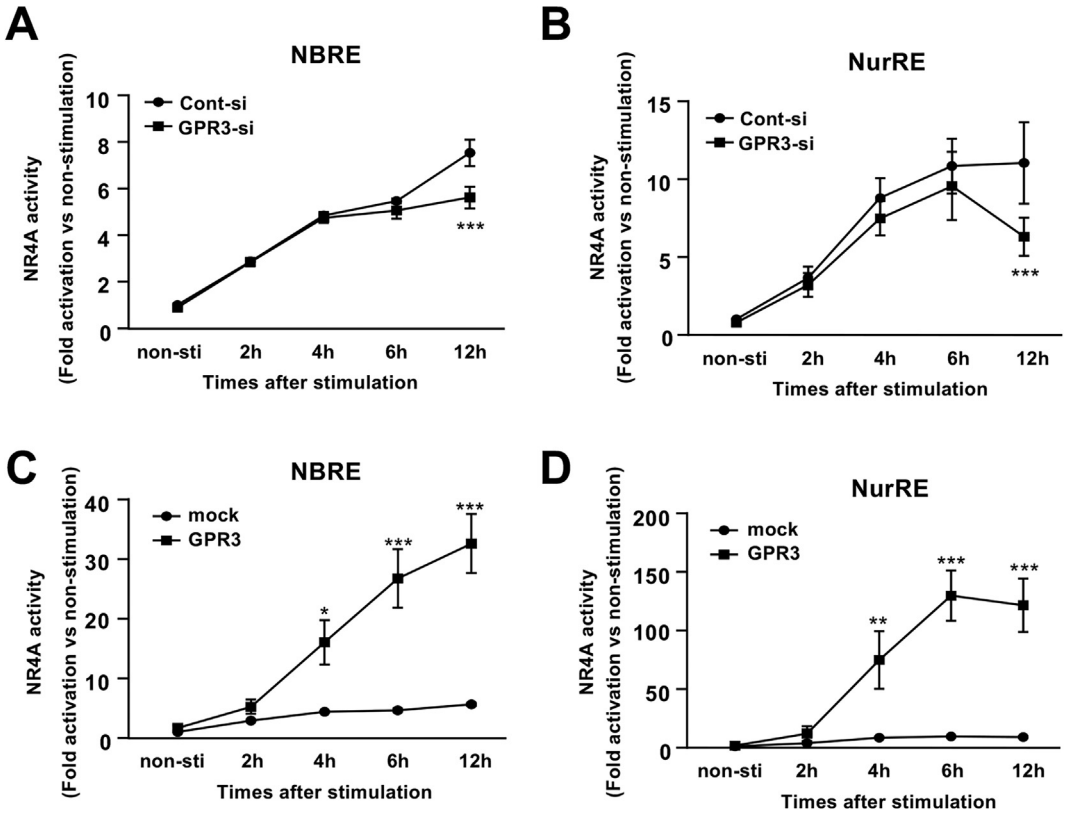

Fig. 3. *NR4A* transcriptional activity was augmented by GPR3 expression in Jurkat cells. A, B) Jurkat cells were transfected with control siRNA or *GPR3* siRNA plus pNL3.2-NBRE/pGL4.54-luc2 vectors (A) or pNL3.2-NurRE/pGL4.54-luc2 vectors (B). One day after transfection, the transfected cells were stimulated by PMA (100 nM) + ionomycin (1 μg/mL) for 2, 4, 6, and 12 h, and the transcriptional activity of *NR4A* was analyzed using a dual luciferase reporter assay. C, D) Jurkat cells were transfected with pC-mAGFL or pC-GPR3mAGFL vectors together with pNL3.2-NBRE/pGL4.54-luc2 vectors (C) or pNL3.2-NurRE/pGL4.54-luc2 vectors (D). Dual luciferase expression assay was performed at 2, 4, 6, and 12 h after stimulation. Values represent the fold activation vs. the non-stimulated control. Results are presented as the mean ± SEM of each condition (three independent replicates). **, $p < 0.01$; ***, $p < 0.001$ vs. control.

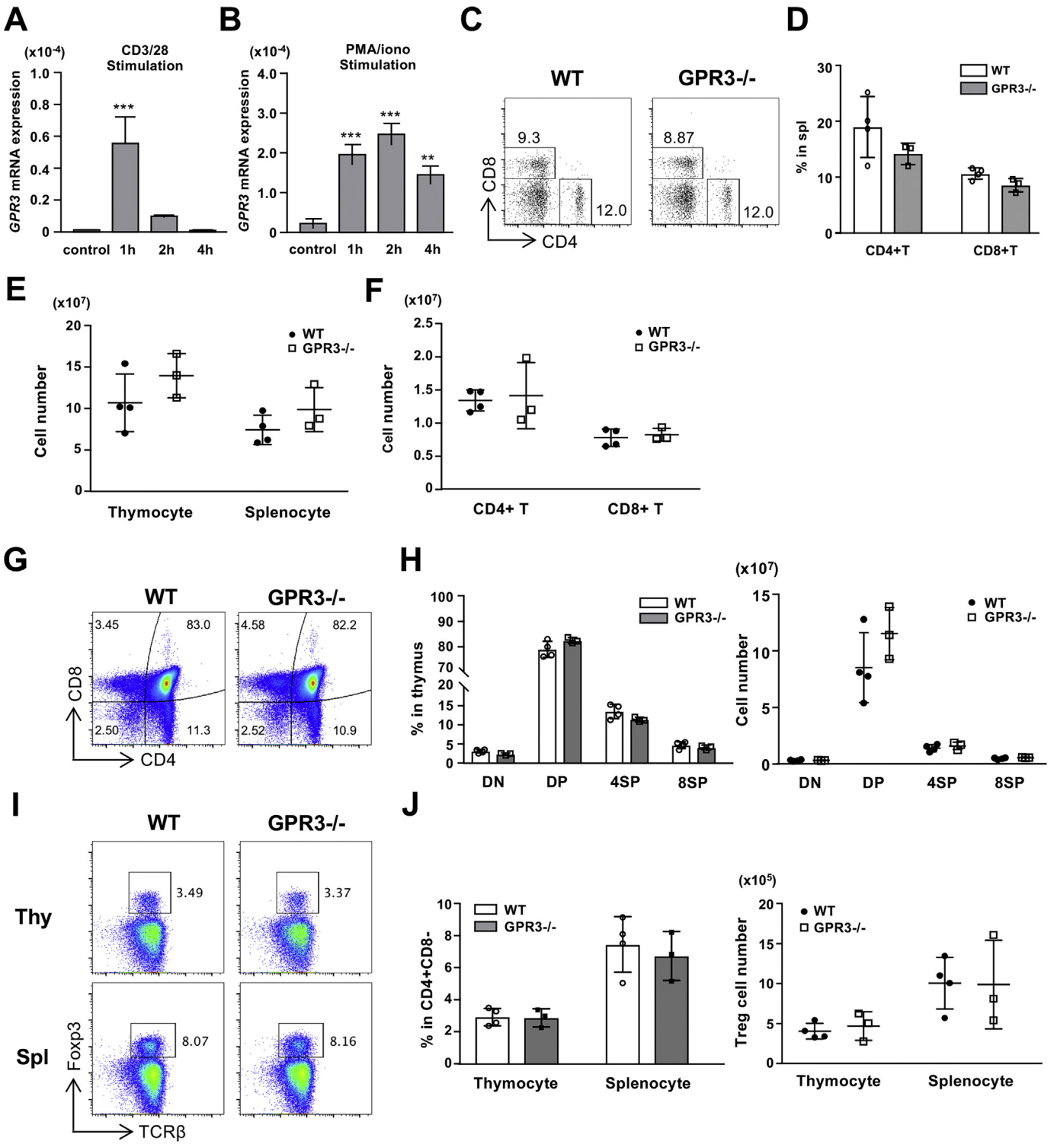

Fig. 4. *GPR3* expression in $CD4^+$ T cell and its effect on the population of T cell and Treg cell in thymocytes and splenocytes. A, B) $CD4^+$ mouse splenocytes were isolated and stimulated by CD3/CD28 (A) or PMA (100 nM) + ionomycin (1 μg/mL) (B). The expression of *GPR3* mRNA was evaluated at 1, 2, and 4 h after stimulation. C, D) Splenocytes were isolated from WT and *GPR3*-knockout ($GPR3^{-/-}$) mice and stained with anti-CD4 and anti-CD8 antibodies, followed by flow cytometry analysis. C) Representative FACS images are shown. D) The percentage of $CD4^+$ and $CD8^+$ splenocytes were compared between WT and *GPR3*-knockout ($GPR3^{-/-}$) mice. E) Total cell number of thymocytes and splenocytes in WT and *GPR3*-knockout (GPR3−/−) mice. F) Total cell numbers of CD4-positive and CD8-poitive splenocytes in WT and *GPR3*-knockout (GPR3−/−) mice. G) Thymocytes were isolated from WT and *GPR3*-knockout (GPR3−/−) mice and stained with anti-CD4 and anti-CD8 antibodies, followed by flow cytometry analysis. Representative FACS image are shown. H) The percentages and total cell number of double-negative (DN; CD4−CD8−), double-positive (DP; CD4+CD8+), CD4+CD8− single-positive (4SP), and CD4−CD8+ single-positive (8SP) thymocytes were compared between WT and *GPR3*-knockout (GPR3−/−) mice. I) Thymocytes and splenocytes were isolated from WT and *GPR3*-knockout (GPR3−/−) mice and stained with anti-CD4, anti-CD8, anti-Foxp3 and anti-TCRβ antibodies, followed by flow cytometry analysis. Representative FACS image are shown. J) The percentages and total cell number of CD4+CD8-Foxp3+ TCRβ+ Treg cell were compared between WT and *GPR3*-knockout (GPR3−/−) mice. Values are displayed as the mean ± SEM (n = 3–4 in each group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

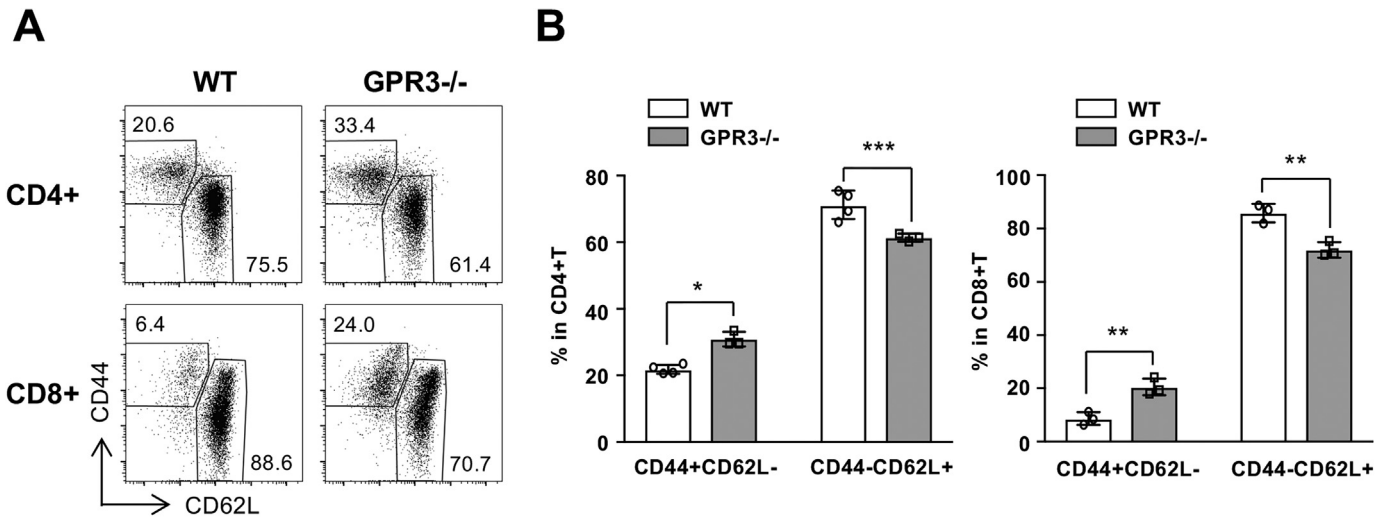

Fig. 5. GPR3 expression modulated effector T cell differentiation. A, B) Splenocytes were isolated from WT and *GPR3*-knockout ($GPR3^{-/-}$) mice and stained with anti-CD4, anti-CD8, anti-CD44, and anti-CD62L antibodies, followed by flow cytometry analysis. A) Representative FACS images are shown. B) The percentage of effector T cells ($CD44^{+}CD62L^{-}$) in $CD4^{+}$ or $CD8^{+}$ splenocytes was compared between WT and *GPR3*-knockout ($GPR3^{-/-}$) mice. Values are displayed as the mean ± SEM (n = 3–4 in each group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

signaling pathways. Crosstalk between cAMP-dependent kinases and MAPK signaling pathways has been reported previously.[39] In cerebellar granular neurons, GPR3 have potential to activate PKA, MAP kinase, and PI3 kinase mediated signaling pathways, thereby enhance neurite outgrowth and neuronal survival.[4,5] Therefore, GPR3-mediated multiple downstream signaling might be involved in *NR4A2* induction in T cell, however precise signaling and regulation of NR4A2 by GPR3 needed to be further examined.

It has been well established that cAMP elevation acts as a potent inhibitor of the activation of T lymphocytes.[11–15] Additionally, the duration of the cAMP elevation induces different T cell functions. Transient elevation in cAMP via segregation of stimulatory G-proteins contributes to T cell sensitization, whereas sustained elevation in cAMP by stronger TCR signaling in dendritic cells contributes to immunosuppression.[10] In addition to TCR signaling, adenosine secreted from tumor cells contributes to elevation in intracellular cAMP via Adenosine A2A receptor in T cells, thereby affecting immunosuppression.[16] Likewise, cAMP elevation via prostaglandin E2 receptor had an inhibitory action on T cell immunity.[40,41] In the current study, we found that GPR3 induction by T cell stimulation and the expression duration were regulated differently depending on the signal input. GPR3 constitutively activated Gs proteins without a ligand in various cells, which resulted in upregulated intracellular cAMP. If GPR3 also upregulates intracellular cAMP in T cells, sustained expression of GPR3 may play a role in immunosuppression. Consequently, GPR3 expression was immediately induced by T cell stimulation, and its expression was sustained or transient depending on the signal input. Sustained GPR3 expression may induce sustained cAMP elevation, resulting in *NR4A2* expression induction via a cAMP-dependent signaling pathway including PKA and MAPK.

The NR4A family receptors are highly enriched in regulatory T cells compared with other types of T cells upon stimulation and play a crucial role in the induction and maintenance of *FOXP3* gene expression, which is required for Treg differentiation.[23,24] However, Treg differentiation remained normal in *GPR3*-knockout thymocytes and splenocytes. By contrast, spontaneous effector T cell activation was observed in *GPR3*-knockout mice. During T cell maturation in thymocytes, positive- and negative-selection, and Treg differentiation occurred depending on the difference in TCR signal strength.[25] NR4A expression is induced by TCR stimulation in $CD4^{+}$ T cells and immature T cells in the developing thymus. Interestingly, the TCR strength is translated into the amount of NR4A expression, namely, high expression of NR4A resulted in Treg differentiation, while the highest expression of NR4A resulted in T cell apoptosis, known as “negative selection”.[23] Furthermore, NR4A family members have functional redundancies, because *NR4A2*-or *NR4A3*-knockout mice show normal Treg differentiation.[23] Therefore, even if NR4A2 suppression by *GPR3* knockout occurs in the thymus, Treg differentiation may develop normally. In such a case, negative selection of cells by apoptosis may be reduced, and consequently escape of self-reactive T cells into the periphery may be increased. However, the potential role of GPR3 in T cell apoptosis remains unclear.

On the other hand, in the physiological condition, NR4A2 expression in T lymphocytes has a beneficial effect in the pathogenesis of multiple sclerosis,[42] and the low expression of GPR3 in patients with multiple sclerosis is highly correlated with a poor prognosis.[8] The potential role of GPR3 in pathological conditions is still unclear, however if a certain level of GPR3 expression is induced under such conditions, GPR3 may have an immunosuppressive function, thereby affecting the development of the pathophysiology of multiple sclerosis.

Clarifying the mechanisms underlying the duration of GPR3 expression in relation to TCR signal intensity and the precise functions of GPR3 in terms of the pathophysiology of GPR3-related diseases may improve our understanding of the potential role of GPR3 in T lymphocytes.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

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