Original Article

Prostaglandin-related immune suppression in cattle

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Abstract

Prostaglandins (PGs) are lipid mediators derived from arachidonic acid by several enzymes including cyclooxygenase (COX)-1 and COX-2. We have previously shown that PGE_2 regulates immune responses, such as Th1 cytokine production and T-cell proliferation, in cattle. However, it is still unclear whether other PGs are involved in the regulation of immune responses in cattle.

Here, immunosuppressive profiles of PGs (PGA_1, PGB_2, PGD_2, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$) were firstly examined using bovine peripheral blood mononuclear cells (PBMCs). In addition to PGE_2, PGA_1 significantly inhibited Th1 cytokine production from PBMCs in cattle. Further analyses focusing on PGA_1 revealed that treatment with PGA_1 in the presence of concanavalin A (con A) downregulated CD69, an activation marker, and IFN-γ expression in both $CD4^+$ and $CD8^+$ T cells. Sorted $CD3^+$ T cells stimulated with con A were cultivated with PGA_1, and IFN-γ and TNF-α concentrations decreased upon PGA_1 treatment. Taken together, these results suggest that the treatment with PGA_1 *in vitro* inhibits T-cell activation, especially Th1 cytokine production, in cattle.

Keywords: cattle; immunosuppression; prostaglandin; prostaglandin A_1; Th1 response

Introduction

Prostaglandins (PGs) are formed from arachidonic acid, which is a 20-carbon unsaturated fatty acid released from the plasma membrane by phospholipases, and metabolized by several enzymes including cyclooxygenase (COX)-1 and COX-2 (Ricciotti and FitzGerald, 2013). PGs regulate a wide variety of physiological processes, including inflammation, immune responses and cell differentiation (Yao et al., 2009; Hirata and Narumiya, 2012; Ricciotti and FitzGerald, 2013). In particular, PGE_2 is a well-known inflammatory mediator, but is also known as an immunosuppressive molecule (Kalinski, 2012). PGE_2 inhibits immune cell activity, such as T cells and natural killer cells, via its receptors E prostanoid (EP) 2 and EP4 (Kalinski, 2012). In cattle, a previous study has shown that PGE_2 suppresses Th1 immune responses by reducing Th1 cytokine production and inducing the expression of programmed death ligand 1 (PD-L1), an immunoinhibitory molecule (Sajiki et al., 2018). In addition, PGE_2 production is upregulated in several chronic bovine infections, such as bovine leukemia virus (BLV) infection, Johne's disease and *Mycoplasma bovis* infection, contributing to the disease progression (Sajiki et al., 2018, 2019; Goto et al., 2020). However, the effects of other PGs on bovine immune cells are still unclear.

Within the PG family, PGA and PGJ are known as cyclopentenone PGs. PGA_1 is formed by dehydration in the cyclopentane ring of PGE_1 (Straus and Glass, 2001). Previous reports have shown the anti-viral effects of cyclopentenone PGs including PGA_1 (Santoro et al., 1982, 1983, 1989; Santoro, 1997). In the veterinary field, Caldas and colleagues have shown that PGA_1 inhibits the replication of bovine viral diarrhea virus (Caldas et al., 2018). Cyclopentenone PGs also have the function to protect cells from thermal injury (Amici et al., 1993). These anti-viral and cytoprotective activities of cyclopentenone PGs are associated with induced expression of heat shock proteins (HSPs), including HSP70 (Morimoto and

Santoro, 1998). In addition, a previous paper has demonstrated that 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2, one of the cyclopentenone PGs, reduces IFN-γ and TNF-α production in T helper cells (Sykes et al., 2012). However, the association of PGA_1 with Th1 responses is still unclear.

In the present study, we firstly examined the immunosuppressive function of six PGs, PGA_1, PGB_2, PGD_2, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$, and found that PGA_1 has immunosuppressive effects in addition to PGE_2. Therefore, we further focused on the effect of PGA_1 and examined whether PGA_1 suppresses Th1 immune responses in cattle.

Materials and methods

Blood collection and cell preparation

Bovine blood samples were obtained by several veterinarians in Hokkaido, Japan. Informed consent was obtained from all owners of cattle sampled in the present study. All experimental procedures using bovine samples were carried out following approval from the local committee for animal studies at Hokkaido University (approval number: 17-0024). As described in a previous paper (Sajiki et al., 2020a), peripheral blood mononuclear cells (PBMCs) were prepared from these blood samples using density gradient centrifugation on Percoll (GE Healthcare, Little Chalfont, UK). As described previously (Nishimori et al., 2017), to isolate $CD3^+$ T cells, bovine PBMCs were incubated with anti-bovine CD3 monoclonal antibody (mAb) (MM1A; Washington State University Monoclonal Antibody Center, Pullman, WA, USA; MacHugh et al., 1998) at 4°C for 30 min. Then, cells were incubated with anti-mouse IgG_1 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C for 15 min. After the second incubation, $CD3^+$ T cells were sorted by using an autoMACS Pro (Miltenyi Biotec) according to the manufacturer's instruction. After sorting,

the purity of $CD3^+$ T cells (> 90%) was confirmed using FACS Verse (BD Biosciences, San Jose, CA, USA).

Cell culture

PBMCs and purified $CD3^+$ T cells were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) including 10% heat-inactivated fetal calf serum (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin (Thermo Fisher Scientific), 100 μg/mL streptomycin (Thermo Fisher Scientific) and 2 mM L-glutamine (Thermo Fisher Scientific). All cultures were grown in a 96-well plate (Corning, Corning, NY, USA) in 250 μL of the culture medium.

To examine whether PGs suppress Th1 immune responses in cattle, PBMCs were cultured with 250 or 25 ng/mL of each PG (PGA_1, PGB_2, PGD_2, PGE_2, $PGF_{1\alpha}$ and $PGF_{2\alpha}$; all from Cayman Chemical, Ann Arbor, MI, USA). Cells were stimulated by 10 μg/mL of concanavalin A (con A) (Sigma-Aldrich). Acetonitrile (Kanto Chemical Co., INC, Tokyo, Japan) was used as a vehicle control. After 24 h, culture supernatants were collected, and the concentrations of Th1 cytokines were measured as described below. PBMCs were cultured with 2,500, 250 or 25 ng/mL of PGA_1 in the presence of 10 μg/mL of con A. After 5 h, the expression of *IFN-γ* and *TNF-α* genes were quantitated by quantitative real-time polymerase chain reaction (qPCR), and CD69 expression was measured by flow cytometry, as described below. To measure intracellular expression of IFN-γ, PBMCs were cultivated with 2,500, 250 or 25 ng/mL of PGA_1 in the presence of 10 μg/mL of con A. Following 19 h incubation, the cells were incubated with 10 μg/ml of brefeldin A (Sigma-Aldrich) for 5 h. Then, the cells were collected, and IFN-γ expression was measured by flow cytometry as described below.

Isolated $CD3^+$ T cells were cultured with 2,500, 250 or 25 ng/mL of PGA_1 in the presence of 10 μg/mL of con A. After 24 h, the concentrations of IFN-γ and TNF-α in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) as described below.

ELISA

The concentrations of IFN-γ, TNF-α and IL-2 in culture supernatants were determined by using Bovine IFN-γ ELISA Development Kit (Mabtech, Nacka Strand, Sweden), Bovine TNF alpha Do-It Yourself ELISA (Kingfisher Biotech, St. Paul, MN, USA) and Bovine IL-2 ELISA Development Kit (Mabtech), respectively, according to the manufacturers' instructions.

qPCR

As described previously (Sajiki et al., 2018), total RNA was extracted from cultured PBMCs using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's protocol. Then, cDNA was synthesized from total RNA by using PrimeScript reverse transcriptase (TaKaRa Bio, Otsu, Japan) following the manufacturer's protocol. The gene expression of *IFN-γ* and *TNF-α* in PBMCs was quantitated using a LightCycler 480 system II (Roche Diagnostics, Mannheim, Germany) and TB Green Premix DimerEraser (TaKaRa Bio) according to the manufacturers' instructions. *β-actin* (*ACTB*) was used as a reference gene, and the relative expression levels were calculated using the ΔΔCt method. The results were shown as relative changes to the no stimulation group. As described in our previous paper (Sajiki et al., 2018), primers were 5′-ATA ACC AGG TCA TTC AAA GG-3′ and 5′-ATT CTG ACT TCT CTT CCG CT-3′ for *IFN-γ*, 5′-TAA CAA GCC AGT AGC CCA

CG-3′ and 5′-GCA AGG GCT CTT GAT GGC AGA-3′ for *TNF-α* and 5′-TCT TCC AGC CTT CCT TCC TG-3′ and 5′-ACC GTG TTG GCG TAG AGG TC-3′ for *ACTB*.

Flow cytometry

To prevent non-specific reaction by Fc blocking, cells were incubated with phosphate buffered saline (PBS) including 10% goat serum (Thermo Fisher Scientific) for 15 min at 25°C. CD69 expression was measured as described previously with slight modifications (Sajiki et al., 2021). Briefly, after Fc blocking, the cells were stained with PerCP/Cy 5.5-conjugated anti-bovine CD3 mAb (MM1A), FITC-conjugated anti-bovine CD4 mAb (CC8, Bio-Rad, Hercules, CA, USA), PE-conjugated anti-bovine CD8 mAb (CC63, Bio-Rad) and Alexa Fluor 647-labeled anti-bovine CD69 mAb (KTSN7A; Kingfisher Biotech) for 20 min at 25°C. MM1A was conjugated by using the Lightning-Link antibody labeling kit (Innova Biosciences, Cambridge, England, UK). KTSN7A was prelabeled with a Zenon Alexa Fluor 647 Mouse IgG_1 Labeling Kit (Thermo Fisher Scientific). After staining, the cells were washed twice with PBS including 1% bovine serum albumin (BSA, Sigma-Aldrich), and then, the cells were assayed immediately by FACS Verse (BD Biosciences). The intracellular staining of IFN-γ was performed as described in a previous paper with slight modifications (Sajiki et al., 2021). Briefly, after Fc blocking, the cells were stained with PerCP/Cy 5.5-conjugated anti-bovine CD3 mAb (MM1A), FITC-conjugated anti-bovine CD4 mAb (CC8) and PE-conjugated anti-bovine CD8 mAb (CC63) for 20 min at 25°C. After surface staining, the cells were fixed and permeabilized using FOXP3 Fix/Perm kit (BioLegend, San Diago, CA, USA). The cells were then stained with biotinylated anti-bovine IFN-γ mAb (MT307; Mabtech) for 20 min at 25°C. Finally, the cells were incubated with APC Streptavidin (BioLegend) for 20 min at 25°C. After the final staining, stained cells were washed twice with PBS including 1% BSA and assayed immediately by FACS Verse.

Statistical analysis

In Figure 1, statistical significances were determined by the Dunnett's test. In Figure 2, statistical significances were determined by the Wilcoxon signed-rank test. In Figures 3–5, statistical significances were determined by the Steel–Dwass test. A p value of < 0.05 was considered statistically significant.

Results

The immunosuppressive profiles of PGs

Our previous study has shown that PGE_2 suppresses Th1 immune responses in cattle (Sajiki *et al*., 2018). In the present study, to examine whether other PGs also suppress immune responses in cattle, PBMCs were cultivated with PGA_1, PGB_2, PGD_2, PGE_2, $PGF_{1\alpha}$ or $PGF_{2\alpha}$ in the presence of con A. Then, Th1 cytokine concentrations in culture supernatants were measured by ELISA. As expected, PGE_2 strongly suppressed IFN-γ, TNF-α and IL-2 production from PBMCs (Figures 1B, 1D and 1F). In addition, IFN-γ, TNF-α and IL-2 production from PBMCs decreased upon PGA_1 treatment compared to the group treated with the vehicle (Figures 1A, 1C and 1E). These results suggested that PGA_1 may potentially impair Th1 immune responses in cattle. In contrast, the other PGs had little or no effects on the production of Th1 cytokines from PBMCs (Figures 1A–1F). Therefore, in this study, we examined the immunosuppressive effects of PGA_1 in detail.

The inhibition of T-cell activation by PGA_1

At first, we measured gene expression of Th1 cytokines in PBMCs cultured with PGA_1. Treatment with PGA_1 downregulated *IFN-γ* and *TNF-α* expression in PBMCs *in vitro* (Figures 2A and 2B). To examine whether PGA_1 inhibits the activation of T cells, the

expression of CD69, a lymphocyte activation marker, was measured on T-cell populations by flow cytometry. The gating strategy and representative plots for CD69 staining are shown in Figures 3A and 3B. Treatment with PGA_1 downregulated CD69 expression in $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in a dose-dependent manner (Figures 3C–3E). We then examined whether PGA_1 decreases IFN-γ expression in T cells. The representative plots for IFN-γ staining are shown in Figure 4A. Flow cytometric analysis revealed that treatment with PGA_1 decreased IFN-γ expression in $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in dose-dependent manner (Figures 4B–4D). Furthermore, we tested whether PGA_1 suppresses the function of T cells directly. $CD3^+$ T cells were isolated from PBMCs and cultivated with PGA_1. As shown in Figure 5, treatment with PGA_1 *in vitro* reduced the secretions of IFN-γ and TNF-α from $CD3^+$ T cells (Figures 5A and 5B). Taken together, these findings suggest that PGA_1 has immunosuppressive effects on T cells in the presence of con A stimulation in cattle.

Discussion

PGA_1 is known as one of the cyclopentenone PGs. Previous studies have shown that cyclopentenone PGs including PGA_1 have several effects, such as anti-viral and cytoprotective effects (Amici et al., 1993; Santoro, 1997). Additionally, a previous study has shown that a cyclopentenone PG, 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2, reduces IFN-γ and TNF-α production in T helper cells (Sykes et al., 2012). However, the association of PGA_1 with Th1 responses was still unclear. In the present study, we revealed the suppressive effect of PGA_1 on bovine Th1 responses in the presence of con A stimulation. PGA_1 inhibited the expression of CD69 and IFN-γ in both $CD4^+$ and $CD8^+$ T cells in a dose-dependent manner. Additionally, PGA_1 directly impaired Th1 cytokine production from $CD3^+$ T cells. To the best

of our knowledge, this is the first report to investigate the inhibition of T-cell activation by PGA_1 not only in cattle but also in other animals.

CD69 is a well-known activation marker of lymphocytes such as T cells and natural killer (NK) cells (Cibrián and Sánchez-Madrid, 2017). In bovine research, Ahn and colleagues have cloned and characterized bovine CD69 molecule (Ahn et al., 2002). In addition, several studies have used it as an activation marker of T cells and NK cells (Lund et al., 2012; Sajiki et al., 2020a, 2020b). Interestingly, a previous study has shown the association of CD69 expression with antigen-specific IFN-γ production (Okagawa et al., 2016). $CD69^+$ T cells highly expressed IFN-γ in the presence of antigens in *Mycobacterium avium* subsp. *paratuberculosis*-infected cattle (Okagawa et al., 2016). In the present study, treatment with PGA_1 *in vitro* suppressed CD69 expression in T cells. Taken together, treatment with PGA_1 might have a potential to suppress Th1 responses in the presence of antigen stimulation. However, in this study, we examined the effects of PGA_1 on immune responses only under the stimulation with con A. This could be one of the limitations of this study. Thus, the experiments using other stimulatory agents, such as antigens or anti-bovine CD3 and bovine CD28 mAbs, is required to evaluate the suppressive function of PGA_1 in detail. Further, additional experiments are also needed in order to elucidate the association of antigen-specific Th1 responses with suppressive function of PGA_1.

In this study, PGA_1 treatment significantly inhibited Th1 cytokine production, such as IFN-γ and TNF-α, *in vitro*. Several studies have previously demonstrated that nuclear factor-kappa B (NF-κB), a transcription factor, plays key roles for in the production of these cytokines (Hayden et al., 2006, Hayden and Ghosh, 2011). NF-κB is important for the upregulation of TNF-α production (Wang et al., 2014; Liu et al., 2017), and NF-κB induction

241 is required for IFN-γ production in Th1 responses (Corn et al., 2003). Additionally, several
242 studies have demonstrated a role of NF-κB in Th1 differentiation (Aronica et al., 1999; Oh
243 and Ghosh, 2013). A previous study has shown that NF-κB signaling is required for Th1
244 differentiation and immune responses rather than Th2 cell-mediated reaction (Aronica et al.,
245 1999). Interestingly, it has been reported that PGA_1 inhibits NF-κB activation (Rossi et al.,
246 1997). Hence, PGA_1 might regulate Th1 immune responses via inhibiting NF-κB activation.
247 The detailed information including Th1/Th2 related transcription factors, for example T-bet
248 and GATA3 (Naito et al., 2011), could be an informative evidence to understand how PGA_1
249 modulates Th1/Th2 differentiation in cattle.
250
251 Previous studies have reported the association of PGD_2 with human Th1/Th2
252 responses (Gosset et al., 2003; Tanaka et al., 2004). PGD_2 exerts biological functions via two
253 different receptors: D prostanoid receptor (DP) and chemoattractant receptor-homologous
254 molecule expressed on Th2 cells (CRTH2; alternative name DP2). Among human T cells, the
255 receptor CRTH2 is preferentially expressed on Th2-type cells (Nagata et al., 1999; Nagata
256 and Hirai, 2003). PGD_2 can promote Th2 polarization and recruitment of Th2 lymphocytes
257 through the inhibition of Th1 cytokine production (Tanaka et al., 2004; Gosset et al., 2005). In
258 this context, it is well known that PGD_2 mainly produced by mast cells is related to asthma
259 (Fajt et al., 2013). However, little information is available on the involvement of PGD_2 in
260 Th1/Th2 responses in cattle. In the present study, treatment with PGD_2 (250 ng/mL)
261 significantly inhibited IFN-γ production in response to con A from PBMCs, and it also tended
262 to inhibit TNF-α ($p = 0.062$) and IL-2 ($p = 0.064$) production, as shown in Figure 1. Although
263 the effect was obscure with the limited sample size, Th1 response might have been dampened
264 by PGD_2. Further studies are required to reveal the effects of PGD_2 on Th2 skewing and Th2
265 polarization.

Conclusions

The present study revealed that PGA_1 inhibits T-cell activation and Th1 cytokine production in the presence of con A stimulation. This is the first study that indicates the association of PGA_1 with the regulation of Th1 responses. Although Th1 responses are essential for the control of bovine chronic infections such as BLV infection and Johne's disease (Kabeya et al., 2001; Stabel, 2006), the kinetic of PGA_1 in cattle is still unclear. Future studies will indicate the relationship between PGA_1 and bovine diseases.

Conflict of interest statement

The authors declare that they have no competing interests.

Acknowledgements

This research was supported by JSPS KAKENHI grant number 19KK0172, AMED under grant number JP19fk0108068, Regulatory research projects for food safety, animal health and plant protection (JPJ008617.17935709) funded by the Ministry of Agriculture, Forestry and Fisheries of Japan, grants from the Project of the NARO, Bio-oriented Technology Research Advancement Institution (Research program on development of innovative technology; number 26058 BC and the special scheme project on regional developing strategy; grant 16817557) and JSPS and CAPES under the Japan-Brazil Research Cooperative Program (CAPES 88881.153222/2017-01) and CNPq under grant numbers 465678/2014-9, 405763/2018-2 and 141395/2016-8. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We are grateful to Dr. Hideyuki Takahashi, Dr. Yasuyuki Mori, and Dr. Tomio Ibayashi for valuable advice and discussions. We would like to thank Enago (www.enago.jp) for the English language review.

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Figure legends

Figure 1. Immunosuppressive profiles of PGs. (A–F) PBMCs (n = 7) were cultured with PGA_1, PGB_2, PGD_2, PGE_2, $PGF_{1\alpha}$ or $PGF_{2\alpha}$ in the presence of con A for 24 h. IFN-γ (A and B), TNF-α (C and D) and IL-2 (E and F) concentrations in culture supernatants were determined by ELISA. Acetonitrile was used as a vehicle control. Data are presented as the mean ± S.D., and statistical significances were determined by the Dunnett's test. *: $p < 0.05$ vs. the vehicle group, ***: $p < 0.001$ vs. the vehicle group, N.D.: not detected.

Figure 2. The decrease in the expression of Th1 cytokine genes by PGA_1. (A and B) PBMCs (n = 6) were cultured with 2,500 ng/mL of PGA_1 in the presence of con A for 5 h. *IFN-γ* (A) and *TNF-α* (B) expression in PBMCs were quantitated by qPCR. Data are indicated as relative expression levels compared to the no stimulation group. Statistical significances were determined by the Wilcoxon signed-rank test.

Figure 3. The decrease in CD69 expression in T cells by PGA_1. (A–E) PBMCs (n = 10) were cultured with PGA_1 in the presence of con A for 5 h. (A and B) The gating strategy and representative plots. (C–E) The percentages of $CD69^+$ cells in $CD3^+$ (C), $CD3^+CD4^+$ (D) and $CD3^+CD8^+$ (E) T cells were measured by flow cytometry. Statistical significances were determined by the Steel–Dwass test.

Figure 4. The suppression of IFN-γ expression in T cells by PGA_1. (A–D) PBMCs (n = 10) were cultured with PGA_1 in the presence of con A for 24 h. (A) Representative plots of IFN-γ staining. (B–D) The percentages of IFN-γ^+ cells in $CD3^+$ (B), $CD3^+CD4^+$ (C) and $CD3^+CD8^+$

(D) T cells were measured by flow cytometry. Statistical significances were determined by the Steel–Dwass test.

Figure 5. The inhibition of Th1 cytokine production from T cells by PGA_1. (A and B) Isolated $CD3^+$ T cells (n = 7) were cultured with PGA_1 in the presence of con A for 24 h. IFN-γ (A) and TNF-α (B) concentrations in culture supernatants were determined by ELISA. Statistical significances were determined by the Steel–Dwass test.

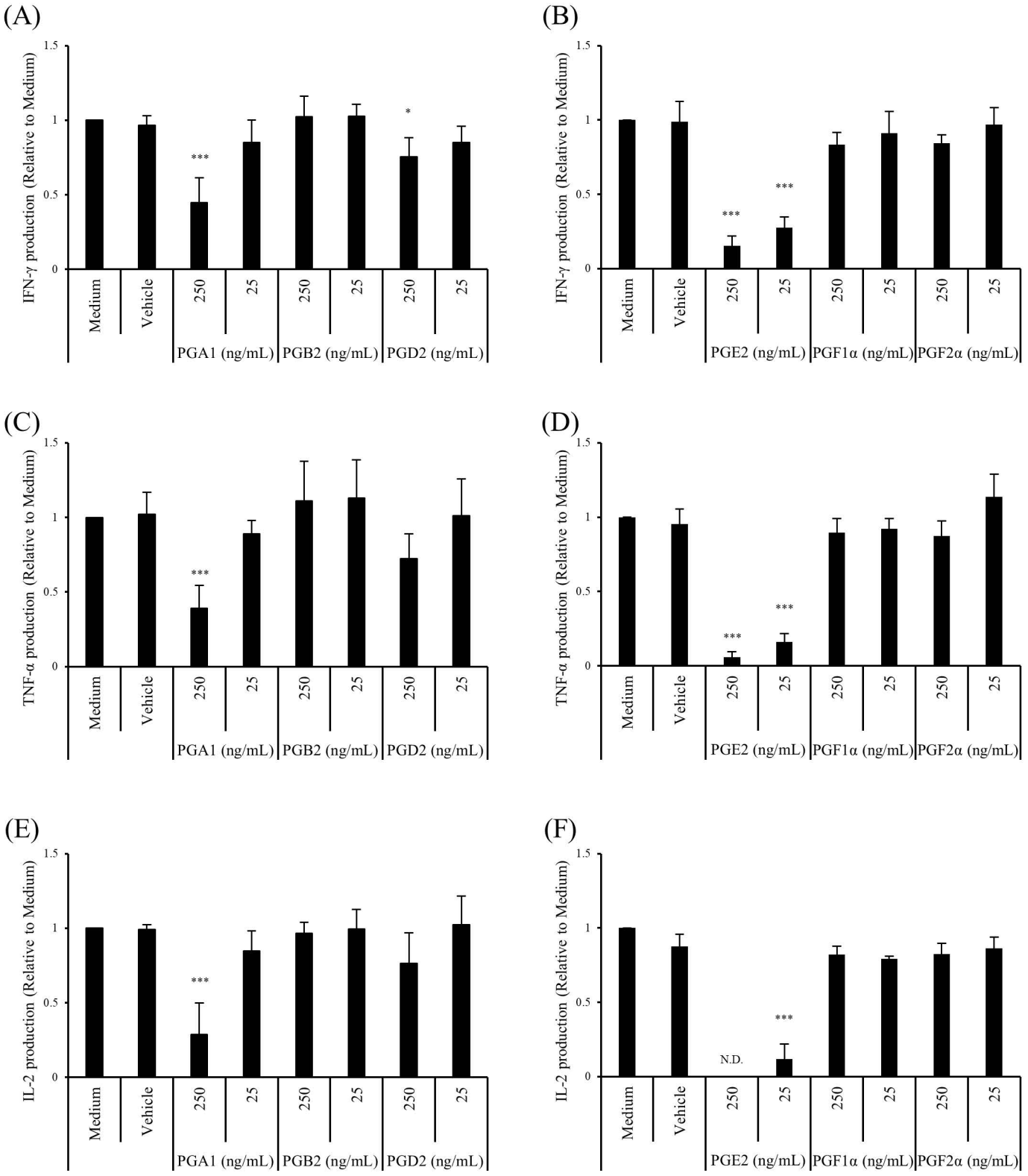
(A)
IFN-γ production (Relative to Medium)
1.5
1
0.5
0
***
*
Medium
Vehicle
250
25
250
25
250
25
PGA1 (ng/mL)
PGB2 (ng/mL)
PGD2 (ng/mL)
(B)
IFN-γ production (Relative to Medium)
***
***
Medium
Vehicle
250
25
250
25
250
25
PGE2 (ng/mL)
PGF1α (ng/mL)
PGF2α (ng/mL)
(C)
TNF-α production (Relative to Medium)
***
Medium
Vehicle
250
25
250
25
250
25
PGA1 (ng/mL)
PGB2 (ng/mL)
PGD2 (ng/mL)
(D)
TNF-α production (Relative to Medium)
***
***
Medium
Vehicle
250
25
250
25
250
25
PGE2 (ng/mL)
PGF1α (ng/mL)
PGF2α (ng/mL)
(E)
IL-2 production (Relative to Medium)
***
Medium
Vehicle
250
25
250
25
250
25
PGA1 (ng/mL)
PGB2 (ng/mL)
PGD2 (ng/mL)
(F)
IL-2 production (Relative to Medium)
N.D.
***
Medium
Vehicle
250
25
250
25
250
25
PGE2 (ng/mL)
PGF1α (ng/mL)
PGF2α (ng/mL)

Fig. 1

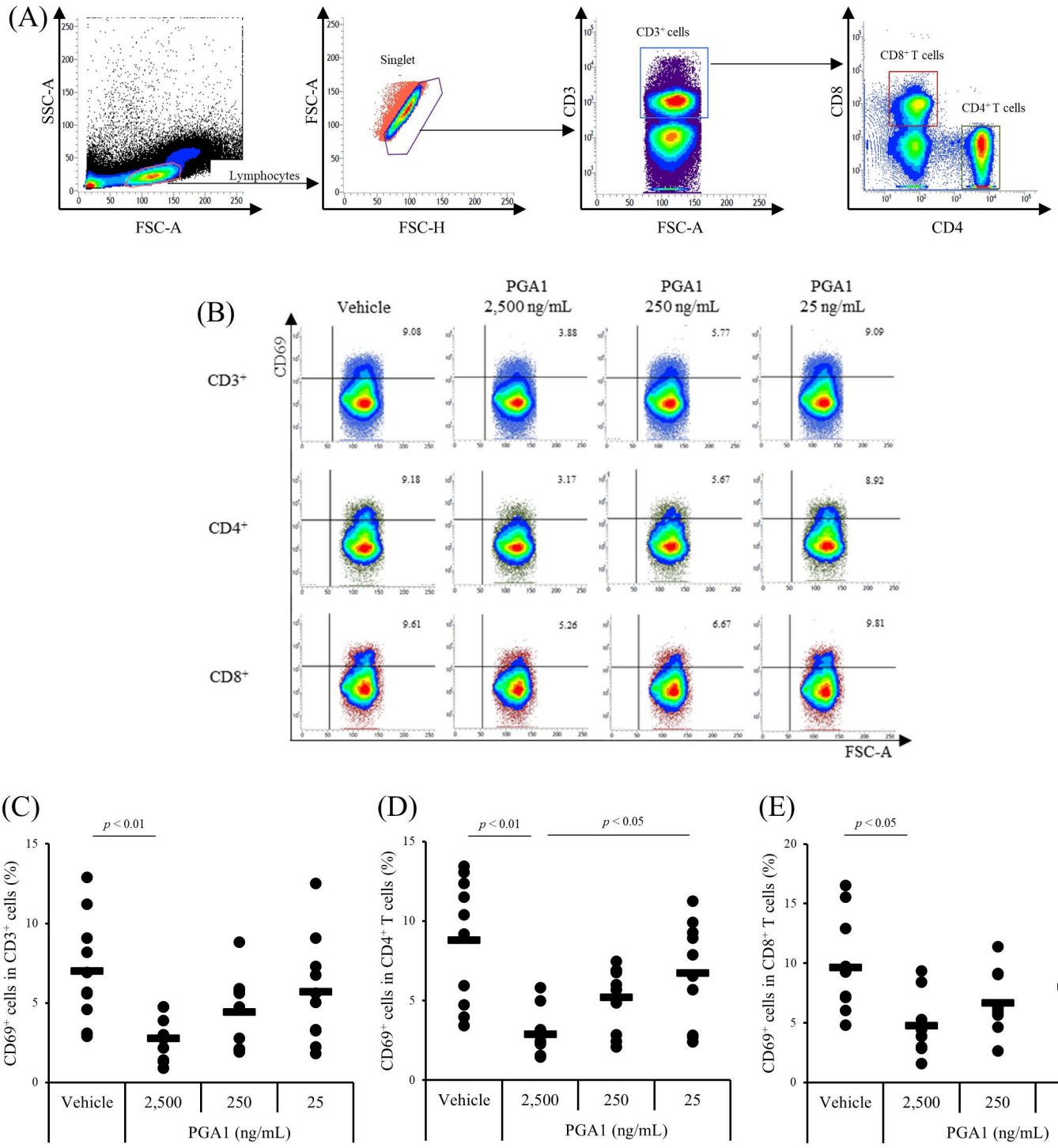
(A)
SSC-A
FSC-A
Lymphocytes
FSC-A
FSC-H
Singlet
CD3
FSC-A
CD3+ cells
CD8
CD4
CD8+ T cells
CD4+ T cells
(B)
Vehicle
PGA1 2,500 ng/mL
PGA1 250 ng/mL
PGA1 25 ng/mL
CD69
CD3+
CD4+
CD8+
9.08
3.88
5.77
9.09
9.18
3.17
5.67
8.92
9.61
5.26
6.67
9.81
FSC-A
(C)
p < 0.01
CD69+ cells in CD3+ cells (%)
Vehicle
2,500
250
25
PGA1 (ng/mL)
(D)
p < 0.01
p < 0.05
CD69+ cells in CD4+ T cells (%)
Vehicle
2,500
250
25
PGA1 (ng/mL)
(E)
p < 0.05
CD69+ cells in CD8+ T cells (%)
Vehicle
2,500
250
25
PGA1 (ng/mL)

Fig. 3

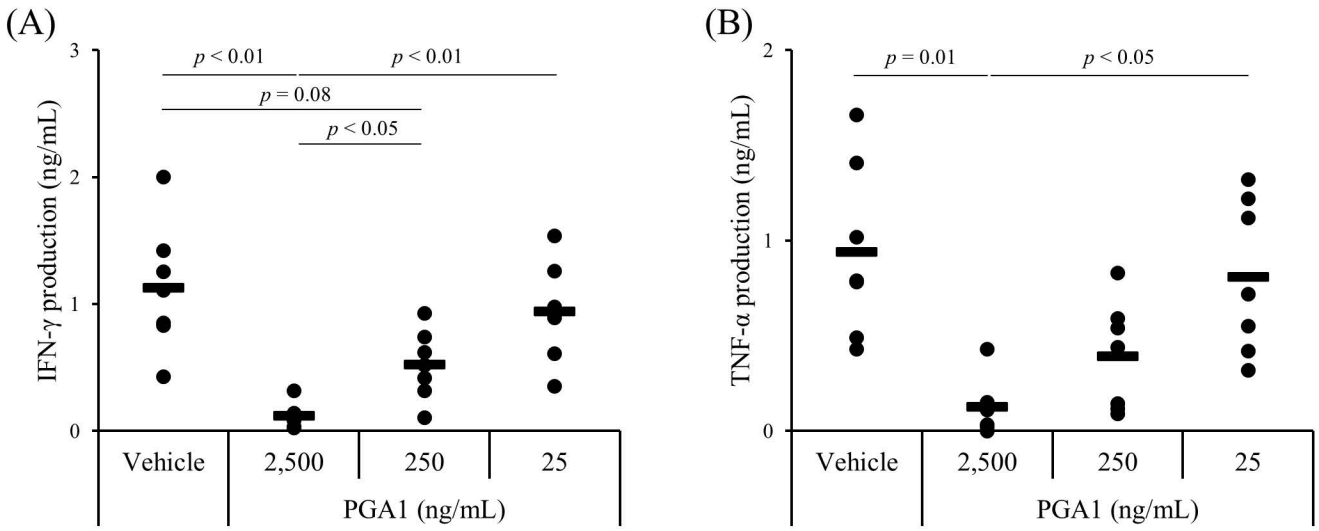
(A)
IFN-γ production (ng/mL)
3
2
1
0
p < 0.01
p < 0.01
p = 0.08
p < 0.05
Vehicle
2,500
250
25
PGA1 (ng/mL)
(B)
TNF-α production (ng/mL)
2
1
0
p = 0.01
p < 0.05
Vehicle
2,500
250
25
PGA1 (ng/mL)

Fig. 5

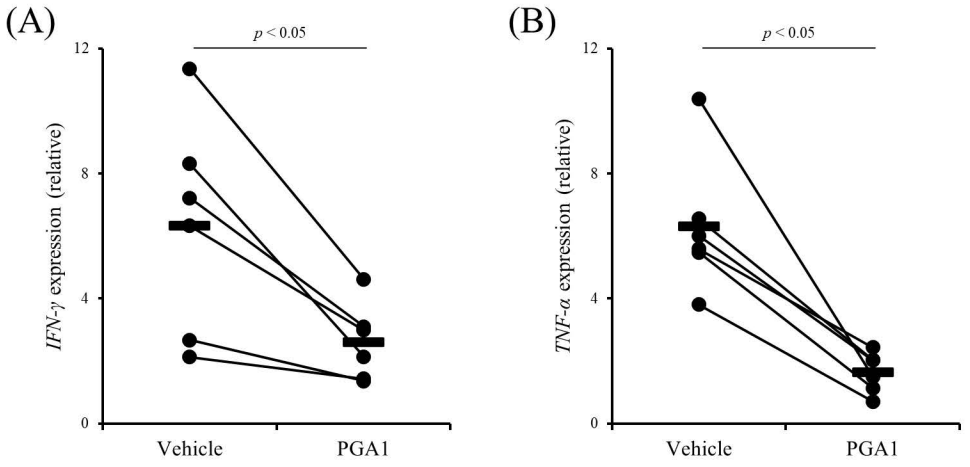
(A)
p < 0.05
12
8
4
0
IFN-γ expression (relative)
Vehicle
PGA1
(B)
p < 0.05
12
8
4
0
TNF-α expression (relative)
Vehicle
PGA1

Fig. 2

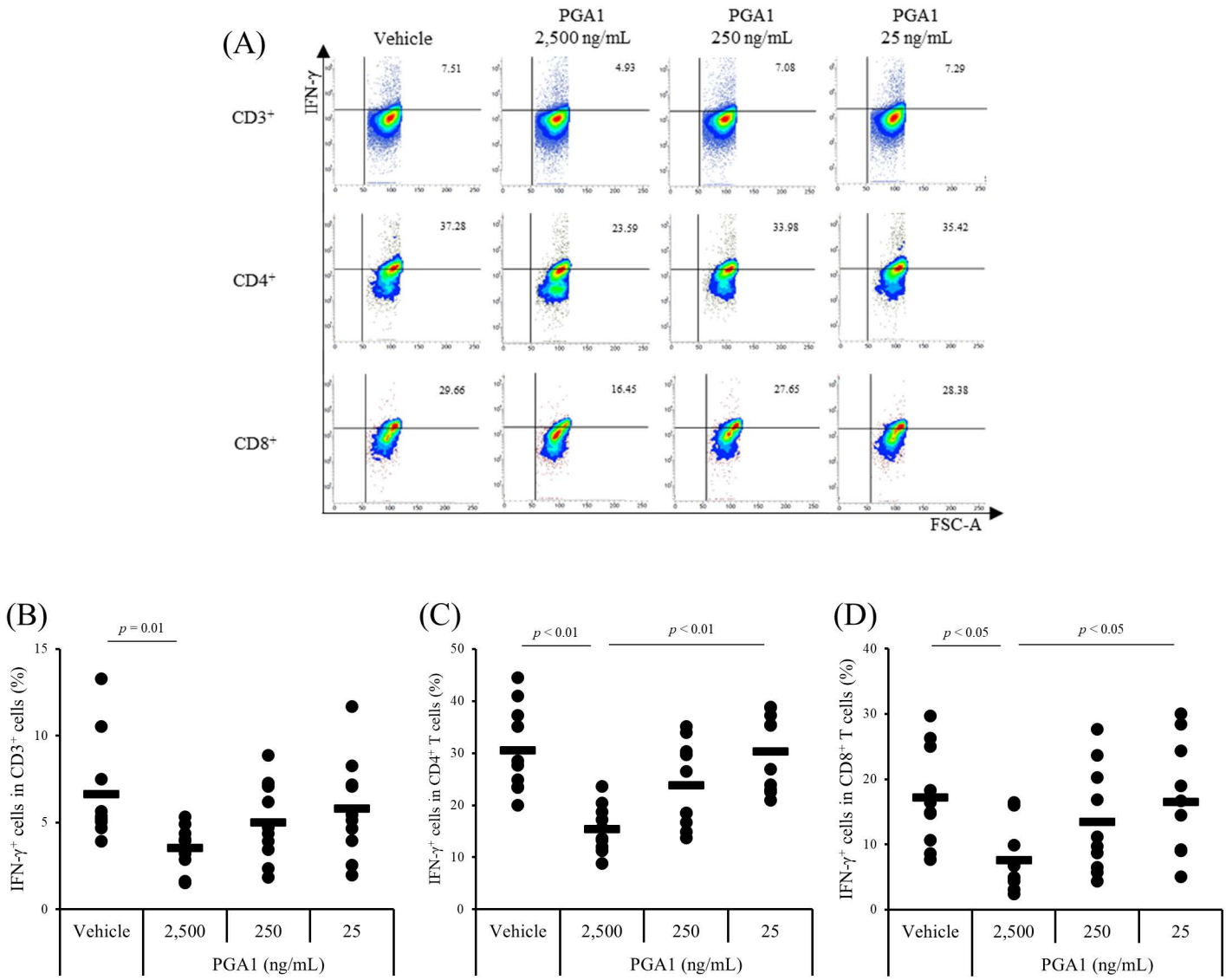
(A)
Vehicle
PGA1 2,500 ng/mL
PGA1 250 ng/mL
PGA1 25 ng/mL
IFN-γ
CD3+
CD4+
CD8+
7.51
4.93
7.08
7.29
37.28
23.59
33.98
35.42
29.66
16.45
27.65
28.38
FSC-A
(B)
p = 0.01
IFN-γ+ cells in CD3+ cells (%)
Vehicle
2,500
250
25
PGA1 (ng/mL)
(C)
p < 0.01
p < 0.01
IFN-γ+ cells in CD4+ T cells (%)
Vehicle
2,500
250
25
PGA1 (ng/mL)
(D)
p < 0.05
p < 0.05
IFN-γ+ cells in CD8+ T cells (%)
Vehicle
2,500
250
25
PGA1 (ng/mL)

Fig. 4