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Original article

The novel immunosuppressant prenylated quinolinecarboxylic acid-18 (PQA-18) suppresses macrophage differentiation and cytotoxicity in xenotransplantation

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ABSTRACT

Innate immunity plays a major role in xenograft rejection. However, the majority of immunosuppressants focus on inhibiting acquired immunity and not innate immunity. Therefore, a novel immunosuppressant suitable for use in conjunction with xenografts continues to be needed. It has been reported that prenylated quinolinecarboxylic acid-18 (PQA-18), a p21-activated kinase 2 (PAK2) inhibitor, exerts an immunosuppressive function on T cells. Hence, the possibility exists that PQA-18 might be used in conjunction with xenografts, which prompted us to investigate the efficacy of PQA-18 on macrophages compared with Tofacitinib, a janus kinase (JAK) inhibitor. Initial experiments confirmed that PQA-18 is non-toxic to swine endothelial cells (SECs) and human monocytes. Both PQA-18 and Tofacitinib suppressed macrophage-mediated cytotoxicity in both the differentiation and effector phases. Both PQA-18 and tofacitinib suppressed the expression of HLA-ABC by macrophages. However, contrary to Tofacitinib, PQA-18 also significantly suppressed the expression of CD11b, HLA-DR and CD40 on macrophages. PQA-18 significantly suppressed CCR7 expression on day 3 and on day 6, but Tofacitinib-induced suppression only on day 6. In a mixed lymphocyte reaction (MLR) assay, PQA-18 was found to suppress Interleukin-2 (IL-2)-stimulated T cell proliferation to a lesser extent than Tofacitinib. However, PQA-18 suppressed xenogeneic-induced T cell proliferation more strongly than Tofacitinib on day 3 and the suppression was similar on day 7. In conclusion, PQA-18 has the potential to function as an immunosuppressant for xenotransplantation.

1. Introduction

Xenotransplantation is an attractive solution to the problem of organ shortages. Due to the numerous molecular incompatibilities between donor and recipient, genetically modified pigs have been used in studies related to the suppression of xenograft rejection. Hyperacute rejection (HAR) is strongly suppressed in α-gal knockout pigs (Niu et al., 2017; Servick, 2017; Cooper et al., 2018a, b). The expression of complement regulatory proteins (C1 esterase inhibitors, decay accelerating factors, and membrane cofactor proteins) and human thrombomodulin down-regulate complement-mediated cytotoxicity and the coagulation system (Miyagawa et al., 1994; Fukuta et al., 2003; Miyagawa et al., 2004, 2006; Miwa et al., 2010; Cardone et al., 2011; Kim et al., 2015). However, there continue to be numerous obstructions to overcoming the xenogeneic innate cellular rejection induced by natural killer (NK) cells, phagocytes (monocytes/macrophages and

Abbreviations: APCs, Antigen presenting cells; CFSE, Carboxyfluorescein succinimidyl ester; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HAR, Hyperacute rejection; IL-2, Interleukin-2; JAK, Janus kinase; MACS, Magnetic-activated cell sorting; M-CSF, Macrophage colony stimulating factor; MHC, Major histocompatibility; MLR, Mixed lymphocyte reaction; MMC, Mitomycin C; NK, cells Natural killer cells; PAK2, P21-activated kinase 2; PBMCs, Peripheral blood mononuclear cells; PI, Propidium Iodide; PI3K, Phosphatidylinositide 3-kinases; PQA-18, Prenylated quinolinecarboxylic acid-18; SECs, Swine endothelial cells; TNF-α, Tumor necrosis factor-α

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neutrophils), and T cells (Cadili and Kneteman, 2008; Wang and Yang, 2012; Vadori and Cozzi, 2015; Meier et al., 2018). NK cytotoxicity in xenografts has been the subject of numerous studies over the years (Matsunami et al., 2001, 2002). Macrophages use a variety of surface receptors to sense self or non-self and to initiate defensive actions that are beneficial to the host. In previous studies, we, and others, have demonstrated that the expression of human leukocyte antigen-E (HLA-E), human CD47, human CD200, and the surfactant protein D masks and suppresses macrophage-mediated cell cytotoxicity (Yang, 2010; Maeda et al., 2013; Jiaravuthisan et al., 2018; Sakai et al., 2018).

Immunosuppressants are used to prevent various immune responses and to enhance tissue tolerance in organ transplantation. However, the majority of immunosuppressants that are used in standard therapeutic strategies only focus on acquired immunity and not innate immunity (Cooper et al., 2018a, b; Choi et al., 2018; Ezzelarab, 2018; Villadiego et al., 2018; Yamamoto et al., 2018; Zhang et al., 2018). Furthermore, most of the immunosuppressants that are used for anti-rejection in transplant patients would likely lead to the development of severe complications such as nephrotoxicity, neurotoxicity, hepatotoxicity and infections by conventional bacteria or viruses (Choi et al., 2018; Krook et al., 2002; Piscianz et al., 2014; Lee et al., 2018). Therefore, the development of a new immunosuppressant capable of preventing both innate and adaptive immunity with a high safety level would be desirable.

Prenylated quinolinecarboxylic acid (PQA-18), a novel immunosuppressant, inhibits the kinase activity of p21-activated kinase 2 (PAK2) in a non-competitive manner and shows suppressive effects on interleukin-2 (IL-2), IL-4, IL-6, and tumor necrosis factor-α (TNF-α) production in human peripheral lymphocytes (Ogura et al., 2016). PAK2 is a serine/threonine protein kinase that undergoes autophosphorylation and activation upon binding to the activated form of the small G proteins that have different functions at different points in hematopoiesis (Kosoff et al., 2015; Reddy et al., 2016). PAK2 regulates the survival and proliferation of hematopoietic progenitor cells and down-regulates granulocyte-monocyte lineage commitment (Zeng et al., 2015, 2017). PAK2 has also been implicated in T and B cell differentiation/maturation in the thymus and bone marrow in vivo (Zeng et al., 2015; O'Hagan et al., 2015). A previous study by Ogura et al. reported that PQA-18 exhibits the suppressive effect on T cells via the inhibition of PAK2 phosphorylation (Ogura et al., 2016), but, to date, the immunoregulatory potential of PQA-18 in innate immune cells has not yet been explored.

In the genetically modified pig xenotransplantation model, the levels of inflammatory cytokines and granulocyte-macrophage colony-stimulating factor (GM-CSF) were significantly and immediately increased, even in the presence of common immunosuppressants (Zhang et al., 2017). The release of cytokines results in immune cells infiltrating the xenograft. The release of GM-CSF promotes the differentiation, proliferation, and survival of macrophages. However, macrophage-mediated immune responses that directly damage xenografts and also promote T cell activation and indirectly disrupt xenografts are the main cause of xenograft rejection (Cadili and Kneteman, 2008). In this study, we report on an examination of the suppressive function of PQA-18 in macrophage-mediated xenogeneic immune responses. The findings indicate that PQA-18 suppresses GM-CSF-mediated macrophage differentiation, indicating that PAK2 plays a role in this process. GM-CSF performs its function after interacting with related receptors. At least two distinct domains of the receptors are phosphorylated via distinct pathways. One of these results in the activation of mitogen-activated protein kinases and the PI3K/AKT/p21 pathway (Comalada et al., 2004), the other induces activation of the JAK/STAT pathway (Sebastian et al., 2008). These two pathways in the immune system suggest that PAKs and JAKs could be used as targets for the design of immunosuppressants.

The JAK/STAT pathways are thought to be related to the development of various autoimmune diseases. The JAKs inhibitor, Tofacitinib, reduces macrophage-mediated inflammatory cytokine production (Ghoreschi et al., 2011; Nishimura et al., 2015). Tofacitinib is extensively used in the treatment of rheumatoid arthritis (Caporali and Zavaglia, 2018), autoimmune diseases (Borman et al., 2018; Fernandez-Clotet et al., 2018), and organ transplantation (Changelian et al., 2003; Betts et al., 2018). Meanwhile, preliminary results show that Tofacitinib has beneficial effects in early periods of pig-to-mouse islet xenotransplantation (Kang et al., 2017). In our study, the PAK2 inhibitor, PQA-18, was found to suppress the GM-CSF mediated differentiation of macrophages. Therefore, the function of PQA-18 was compared to that of Tofacitinib in macrophage differentiation, cytotoxicity ability, and the subsequent T-cell responses under conditions of xenogeneic stimulation.

2. Materials and methods

2.1. Cells and reagents

Swine endothelial cells (SECs), MYP30 (Miyagawa et al., 1994), were cultured in Dulbecco's modified Egale's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS). The human monocytic cell line (THP-1) was cultured in Roswell Park Memorial Institute (RPMI)1640 with 10% FBS. Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteer donors, followed by differential density gradient separation (Lymphocyte Separation Solution; Nacalai Tesque, Kyoto, Japan) and cultured in RPMI1640 complete medium (medium contained 10% FBS, 50 μM 2-mercaptoethanol (2-ME), 0.1 mM non-essential amino acids, 10 mM HEPES, and 1 mM sodium pyruvate) supplemented with 100 ng/ml recombinant GM-CSF (rGM-CSF) (Peprotech, Rocky Hill, NJ). Tofacitinib was purchased from AdipoGen Life Sciences (CA, USA). APC conjugated anti-human CD14 antibody, PE-conjugated anti-human CD3, PE-conjugated anti-human CD11b antibody, PE-conjugated anti-human HLA-DR antibody, FITC conjugated anti-human CCR7 antibody, and FITC anti-mouse IgG antibody were purchased from BioLegend, Tokyo, Japan. PE-conjugated anti-CD40 was purchased from Invitrogen, CA, USA. Anti-HLA-ABC class I antibody was purchased from Beckman Coulter Life Sciences, CA, USA.

2.2. Drug toxicity tests

SECs were plated in triplicate at a concentration of 1×10^4 cells/well in a flat-bottom gelatin-coated 96-well plate overnight. To evaluate the toxicity of PQA-18, SECs were incubated in 0–80 μM PQA-18 medium for 24 h at 37 °C. Each experiment was replicated four times. After exposure to PQA-18, the viability of the cells was evaluated using the WST-8 reagent solution (10 μl; Nakalai Tesque, kyoto, Japan) for 30 min. Color conversion was measured by a microplate reader (Corona, Tokyo, Japan) at a wavelength of 450 nm. The % viability was calculated as follows, % Viability = [OD (Target cells + PQA-18) - OD (Medium + PQA-18)]/[OD (Target cells) - OD (Medium)] × 100 (%).

2.3. Annexin V and propidium iodide (PI) staining

SECs or human PBMCs were cultured in 24 well plates in the presence and absence of PQA-18 (respectively 4×10^4 cells/well and 1×10^6 cells/well). After culturing for 24 h, the cells were harvested and suspended in 500 μl of Annexin V staining buffer, followed by Annexing V staining. PI (2 μg) was added to each sample before the flow cytometry experiments.

2.4. Generation of human macrophages

M1 and M2 macrophages were generated from human PBMCs in the presence of rGM-CSF, or in the presence of rM-CSF (R&D). The PBMCs, which were isolated from healthy volunteers, were incubated for 1 h at

37 °C in culture dishes. To isolate monocytes, non-adherent cells were removed and washed twice with PBS supplemented with 5% FBS and 2 mM EDTA. The adherent PBMCs were then cultured in RPMI 1640 complete medium, supplemented with 10% heat-inactive FBS and 100 ng/ml rGM-CSF or rM-CSF in the presence and absence of PQA-18 and Tofacitinib. In some experiments, the $CD14^+$ cells were purified from PBMCs by positive selection using magnetic-activated cell sorting technology (MACS) (Miltenyi Biotec, Germany) and then cultured in RPMI1640 complete medium supplemented with 100 ng/ml rGM-CSF for 5 days.

2.5. In vitro Cytotoxicity assay

SECs, at a concentration of 1×10^4 cells/well, were plated in triplicate in a flat-bottom gelatin-coated 96-well plate, followed by incubation overnight. To assess the cytotoxic activity of the human macrophages in the effector phase, 4×10^4 adherent GM-CSF-differentiated $CD14^+$ macrophages were added to each well in the presence and absence of PQA-18 (5 μM) or Tofacitinib (100 nM). To assess the efficacy of PQA-18 and Tofacitinib in the macrophage differentiation phase, 4×10^4 $CD14^+$ cells that had been pre-incubated with DMSO, PQA-18 (5 u M), or Tofacitinib (100 nM) were added to each well. WST-8 reagent solution was added to each well after 24 h, and the plate was then incubated for 30 min at 37 °C. Color conversion was measured by a microplate reader at a wavelength of 450 nm. The % Cytotoxicity was calculated as follows. % Cytotoxicity = [OD (SECs + Macrophages) - OD (Macrophages)]/[OD (SECs) - OD (Medium)] × 100 (%).

2.6. Mixed lymphocyte reaction (MLR)

For the xenogeneic T cell proliferation assay, SECs were treated with a 100 μg/ml mitomycin C (MMC) solution for 45 min at 37 °C to serve as stimulator cells. Human PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE) and co-cultured with the MMC-stimulated SECs at 37 °C in a 96 well-round bottom plate in RPMI complete medium, supplemented with 10% heat-inactive human serum and 50 μM 2-ME in the presence and absence of PQA-18 and Tofacitinib. In this study, 5×10^4 CFSE-labeled PBMCs were co-cultured with 5×10^4 MMC-treated SECs in 100 μl of complete medium/well. After 3 or 7 days of co-culture, the cells were harvested and stained with a PE-conjugated anti-human CD3 antibody. The proliferation of T cells was analyzed by flow cytometry. The % mean fluorescence intensity (MFI) was calculated as follows, % MFI = MFI [(Control group + MMC-treated SECs)/MFI (Drug treated PBMC groups + MMC-treated SECs)] × 100 (%). To compare the effects of PQA-18 and Tofacitinib in APC-independent T cell proliferation, 2×10^5/100 μl of PBMCs were cultured with 100 U/well of recombinant human IL-2 (rhIL-2) in a 96well round bottomed plate in the presence and absence of PQA-18 or Tofacitinib. After a 48 h period of cultivation, the cells were stained with PE-conjugated anti-human CD3 and T cell proliferation was determined by flowcytometry. % MFI = [MFI (Control groups + rhIL-2)/MFI (Drug treated PBMC group + rhIL-2)] × 100 (%).

2.7. Flow cytometry

FACS Verse and FACS Canto II (BD Biosciences, Heidelberg, Germany) were used to perform the flow cytometry and the data were analyzed by means of the FlowJo software (TreeStar, USA).

2.8. Statistics

Data are presented as the Mean ± SEM and statistical significances of differences between two groups were analyzed with the two-side Student's t-test. One-way ANOVA with the bonferroni post hoc test were used in more than two groups by using GraphPad prism software (La Jolla, CA). Results were considered to be significant at $P < 0.05$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control. † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ vs. each indicated group.

3. Results

3.1. At concentrations below 10 μM, PQA-18 has no observable toxic effect on SECs and human monocytes

To determine the optimal dose of PQA-18 that did not cause a significant loss of cell viability in SECs and human monocytes, we analyzed the toxic effect of PQA-18 on SECs and human monocytes. Cells were treated with various concentrations of PQA-18 (0 μM–80 μM) and cell viability was assessed by a WST-8 assay. No reduction in cell viability for both SECs and human monocytes was observed at concentrations of PQA-18 below 10 μM (Fig. 1A). We also examined cell toxicity by PI/Annexin V staining. The results showed that no reduction in PI and Annexin V double negative cells when treated with PQA-18 at concentrations of 2 μM–20 μM (Fig. 1B, 1C). This indicates that 5 μM PQA-18 is not toxic to SECs and human monocytes.

3.2. Both PQA-18 and Tofacitinib suppress macrophage-mediated xenogeneic cytotoxicity

Macrophages have been frequently been reported to be the main innate immune cells that kill xenogeneic target cells by macrophage-mediated cytotoxicity. Therefore, the efficacy of PQA-18 and Tofacitinib in macrophage-mediated xenogeneic cytotoxicity was examined. The adherent PBMCs were cultured in the presence of rGM-CSF for 6 days to allow them to differentiate into mature macrophages. The mature $CD14^+$ macrophages were isolated by MACS positive selection. The serum concentration of Tofacitinib reached around 100-300 nM on oral administration of 5 or 10 mg twice per day as an effective concentration (Kubo et al., 2014). We therefore compared the effect of 5 μM PQA-18 to 100 nM Tofacitinib. To assess the effect of PQA-18 in the effector phase, mature $CD14^+$ macrophages were co-cultured with SECs in the presence and absence of 5 μM PQA-18 or 100 nM Tofacitinib for 24 h (Fig. 2A). While Macrophages killed SECs effectively this killing was suppressed in the presence of 5 μM PQA-18 or 100 nM Tofacitinib in the effector phase. To further examine the effect of PQA-18 and Tofacitinib on macrophages differentiation, macrophages were developed with rGM-CSF in the presence and absence of 5 μM PQA-18 or 100 nM Tofacitinib before the cytotoxicity assay for 3 days. The PQA-18- or Tofacitinib-treated $CD14^+$ macrophages were washed, isolated and then cultured with SECs for 24 h in the absence of drugs. Both the PQA-18- and Tofacitinib-treated $CD14^+$ macrophages maybe exhibited reduced cytotoxicity for SECs in macrophage-mediated xenocytotoxicity (Fig. 2B). The results showed that, not only PQA-18, but also Tofacitinib pretreatment during differentiation significantly suppressed macrophage-mediated xenogeneic cytotoxicity, even in the differentiation phase.

Complement receptor CR3 (CD11b/CD18) which is expressed on macrophages, plays an important role in phagocytosis, cellular adherence and migration (Lukacsi et al., 2017; Fossati-Jimack et al., 2013). In this study, we examined the expression of CD11b as a marker for phagocytosis ability in the effector phase and in the differentiation phase. To examine the expression of CD11b in the effector phase, the adherent monocytes were cultured for 6 days in the presence of rGM-CSF. The mature macrophages were isolated and cultured for 24 h in the presence and absence of 5 μM PQA-18 or 100 nM Tofacitinib and the expression of CD11b in the cells was then examined. While a high level of CD11b expression on $CD14^+$ macrophages was observed in the control group, 5 μM PQA-18, but not 100 nM Tofacitinib, significantly suppressed the expression of CD11b in the effector phase (Fig. 2C, D). To examine the expression of CD11b in the differentiation phase, the macrophages were incubated with rGM-CSF in the presence and absence of 5 μM PQA-18 or 100 nM Tofacitinib for 3 days and the

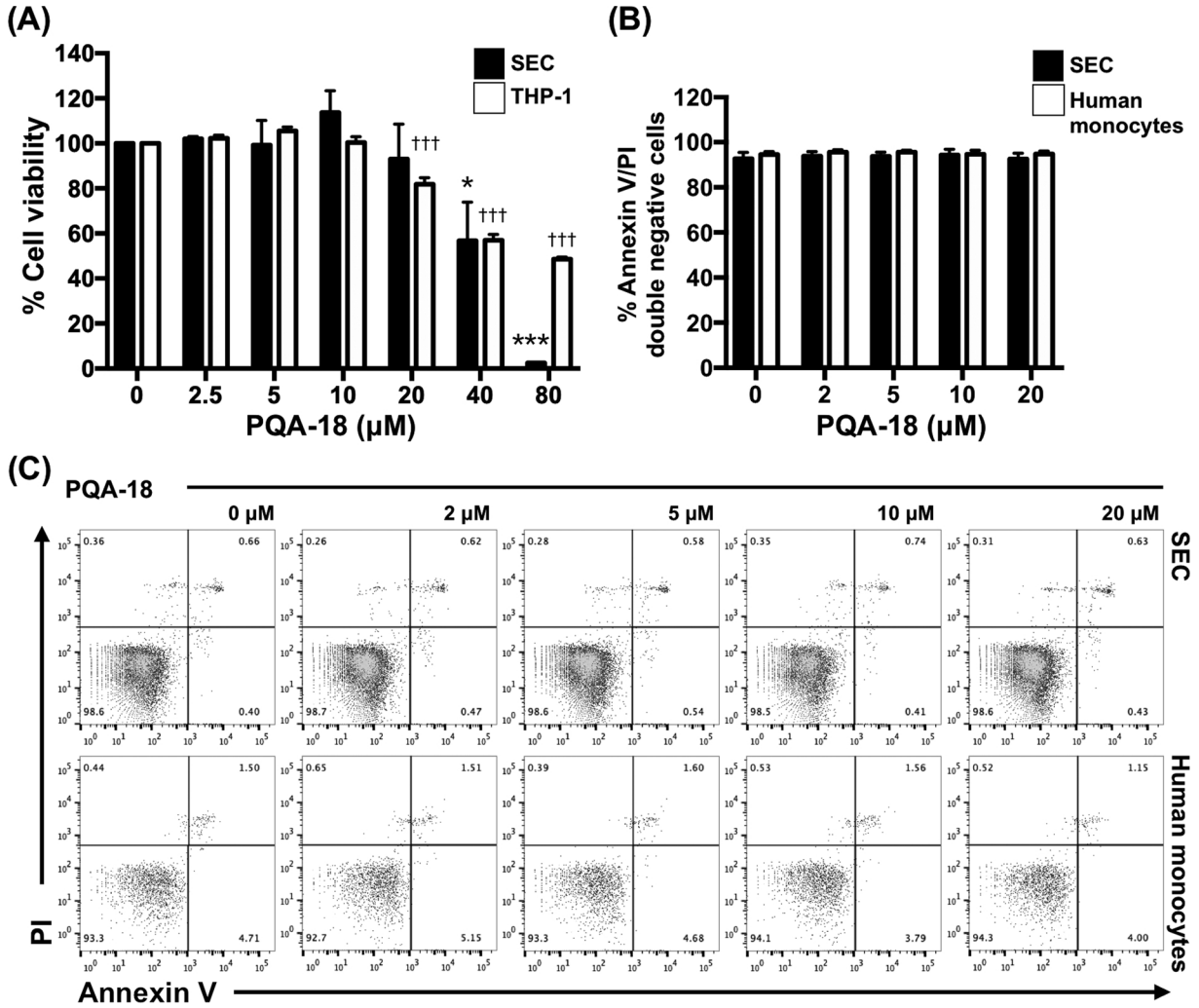

Fig. 1. Cell viability of PQA-18 treated SECs and human monocytes.
(A) Various concentrations of SECs or THP-1 cells were cultured in triplicate (0-80 μM) of PQA-18. The percentages of viable cells were measured by a WST-8 assay after a 24 h culture period. Data represent the Mean ± SEM, N = 4. *p < 0.05,*** P < 0.001 of SEC vs. control. ††† P < 0.001 of THP-1 vs. control. SECs or human monocytes were cultured with (0-20 μM) PQA-18 for 24 h and stained with Annexin V/PI. The double negative cells were considered to be live cells. **(B)** The % of Annexin V/PI double negative cells is represented by a histogram. Data represent the Mean ± SEM, N = 4. No significant differences were found. **(C)** The representative data showed the expression of annexin V and PI in SECs and human monocytes.

expression of CD11b was then examined. While a high level of CD11b expression was observed by $CD14^+$ macrophages in the DMSO group, PQA-18, but not Tofacitinib, strongly suppressed CD11b expression in the case of the three-day pretreated macrophages (Fig. 2E, F).

3.3. PQA-18 suppresses the differentiation of M1 but not M2 macrophages

Macrophages are crucial not only for innate immunity but also as antigen presenting cells (APCs) for host defense. The expression of MHC class II molecules at the cell surface of macrophages is required for their function and for the activation of T cells. GM-CSF and M-CSF are the main activators of the expression of MHC class II molecules in macrophages and mediate the differentiation of monocytes into M1 and M2 macrophages. The rGM-CSF-induced differentiation of M1 macrophages has been reported to be mediated by the PI3K/AKT/p21 pathway and the JAK signal pathway. We therefore reasonably expected that both PQA-18 and Tofacitinib would effectively suppress M1 macrophage differentiation. The PBMCs were cultured with rGM-CSF in the presence and absence of PQA-18 or Tofacitinib for 3 or 6 days. We found that PQA-18 suppressed the expression of the differentiation marker, HLA-DR, on rGM-CSF mediated M1 macrophages on day 3 and day 6. However, Tofacitinib had no ability to suppress HLA-DR expression on either day 3 or day 6 (Fig. 3A-C). Furthermore, both PQA-18 and Tofacitinib failed to suppress M-CSF mediated M2 macrophage differentiation (Fig. 3D, E). We also found that PQA-18 had a stronger efficiency for suppressing the expression of HLA-ABC than Tofacitinib in M1 macrophages (Fig. 3F, G).

Next, the expression of the M1 polarization marker, a C-C chemokine receptor type 7 (CCR7), on GM-CSF-mediated macrophages was evaluated. PQA-18 was found to suppress the expression of CCR7 on M1 macrophages on both day 3 and day 6, whereas Tofacitinib suppressed CCR7 expression on M1 macrophages only on day 6 but not on day 3 (Fig. 4A-C). These collective findings indicate that PQA-18 and Tofacitinib strongly suppress the differentiation and polarization of M1 but not M2 macrophages.

3.4. PQA-18 suppresses the expression of costimulatory molecule on M1 macrophages

CD40 is a costimulatory molecule that is constitutively expressed on APCs. The ligand for CD40 (CD40 L) is inducible on activated T cells. Co-stimulation through the CD40-CD40 L pathway is essential for T cell

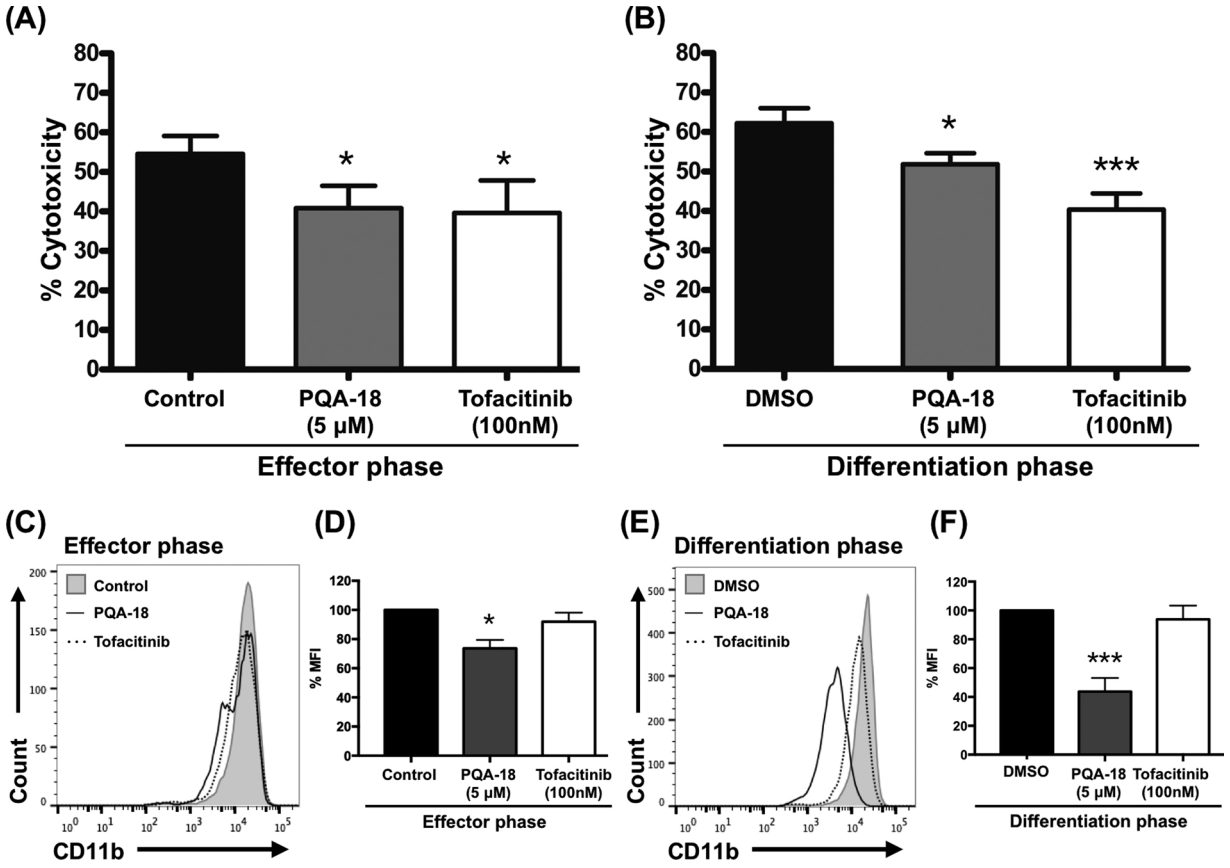

Fig. 2. Effect of PQA-18 and Tofacitinib on macrophage-mediated xenogeneic cytotoxicity in the effector phase or in the differentiation phase. SECs were plated in triplicate at a concentration of 1×10^4 cells/well as target cells in 96-well plates and cultured for 24 h. **(A)** To examine the function of macrophages in the effector phase, the $CD14^+$ macrophages were positively sorted from the day 5 GM-CSF-differentiated macrophages by MACS and the $CD14^+$ cells (4×10^4) were co-cultured with SECs, in the absence (control) and presence of 5 μM PQA-18 or 100 nM Tofacitinib for 24 h. **(B)** To examine the function of macrophages in the differentiation phase, macrophages were developed with rGM-CSF in the presence of DMSO, 5 μM PQA-18 or 100 nM Tofacitinib for 3 days. Macrophages were harvested and washed with PBS to remove drugs, followed by $CD14^+$ cell sorting. $CD14^+$ cells (4×10^4) were cultured with SECs for 24 h in the absence of drugs. After co-culturing, a WST-8 assay was performed to evaluate the % cytotoxicity. Data represent the Mean ± SEM, N = 4 -8. *p < 0.05, *** P < 0.001 vs. control. **(C, D)** The CD11b expression of rGM-CSF differentiated $CD14^+$ cells in the absence (control) and presence of 5 μM PQA-18 or 100 nM Tofacitinib for 24 h were examined. **(E, F)** The expression of CD11b by $CD14^+$ cells from drug-pre-treated human PBMCs was examined on day 3. Data represent the Mean ± SEM, N = 6. *p < 0.05, *** P < 0.001 vs. control.

dependent immune responses and the function of APCs. Inhibition of the CD40-CD40 L co-stimulatory pathway was reported to induce tolerance and prolong allograft survival in preclinical models (Ristov et al., 2018; Larsen et al., 2011; Zhang et al., 2015). Therefore, we examined the efficiency of PQA-18 and Tofacitinib on the expression of CD40 by macrophages. Human PBMCs were cultured for 3 days with rGM-CSF in the presence and absence of PQA-18 or Tofacitinib. We found that PQA-18 suppressed the expression of CD40 but Tofacitinib failed to suppress the expression of CD40 (Fig. 5).

3.5. PQA-18 shows stronger suppression activity in xenogeneic-induced T cell proliferation than Tofacitinib

A previous study reported that PQA-18 suppresses the expression of IL-2 in ConA-stimulated Jurkat cells (Ogura et al., 2016). However, in-depth information regarding the efficacy of PQA-18 on the relationship between macrophages and T cells in xenotransplantation is not currently available. We first compared the efficacy of PQA-18 and Tofacitinib in IL-2-induced T cell proliferation. Human PBMCs were fluorescently labeled at day 0 and cultured with rhIL-2 in the presence and absence of PQA-18 or Tofacitinib for 48 h. T cells were stimulated to proliferate by treatment with rhIL-2, which is strongly suppressed in the presence of both a low and a high dose of Tofacitinib (dose independent). In contrast, 5 μM PQA-18 showed a weaker suppression compared to Tofacitinib (Fig. 6A and B). We further compared the efficacy of PQA-18 and Tofacitinib by means of a xenogeneic-induced T cell proliferation assay on day 3 and day 7. The fluorescently labeled human PBMCs were stimulated with MMC-treated SECs for 3 or 7 days. While a significant level of xenogeneic T cell proliferation was induced in the control cells, this proliferation was significantly suppressed both on day 3 (Fig. 6C, E) and day 7 (Fig. 6D, F) by PQA-18 and Tofacitinib. Furthermore, while the suppression of xenogeneic MLR on day 7 by 5 μM PQA-18 was comparable to that for 100 nM Tofacitinib, PQA-18 showed a significantly stronger suppression than Tofacitinib on day 3 (Fig. 6C, E). Although 5 μM PQA-18 exhibited a more gentle suppression of IL-2 induced T cell proliferation compared to 20 and 100 nM Tofacitinib, 5 μM PQA-18 induced a stronger expression of xenogeneic T cell proliferation on day 3 than 20 and 100 nM Tofacitinib, indicating that the PQA-18-induced suppression of APCs mediated T cell proliferation is more significant than Tofacitinib.

4. Discussion

Xenograft rejection by the host immune system induces severe damage to grafts. Macrophages destroy xenografts by migrating to the tissues, followed by phagocytosis and antigen presentation to the cells

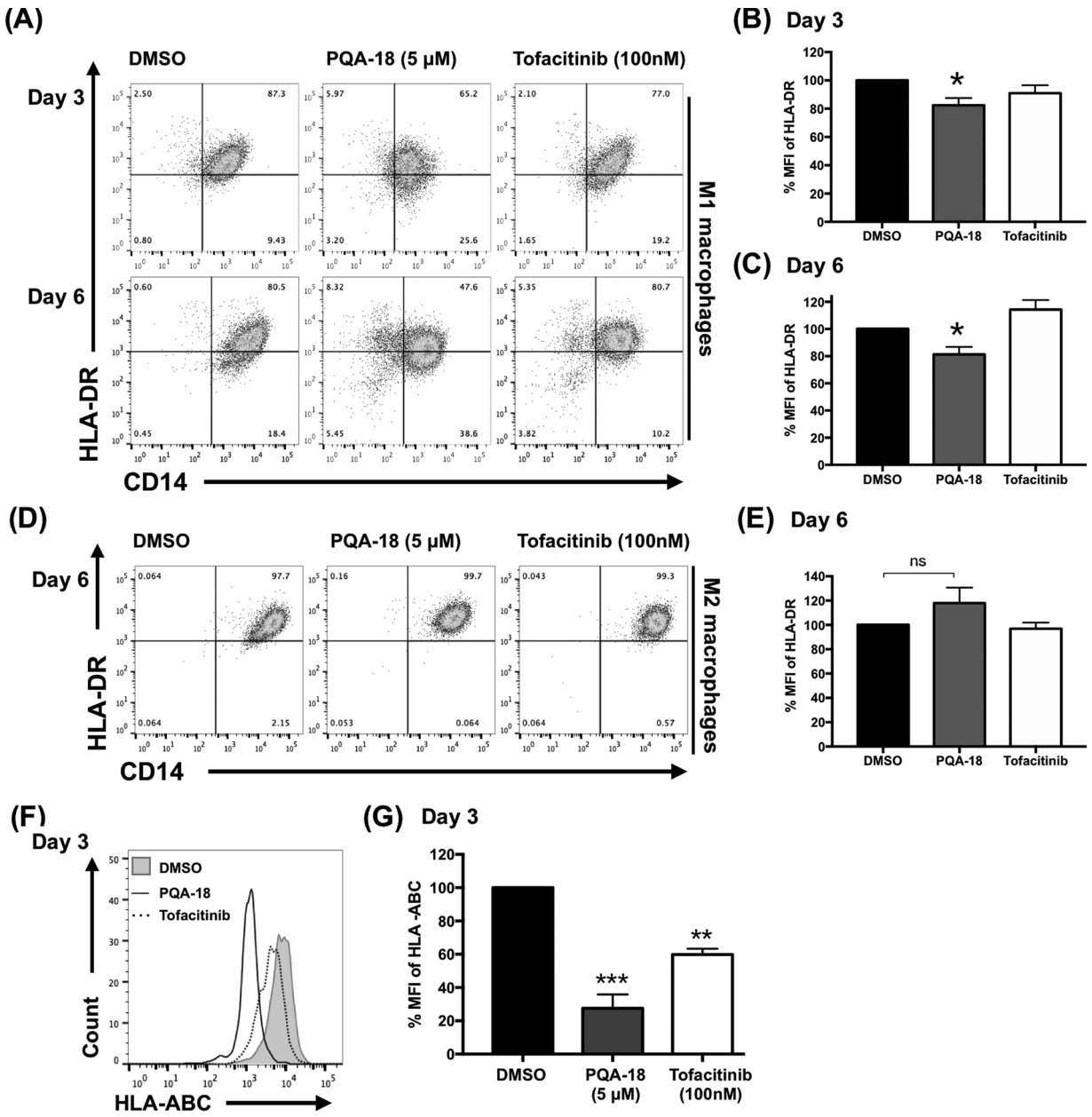

Fig. 3. Effect of PQA-18 or Tofacitinib on macrophage differentiation.
PBMCs were cultured with rGM-CSF in the presence of DMSO, PQA-18 or Tofacitinib at the indicated doses. **(A)** The expression of CD14 and HLA-DR in M1 macrophages was examined on day 3 and day 6 by flow cytometry. The % MFI of HLA-DR in M1 macrophages is represented by histogram on day 3 **(B)** and day 6 **(C)**. The expression of CD14 and HLA-DR in rM-CSF differentiated M2 macrophage in the presence of DMSO, 5 μM PQA-18 or 100 nM Tofacitinib on day 6 **(D)**. The % MFI of HLA-DR in M2 macrophages was represented by histogram on day 6 **(E)**. Data are the Mean ± SEM, N = 9. *p < 0.05 vs. control. **(F)** The expression of HLA-ABC in M1 macrophages was examined by flow cytometry. **(G)** The % MFI of HLA-ABC in M1 macrophages is represented by histogram. Data are the Mean ± SEM, N = 9. *p < 0.05 vs. control. ns = not significant. (**B, C, E**) N = 4. **p < 0.01, *** P < 0.001 vs. control. (**G**)

of the adaptive immune system, followed by boosting T cell xenoresponses. Phagocytes cause cytolysis and xenograft damage by generating reactive nitrogen species, reactive oxygen species and by producing inflammatory cytokines. The signaling pathways leading to macrophage functions are tightly regulated by the modulation of signaling cascades.

GM-CSF induces the proliferation of macrophages via the ERK pathway, macrophage survival via the PI3K/AKT/p21 pathway, and macrophage differentiation via the JAK/STAT pathways (Comalada et al., 2004; Sebastian et al., 2008). During chemotaxis and subsequent phagocytosis, macrophage polarization, recruitment and activation occur in areas that are inflamed through the PI3K/Rho/PAK2 pathway (Weiss-Haljiti et al., 2004; Di Marzio et al., 2005; O'Hayre et al., 2008). It has also been reported that the PI3K/Rho/PAK2 pathway is related to T cell polarization (Krummel and Macara, 2006), neutrophil migration (Itakura et al., 2013), and mast cell degranulation (Kosoff et al., 2013). The findings reported herein serve to demonstrate that PQA-18 and Tofacitinib suppress macrophage-mediated xenogeneic cytotoxicity, indicating that both the PAK and JAK related pathways contribute to macrophage-mediated phagocytosis and cell lysis.

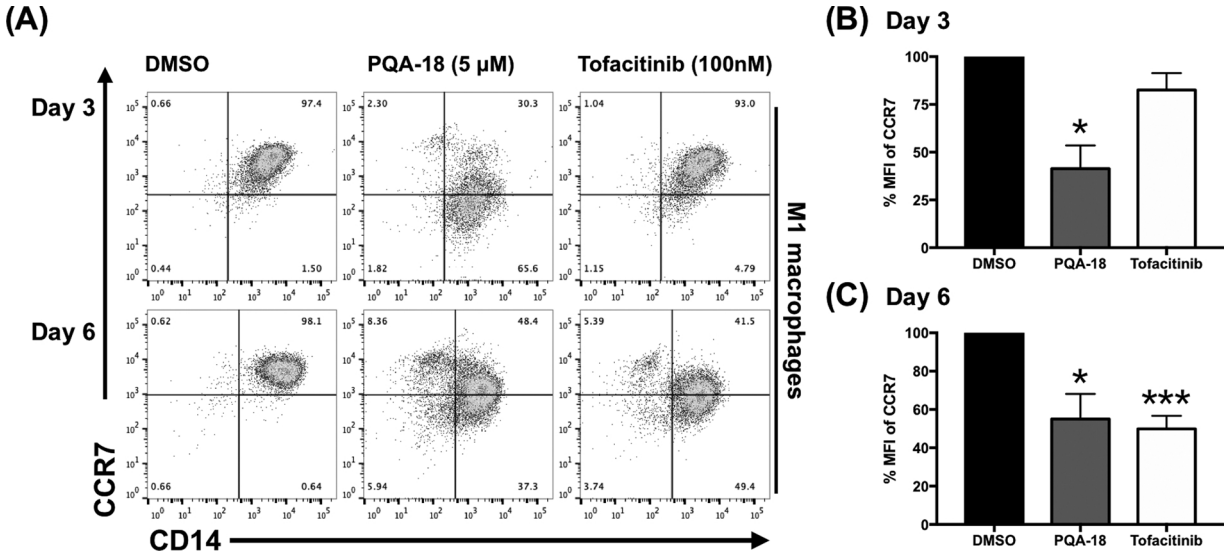

Fig. 4. Effect of PQA-18 or Tofacitinib on macrophage polarization.
(A) Dot plots representing the expression of CD14 and CCR7 in rGM-CSF differentiated M1 macrophages in the presence of DMSO, 5 μM PQA-18 or 100 nM Tofacitinib on day 3 and day 6. The % MFI of CCR7 on M1 macrophages is represented by histograms on day 3 **(B)** and day 6 **(C)**. Data are Mean ± SEM, N = 9. *p < 0.05 ***p < 0.001 vs. control.

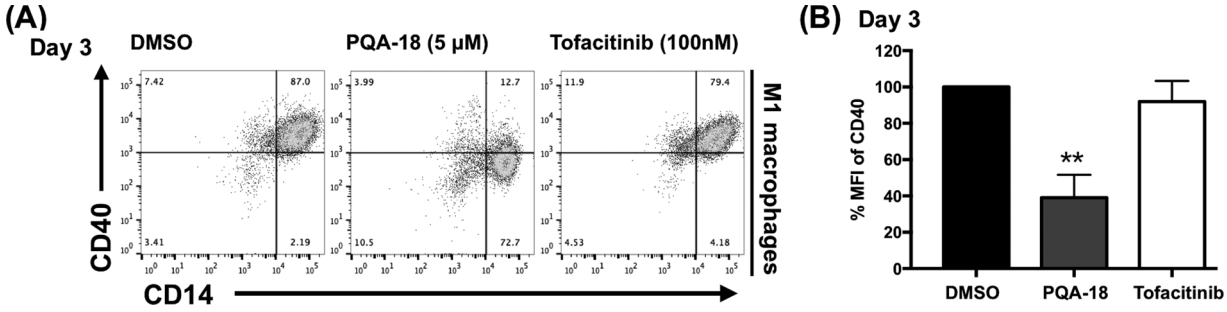

Fig. 5. Effect of PQA-18 or Tofacitinib in macrophage co-stimulatory molecule expression.
PBMCs were cultured with rGM-CSF for 3 days in the presence of DMSO, PQA-18 or Tofacitinib at the indicated doses. **(A)** The expression of CD14 and CD40 in M1 macrophages was examined by flow cytometry. **(B)** The % MFI of CD40 in $CD14^+$ macrophages is represented by a histogram. Data are the Mean ± SEM, N = 4. **p < 0.01 vs. control.

During macrophage differentiation, GM-CSF induced the activation of STAT5 via JAK/STAT5 pathway and PAK2-dependent STAT5 in an BCR-ABL-induced leukemogenesis model (Wang et al., 2007; Lehtonen et al., 2002; Berger et al., 2014). However, in our study, PQA-18, but not Tofacitinib, failed to suppress the GM-CSF induced phosphorylated-STAT5 expression (data not shown). Furthermore, only PQA-18 has the potential to suppress the phagocytosis of macrophages in both the effector phase and the differentiation phase (Fig. 2C-F). These findings suggest that PQA-18 and Tofacitinib suppress macrophage functions via different mechanisms.

PAK2 is expressed ubiquitously in various cells, such as hematopoietic and endothelial cells and acts downstream of the Rho family, Rac2, and Cdc42 that regulate numerous cellular processes. PAK2 inhibitors appear to have the potential to suppress immune function on macrophages (Comalada et al., 2004; Weiss-Haljiti et al., 2004), T cells (O'Hagan et al., 2015; Krummel and Macara, 2006), neutrophils (Itakura et al., 2013), and mast cells (Kosoff et al., 2013). However, Radu et al. reported that the depletion of PAK2 inhibited the proliferation and survival of adult murine endothelial cells (Radu et al., 2015). We demonstrated here that, at PQA-18 concentrations higher than 20 μM, the cell viability of SECs is reduced. In contrast, the toxic effect of 20 μM PQA-18 on SECs was negligible. Five micromolar PQA-18 showed significant immunosuppressive effects and was considered to be a safe and effective dose in this system.

The level of macrophage differentiation/polarization and the subsequent adaptive immune responses are intimately related to antigen presentation ability, and this process is closely associated with the level of expression of HLA-ABC and HLA-DR (Lin et al., 2016). Our results showed that PQA-18 caused a decrease in HLA-DR expression in M1 but not in M2 macrophages on day 3 and 6, whereas Tofacitinib failed to show suppressive effects (Fig. 3A-C). A similar finding showing that Tofacitinib failed to reduce HLA-DR expression on monocyte-derived dendritic cells was reported in a previous study by Kubo et al. (Kubo et al., 2014). On the other hand, the CCR7 expression could be regulated by PQA-18 and Tofacitinib on diverse phases (Fig. 4), indicating that the PAK2 and JAK pathways influence macrophage differentiation in different phases. Thus, we conclude that PAK2 might be an earlier factor for regulating macrophage polarization compared to the JAK pathway.

Our results showed the PQA-18 exerted less suppressive effects on IL-2-stimulated T cell proliferation than Tofacitinib (Fig. 6A and B). However, PQA-18 showed a stronger suppression of xenogeneic T cell proliferation than Tofacitinib on day 3 suggesting that PQA-18 suppresses T cell proliferation via the APC-dependent and APC-independent pathways. Taking into consideration the results showing IL-2 induced T cell proliferation, it can be assumed that PQA-18 is more effective in suppressing APC-mediated T cell proliferation and is less effective in suppressing APC-independent T cell proliferation compare

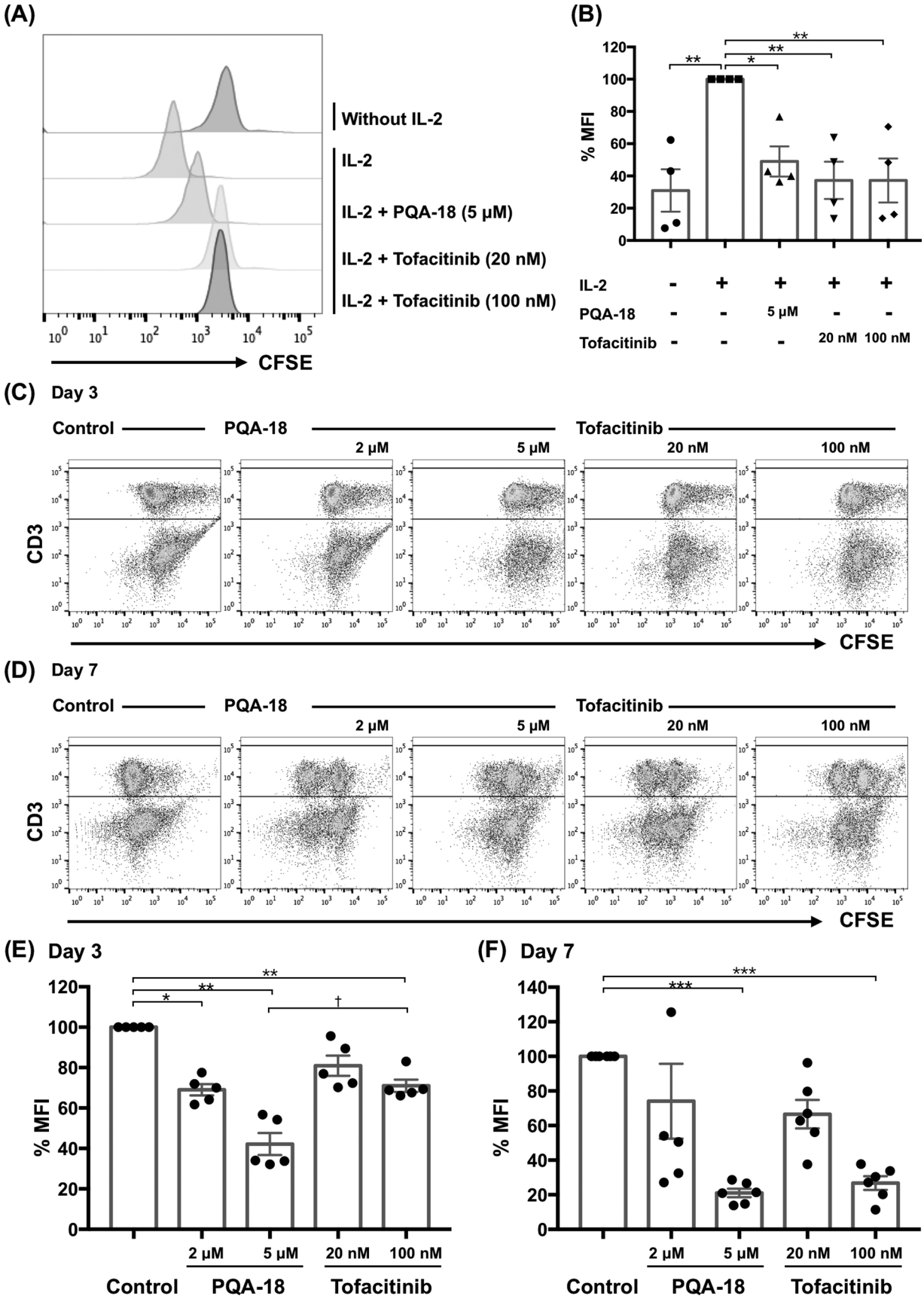

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Fig. 6. Effect of PQA-18 and Tofacitinib on IL-2 stimulated and xenogeneic-induced CD3$^+$T cell proliferation.
(A) CFSE-labeled human PBMC were stimulated by rhIL-2 in the absence (control) and the presence of PQA-18 or Tofacitinib at the indicated doses, and proliferation was evaluated at day 2 by flow cytometry. **(B)** T cell proliferation rates were calculated by setting MFI (rhIL-2 group)/MFI (drug-treated group) × 100% as a control. CSFE-labeled human PBMCs were co-cultured with MMC-treated SECs in the absence (control) and the presence of PQA-18 and Tofacitinib at the indicated doses, and proliferation was evaluated after culturing for 3 **(C)** or 7 **(D)** days. Cells were harvested and stained with anti-CD3-PE. CD3$^+$ T cells were gated and analyzed for proliferation by flow cytometry. **(E, F)** T cell proliferation were calculated by setting positive control as 100% proliferation. The percentage of MFI for each group is indicated. Data represent the Mean ± SEM, N = 4-6. ** $p < 0.01$, *** $p < 0.001$ vs. control. †$p < 0.05$ vs. each indicated group.

to Tofacitinib. In conclusion, the novel immunosuppressant, PQA-18 has the potential to suppress macrophage differentiation/polarization, macrophage-mediated cytotoxicity, macrophage-dependent xenogeneic T cell activation, and macrophage-independent T cell activation. It should be noted here that the mechanism responsible for the suppression of macrophage-mediated cytotoxicity by PQA-18 is still unclear. Further investigations will be needed for developing a better understanding of PQA-18 mediated suppression.

Disclosure

The authors declare no conflicts of interest.

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Authors' contributions

PCL and AM conceived and designed the study, performed the research, analyzed data, and wrote the manuscript. TK, CT and TY performed the correlative research and analyzed data. RS, YN, RM, and HE contributed to statistical analysis. AM, HO, and SM were involved in the critical editing of content and approval of the final version.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.04.003.

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