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The PI3Kδ selective inhibitor AS2541019 suppresses donor-specific antibody production in rat cardiac and non-human primate renal allotransplant models

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ABSTRACT

Long-term graft survival after organ transplantation is difficult to achieve because of the development of chronic rejection. One cause of chronic rejection arises from antibody-mediated rejection (AMR), which is dependent on the production of donor-specific antibodies (DSA). Current immunosuppression in organ transplantation is effective in preventing acute T cell-mediated rejection, but the risk of DSA production and graft loss due to AMR remains unchanged. Phosphatidylinositol-3-kinase p110δ (PI3Kδ), a member of the family of PI3K lipid kinases, is a key mediator of B cell activation, proliferation and antibody production. AS2541019 is a novel PI3Kδ selective inhibitor that prevents antibody production by inhibiting B cell immunity. The purpose of this study was to evaluate the inhibitory effect of AS2541019 on DSA production in preclinical rodent and non-human primate allotransplant models. Concomitant administration of AS2541019 with tacrolimus and mycophenolate mofetil (MMF) inhibited de novo DSA production in an ACI-to-Lewis rat cardiac allotransplant model. To predict the efficacy of AS2541019 in clinical practice, we evaluated its effects in cynomolgus monkeys. AS2541019 inhibited B cell proliferation and major histocompatibility complex (MHC) class II expression on B cells in cynomolgus monkeys. Oral administration of AS2541019 inhibited MHC class II expression on peripheral B cells and anti-tetanus toxoid antibody production. In cynomolgus monkey renal allotransplant model, concomitant administration of AS2541019 with tacrolimus and MMF significantly inhibited de novo DSA production. Together, our findings indicate that the PI3Kδ selective inhibitor AS2541019 is a potential candidate for preventing AMR development by inhibiting DSA production.

1. Introduction

Organ transplantation is the only curative therapy for overcoming end stage organ dysfunction. Acute T cell-mediated rejection is successfully controlled using current immunosuppressants, which generally comprise calcineurin inhibitors (tacrolimus or cyclosporine) and an inosine monophosphate dehydrogenase inhibitor (mycophenolate mofetil; MMF). However, long-term graft survival is affected by chronic rejection and remains unchanged with calcineurin inhibitors and MMF [1,2]. One of the important causes of chronic rejection is antibody-mediated rejection (AMR), which is due to the production of donor-specific antibodies (DSA) [3,4]. Clinical reports indicate that detection of de novo DSA in a patient's peripheral blood is correlated with a decrease in the graft survival rate [5–7]. Several modalities of treatment are in use such as plasmapheresis (PP) and intravenous immunoglobulin (IVIG) to decrease serum DSA levels and counter the risks of AMR and graft loss [8]. PP and IVIG administration remove the existing DSA in serum but have no direct effect on the production of DSA from immune cells [9]. Currently, there are no effective drugs or treatment available to reduce DSA production and this remains an unmet medical need. Current immunosuppressants including tacrolimus and MMF successfully inhibit T cell activation, but ineffective to inhibit DSA production which is mediated by B cell-dependent processes, including B cell proliferation and differentiation into plasma cells [10,11]. Although MMF has also inhibitory effect on B cell expansion [12], AMR development in clinical cases shows that the inhibitory effect on B cells is inadequate. Therefore, a drug that inhibits B cell activation is much needed to improve long term survival in organ transplant patients.

Phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases that

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regulate various biological functions. PI3Kδ is a class IA PI3K composed of p110δ, a 110 kDa catalytic subunit, and regulatory subunits, such as p85α [13]. Class I PI3K enzymes phosphorylate phosphatidylinositol 4,5-bisphosphate to generate phosphatidylinositol 3,4,5-trisphosphate in the cell membrane, where it acts as a docking domain for various signaling proteins [14]. PI3Ks mediate important functions through this pathway, including cell proliferation, growth, survival and metabolism [15]. Given that PI3Kδ is mainly expressed in leukocytes, including B cells [16], inhibition of PI3Kδ may be an effective approach for inhibiting excess antibody production via B cell inactivation. In addition, we previously reported that AS2541019 was a novel PI3Kδ selective inhibitor that prevents antibody production by inhibiting B cell immunity [17].

To evaluate the inhibitory effect of a clinical candidate agent on DSA production, it is important to prove its effectiveness in allotransplant models and asses the predictability of its efficacy in clinical practice. AMR occurs more frequently in patients with de novo DSA production in clinical settings [5], suggesting that inhibiting de novo DSA production may prevent AMR development. Currently no reliable animal model for the evaluation of AMR development has been reported. Here, we evaluated the inhibitory effect of AS2541019 on de novo DSA production using rodent and non-human primate allotransplant models.

2. Materials and methods

2.1. Animals

Lewis and ACI rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and Japan SLC, Inc. (Shizuoka, Japan), respectively. Cynomolgus monkeys (*Macaca fascicularis*) free of specific pathogens (salmonella bacteria, dysentery bacteria, simian immunodeficiency virus, simian retrovirus and herpes B virus) were purchased from Hamri Co., Ltd. (Ibaraki, Japan). All animals were treated in accordance with the guidelines of the Institutional Animal Care and Use Committee of Astellas Pharma Inc. (Tokyo, Japan). Tsukuba Research Center of Astellas Pharma Inc. is accredited by AAALAC International.

2.2. Test compounds

AS2541019, tacrolimus, and MMF were synthesized at Astellas Pharma, Inc. AS2541019 was dissolved in dimethyl sulfoxide for in vitro experiments or in 0.5% methylcellulose solution for in vivo experiments. Tacrolimus was diluted in saline for intramuscular administration. For oral administration, tacrolimus (20% solid dispersion formulation) was suspended in water. MMF was suspended in 0.5% methylcellulose solution. For the in vivo study, 0.5% methylcellulose solution was used as the vehicle.

2.3. Rat cardiac allotransplant model

Male 7-week-old Lewis and ACI rats were used as recipients and donors, respectively. Abdominal vascularized heterotopic cardiac transplantation was performed as previously described [18]. Recipients received tacrolimus at 0.02 mg/kg by intramuscular injection and MMF at 15 mg/kg by oral administration. In addition, recipients concomitantly received AS2541019 at 0.5, 1, or 2 mg/kg by oral administration. All test compounds were administered once daily for 21 consecutive days after transplantation regardless of graft survival. MMF was administered at least 30 min before AS2541019. Graft survival was monitored by daily palpation until 21 days post-transplantation, and graft rejection was defined as cessation of palpable graft beats. Blood samples were taken from recipients, and DSA levels were evaluated using flow cytometric analysis. Each plasma sample was incubated with ACI rat splenocytes (1×10^5 cells/well) for 30 min at 4 °C, and subsequently stained with fluorescein isothiocyanate (FITC)-conjugated anti rat IgG1/2a (BD Biosciences, CA, USA). The mean fluorescence intensity (MFI) was evaluated by flow cytometry.

2.4. Memory-recall antibody production

Male 6-week-old Lewis rats were intraperitoneally administered 50 μg dinitrophenylated keyhole limpet hemocyanin (DNP-KLH; Cosmo Bio Co., Ltd., Tokyo, Japan) with alum adjuvant (Cosmo Bio Co., Ltd) on day 1. On day 15 after this first immunization, anti-DNP IgG levels in plasma were measured using an enzyme-linked immunosorbent assay (ELISA), and rats were grouped (8 animals per group) based on their IgG level. On day 21, DNP-KLH dissolved in saline was intraperitoneally administered without adjuvant for the second immunization (50 μg/rat). From the day of the second immunization, AS2541019 (5 mg/kg) or MMF (40 mg/kg) was orally administered once daily. On day 28, blood samples were taken from rats and plasma anti-DNP IgG levels were determined by ELISA. On day 36, splenocytes were isolated from each rat and stained with R-Phycoerythrin-conjugated anti rat CD45RA (BD Biosciences) and FITC-conjugated anti rat IgG1/2a (BD Biosciences). Both of the rate of B cells in total splenocytes and the class-switched B cells in splenic B cells were evaluated by flow cytometry.

2.5. B cell proliferation assay in non-human primates

Peripheral blood mononuclear cells (PBMCs) were isolated from cynomolgus monkey blood by centrifuging followed by removal of the layer of cells above the Ficoll-Paque plus (GE Healthcare, IL, USA) layer. Isolated PBMCs (1.0×10^5 cells/well) were stimulated with 10 μg/ml goat F(ab')2 anti-human IgM (Jackson ImmunoResearch Laboratories, Inc., PA, USA) in RPMI1640 medium containing 10% fetal bovine serum, penicillin, streptomycin and 50 μM 2-mercaptoethanol, and incubated with or without AS2541019 for 3 days at 37 °C in a humidified atmosphere containing 5% CO_2. During the final 4 h of incubation, 5 μCi of ^{3}H-thymidine/well was added. The cells were then harvested using a Unifilter GF/C plate (PerkinElmer, Inc., MA, USA) and radioactivity was measured using a liquid scintillation counter. For the control, PBMCs were incubated with anti-μ stimulation in the absence of the compound. For the no stimulation control, PBMCs were incubated without either anti-μ stimulation or the compound. The inhibitory activity of AS2541019 on B cell proliferation was calculated using the following formula: Inhibition (%) = 100 − (sample − no stimulation) / (control − no stimulation) × 100.

2.6. Major histocompatibility complex (MHC) class II expression

For in vitro experiments, 50 μl of peripheral blood from cynomolgus monkeys was stimulated with 30 μg/ml goat F(ab')2 anti-human IgM (Jackson ImmunoResearch Laboratories) in RPMI1640 medium containing 10 mM L-glutamine, penicillin, streptomycin and 55 μM 2-mercaptoethanol, and incubated with or without AS2541019 for 21 h at 37 °C in a humidified atmosphere containing 5% CO_2. MHC class II expression on B cells was evaluated by flow cytometry. Stimulated peripheral blood was stained with FITC-labeled mouse anti-human leukocyte antigen-DP, DQ and DR antibody (BD biosciences) and allophycocyanin (APC)-labeled mouse anti-human CD20 antibody (BD biosciences). MHC class II expression was defined as the MFI in CD20-moderate positive B cells by flow cytometry. The inhibition rate was calculated using the following formula: Inhibition (%) = 100 − (sample − no stimulation)/(control − no stimulation) × 100.

For ex vivo experiments, two monkeys were orally administered 10 mg/kg of AS2541019, and the same monkeys were later administered 3 mg/kg of AS2541019. The interval between the 10 mg/kg and 3 mg/kg doses was approximately 1 month to avoid the effects of first administration. Peripheral blood was taken before and at 16 h after

Table 1
Effects of concomitant administration of AS2541019 with tacrolimus and MMF in a rat cardiac allotransplant model.

Treatment (mg/kg)			n	Graft survival time (days)	Median survival time (days)	Body weight (g) ± S.E.M. (21 days post-transplantation)
Tacrolimus	MMF	AS2541019				
Control (no treatment)			12	5 × 10, 6 × 2	5	291.6 ± 4.2
0.02	15	0	11	7 × 2, 13 × 2, 19, ≥21 × 6**	≥21	270.5 ± 3.2##
0.02	15	0.5	13	13, 14 × 2, 15, 18, ≥21 × 8 NS	≥21	269.2 ± 3.0 NS
0.02	15	1	13	10 × 2, 12, 13, 14, ≥21 × 8 NS	≥21	271.1 ± 3.3 NS
0.02	15	2	13	10 × 2, 13, 14 × 2, 16, ≥21 × 7 NS	≥21	275.9 ± 2.8 NS

NS: no significant difference between tacrolimus + MMF combination therapy and concomitant AS2541019 administration groups by the Log-rank test (graft survival time) or Dunnett's multiple comparisons test (body weight).
** Significant difference between control and tacrolimus + MMF combination therapy group by the Log-rank test ($P < 0.01$).
Significant difference between control and tacrolimus + MMF combination therapy group by Student's t-test ($P < 0.01$).

administration, and stimulated with 30 μg/ml goat F(ab')2 anti-human IgM (Jackson ImmunoResearch Laboratories) and incubated for 21 h. Stimulated peripheral blood was stained with FITC-labeled mouse anti-human leukocyte antigen-DP, DQ and DR (BD biosciences) and APC-labeled mouse anti-human CD20 (BD biosciences). MHC class II expression was defined as the MFI in CD20-moderate positive B cells by flow cytometry.

2.7. Tetanus toxoid immunization model

To generate the non-human primate antibody production model, male cynomolgus monkeys were administered tetanus toxoid (Denka Seiken Co. Ltd., Tokyo, Japan) without dilution (< 10 Lf/ml). On day 0, monkeys were intradermally administered total 0.6 ml of tetanus toxoid solution at 12 spots on their back separately. At the same time, cynomolgus monkeys were intramuscularly administered 0.6 ml of tetanus toxoid solution. From day 0, AS2541019 (1 or 3 mg/kg) was concomitantly administered with tacrolimus 1 mg/kg and MMF 20 mg/kg. All compounds were orally administered once a day. Blood samples were taken from monkeys on day 0, 7, 10, 14, 21 after tetanus toxoid administration and plasma anti-tetanus toxoid IgG levels were determined by ELISA.

2.8. Non-human primate renal transplantation

Cynomolgus monkeys underwent renal transplantation according to previous studies [19]. Each animal in this study acted as both a donor and recipient. The donor and recipient combinations were determined by ABO blood type compatibility and the 1-way mixed lymphocyte reaction assay (a stimulation index > 2.5) before transplantation. Monkeys were anesthetized with isoflurane inhalation, and a midline incision was made to open the abdominal cavity. The left kidney and ureter were removed and exchanged between paired monkeys. The renal allograft was implanted by end-to-side anastomoses of the renal artery to the aorta and renal vein to the vena cava, and end-to-end anastomosis of donor and recipient ureters. The transplanted kidney was immediately reperfused after implantation. The right native kidney was not removed to avoid recipient death due to dysfunction of the grafted kidney, allowing adequate detection of DSA production after graft rejection. From day 0, AS2541019 (2 or 3 mg/kg) was concomitantly administered with tacrolimus 1 mg/kg and MMF 20 mg/kg. All compounds were orally administered once daily. Blood samples were taken from recipient monkeys, and donor-specific IgG levels were evaluated using flow cytometric analysis. PBMCs were isolated from donor cynomolgus monkey blood and stored at −80 °C before transplantation. Each plasma sample was incubated with donor PBMCs (2.5×10^5 cells/well) for 30 min at 4 °C. DSA bound to PBMCs was stained with FITC-conjugated anti-human IgG (Dako, CA, USA) and the MFI was evaluated by flow cytometry.

2.9. Statistical analysis

All statistical analyses were performed using Graph Pad Prism 7 software (Graph Pad Software, CA, USA). IC_{50} values were determined using sigmoid Emax non-linear regression analysis. Statistically significant differences were determined using Dunnett's multiple comparison test or Student's t-test. Graft survival time in the rat cardiac transplantation model was compared using the Log-rank test.

3. Results

3.1. Effect on DSA production in the rat cardiac allotransplant model

Our previous study demonstrated that AS2541019 is a potent PI3Kδ selective inhibitor that prevents antibody production through inhibiting B cell immunity in rats [17]. In this study, to evaluate the effect of AS2541019 on de novo DSA production, we examined its inhibitory effect in an ACI-to-Lewis rat cardiac allotransplant model. Given that combination therapy comprising tacrolimus and MMF is a standard treatment regimen for organ transplantation, we concomitantly administered each oral dose (0.5, 1, 2 mg/kg) of AS2541019 with a 0.02 mg/kg intramuscular dose of tacrolimus and 15 mg/kg oral dose of MMF. Tacrolimus and MMF treatment markedly prolonged the graft survival time (median survival time was over 21 days), and none of the concomitant doses of AS2541019 affected the graft survival time during the experimental period (Table 1). Regarding DSA production, tacrolimus and MMF treatment partially inhibited donor-specific IgG production. Donor-specific IgG levels were considerably lower following concomitant treatment with AS2541019 compared to tacrolimus and MMF combination therapy (Fig. 1A). Further, concomitant AS2541019 administration reduced the area under the curve (AUC) for donor-specific IgG, and the effects were statistically significant compared to the tacrolimus and MMF combination therapy group (Fig. 1B). Administration of AS2541019 had no influence on the recipients' body weights by the end of the study (at 21 days post-transplantation) (Table 1). Taken together, these findings suggest that AS2541019 had potent inhibitory effects against DSA production when concomitantly administered with tacrolimus and MMF without severe adverse effects.

3.2. Inhibitory effect on rat memory-recall antibody production

Recall DSA production is one of the factors that induces graft rejection [10]. Because the involvement of PI3Kδ in memory-recall antibody production has not been clarified, we evaluated the effect of AS2541019 on DNP-KLH-induced memory-recall antibody production. Lewis rats were immunized with DNP-KLH and grouped based on their anti-DNP IgG level. DNP-KLH was administered again, and AS2541019 (5 mg/kg) or MMF (40 mg/kg) were orally administered after the second immunization. AS2541019 administration inhibited anti-DNP IgG production (Fig. 2A) induced by twice immunizations of DNP-KLH,

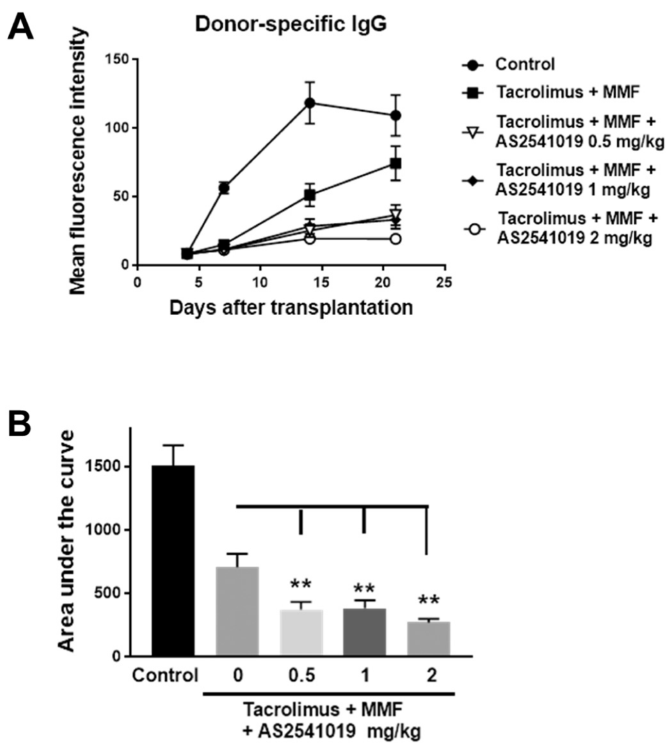

Fig. 1. Effect of AS2541019 on DSA production in a rat cardiac transplantation model. Recipient Lewis rats were transplanted with cardiac grafts from ACI rats. Each oral dose of AS2541019 was administered concomitantly with a 0.02 mg/kg intramuscular dose of tacrolimus and a 15 mg/kg oral dose of MMF once daily from 0 to 20 days after transplantation (n = 11–13 for each group). Peripheral blood was collected from recipients, and plasma samples were used for DSA detection. (A) Changes in donor-specific IgG levels were evaluated by detecting peripheral antibodies bound to donor splenocytes using flow cytometry. Values are expressed as the mean ± S.E.M. (B) The AUC values for donor-specific IgG were calculated and the effect AS2541019 administration was compared with the tacrolimus and MMF combination therapy group. Values are expressed as the mean ± S.E.M. Differences between the AS2541019 administration groups and the tacrolimus and MMF combination therapy group were analyzed by Dunnett's multiple comparisons test. $^{**}P < 0.01$. MMF, mycophenolate mofetil.

suggesting that PI3Kδ is involved in recall DSA production. To elucidate the mechanism of antibody production, splenocytes on day 36 were collected and the IgG expression on the B cells was detected. Flow cytometric analysis showed that MMF decreased the number of B cells (Fig. 2B) without affecting the class switching to IgG (Fig. 2C). On the other hand, AS2541019 administration notably inhibited the class switching to IgG in B cells (Fig. 2C) without decreasing the number of B cells (Fig. 2B), indicating AS2541019 inactivated B cells through different mechanisms from a current immunosuppressant, MMF.

3.3. Inhibitory effects on non-human primate B cells

To predict the efficacy of AS2541019 in clinical settings, we performed further studies using non-human primates, which have relatively similar immune systems to humans. One of the purposes of the current study was to verify the effect of AS2541019 on non-human primates. To accomplish this, we first examined the inhibitory effect of AS2541019 on non-human primate B cell proliferation. PBMCs were isolated from cynomolgus monkeys and stimulated with anti-human IgM in the presence of AS2541019. AS2541019 inhibited monkey B cell proliferation with an IC_{50} value of 16.1 nM (Fig. 3A). In addition, B cells play an important role in antigen presentation to $CD4^+$ helper T cells via MHC class II on their surface [20]. We therefore verified the effect of AS2541019 on antigen presentation by examining its inhibitory effect on B cell MHC class II expression. In the in vitro experiment, we stimulated peripheral blood from cynomolgus monkeys with anti-human IgM. Flow cytometric analysis of MHC class II upregulation on B cells showed that AS2541019 inhibited B cell receptor signal-induced MHC class II expression with an IC_{50} value of 60.6 nM (Fig. 3B). These results indicate that AS2541019 inhibits monkey B cell function by inhibiting proliferation and MHC class II expression on B cells.

3.4. Ex vivo effects on peripheral B cell MHC class II expression

The activity of oral AS2541019 administration was evaluated by examining MHC class II expression on circulating B cells from cynomolgus monkeys. Cynomolgus monkeys were orally administered AS2541019 (3 or 10 mg/kg), and their peripheral blood was collected 16 h after administration and incubated for one day with or without anti-human IgM stimulation. Flow cytometric analysis of MHC class II expression showed that in the absence of stimulation with anti-IgM, AS2541019 administration reduced spontaneous MHC class II expression (MFI) by 25.9 or 32.3% at 3 mg/kg, and 44.2 or 52.9% at 10 mg/kg in each monkey (Fig. 4). We also stimulated peripheral blood with an anti-IgM antibody and evaluated the resulting upregulation in MHC class II expression. Under stimulation condition, AS2541019 inhibited anti-IgM-induced MHC class II expression on B cells 16 h after administration by 14.1 or 38.6% at 3 mg/kg, and 29.9 or 58.5% at 10 mg/kg in each monkey (Fig. 4). These results demonstrate that AS2541019 administration effectively inhibited both of spontaneous MHC class II expression and B cell receptor signal-induced MHC class II upregulation on peripheral B cells. Further, the inhibitory effect was maintained for up to 16 h after oral administration, indicating the ex vivo profile of AS2541019 is probably enough to exert a beneficial effect on in vivo studies using cynomolgus monkey.

3.5. Antibody production in a tetanus toxoid immunization model

The effects of AS2541019 administration on cynomolgus monkey antibody production was examined. Tetanus toxoid is an antigen produced by *Clostridium tetani* and induces antibody production in cynomolgus monkeys [21]. Tacrolimus and MMF are established immunosuppressants, therefore we first evaluated this combination therapy on antibody production. Oral administration of tacrolimus 1 mg/kg and MMF 20 mg/kg showed no effect on anti-tetanus antibody production (Fig. 5A, B). AS2541019 was concomitantly administered with tacrolimus 1 mg/kg and MMF 20 mg/kg once daily after tetanus toxoid immunization. Peripheral blood was taken at 0, 7, 10, 14 and 21 days after immunization and anti-tetanus antibody levels were investigated. Anti-tetanus antibody levels were considerably lower following concomitant treatment with AS2541019 compared to tacrolimus and MMF combination therapy (Fig. 5A). Further, concomitant AS2541019 administration reduced the area under the curve (AUC) for anti-tetanus antibody, and the effects were statistically significant compared to the tacrolimus and MMF combination therapy group (Fig. 5B). These results show that concomitant administration of AS2541019 with tacrolimus and MMF markedly inhibited remaining de novo anti-tetanus toxoid antibody production following administration with current immunosuppressants in non-human primates.

3.6. Effects on a non-human primate renal transplant model

To investigate the inhibitory effect of AS2541019 on DSA production in non-human primates, we examined its effects in a cynomolgus monkey renal transplant model. One kidney was removed from the donor and transplanted into the recipient. The other kidney was left in the recipient to avoid recipient death and to allow adequate detection of DSA production in the event of graft dysfunction. A previous study demonstrated that the combination of tacrolimus 1 mg/kg and MMF 20 mg/kg prolonged graft survival in a cynomolgus monkey renal

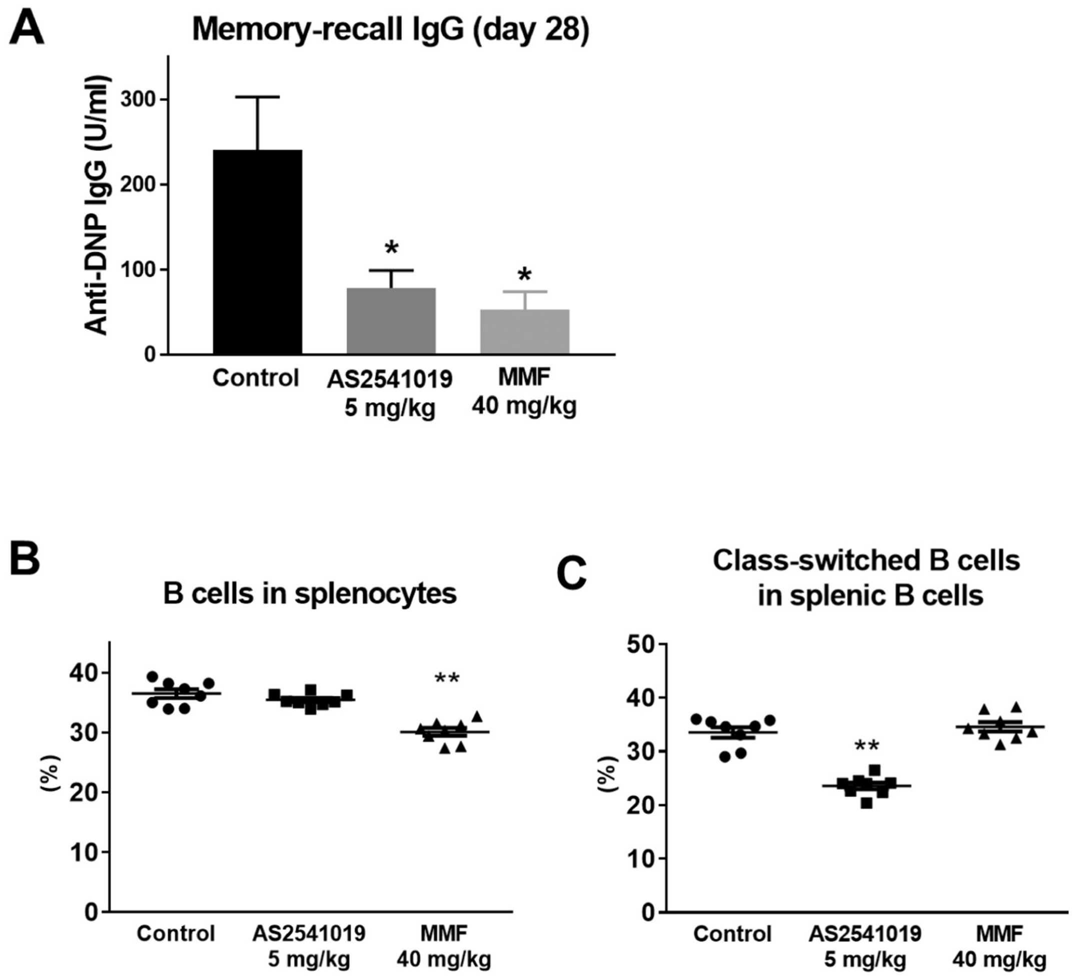

Fig. 2. Effect of AS2541019 on memory-recall antibody production in a rat DNP-KLH immunization model. Lewis rats were intraperitoneally administered 50 μg DNP-KLH with alum adjuvant and grouped ($n = 8$ for each group) based on their IgG level on day 15. On day 21, DNP-KLH was intraperitoneally administered for a second immunization (50 μg/rat) and AS2541019 (5 mg/kg) or MMF (40 mg/kg) was orally administered once a day. (A) Anti-DNP IgG levels on day 28 were evaluated and the effect of AS2541019 administration was compared with the control group. Splenocytes were isolated from each rat on day 36 and evaluated the rate of (B) total B cells in splenocytes and (C) class-switched B cells in splenic B cells. Values are expressed as the mean ± S.E.M. Differences between the treatment groups and control were analyzed by Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

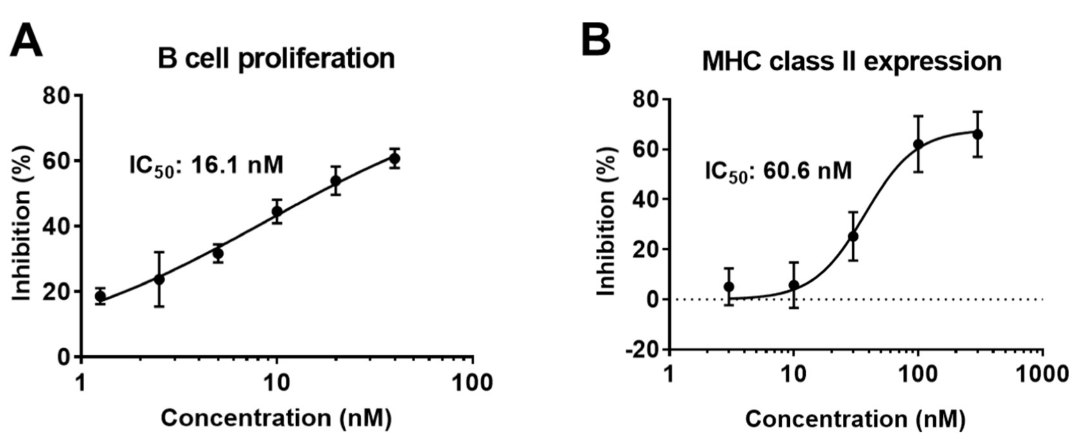

Fig. 3. In vitro inhibitory effects of AS2541019 on cynomolgus monkey B cells. (A) Inhibition of the proliferation of cynomolgus monkey PBMCs by AS2541019. Cynomolgus monkey PBMCs were stimulated with anti-human IgM for 3 days. Proliferation was evaluated by thymidine uptake. Values are expressed as mean ± S.E.M of three experiments. (B) Effects of AS2541019 on the in vitro expression of MHC class II on circulating B cells from cynomolgus monkeys. Cynomolgus monkey peripheral blood was stimulated with anti-IgM for 21 h in the presence of AS2541019. MHC class II on B cells was detected by flow cytometry. Values are expressed as the mean ± S.E.M of six experiments. IC_{50} values were calculated by non-linear regression analysis in both studies.
MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell.

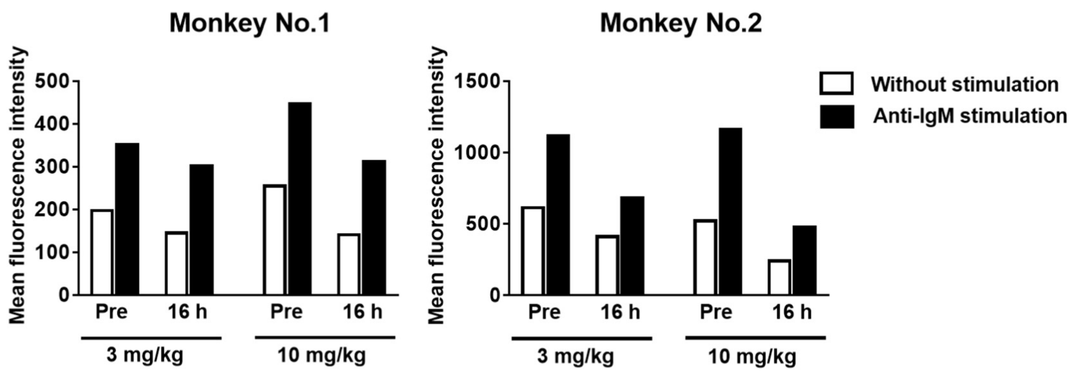

Fig. 4. Effects of AS2541019 on the ex vivo expression of MHC class II on cynomolgus monkey B cells. MHC class II expression on B cells was evaluated before and at 16 h after oral administration of AS2541019 ($n = 2$). Peripheral blood was collected and MHC class II expression on B cells with or without anti-IgM stimulation was detected by flow cytometry. Values are expressed as the mean fluorescence intensity with duplicate measurement.

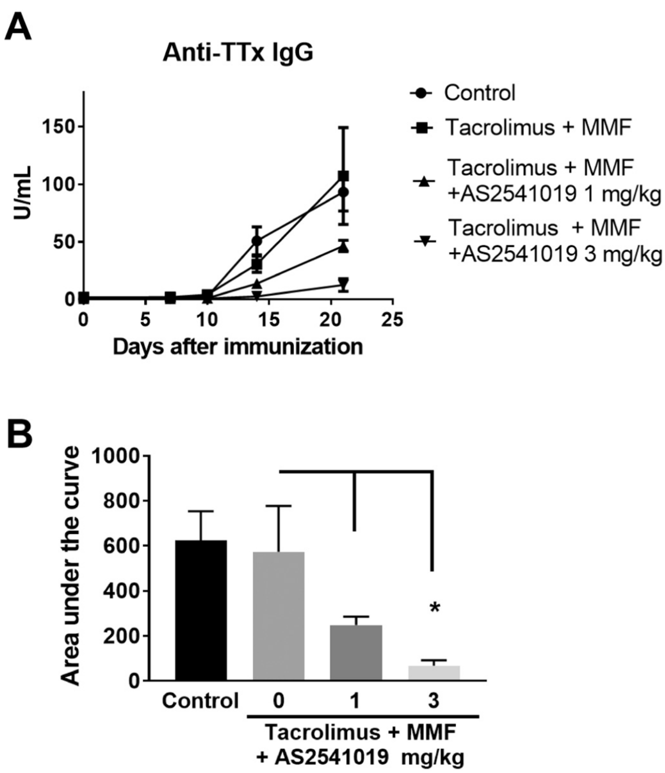

Fig. 5. Effect of AS2541019 in an in vivo cynomolgus monkey antibody production model. Tetanus toxoid was used as the antigen and was administered on day 0 of the experiment. Each dose of AS2541019 was concomitantly administered with tacrolimus 1 mg/kg and MMF 20 mg/kg. All compounds were orally administered once daily from day 0 to day 21 ($n = 3$ for each group). (A) Changes in anti-tetanus toxoid IgG levels were measured by ELISA. (B) The AUC values for anti-tetanus toxoid IgG were calculated and the effect of AS2541019 administration was compared with tacrolimus and MMF combination therapy group. Values are expressed as the mean ± S.E.M. Differences between AS2541019 treatment groups and the tacrolimus and MMF combination therapy group were analyzed by Dunnett's multiple comparisons test. $^*P < 0.05$.
MMF, mycophenolate mofetil; TTx, tetanus toxoid.

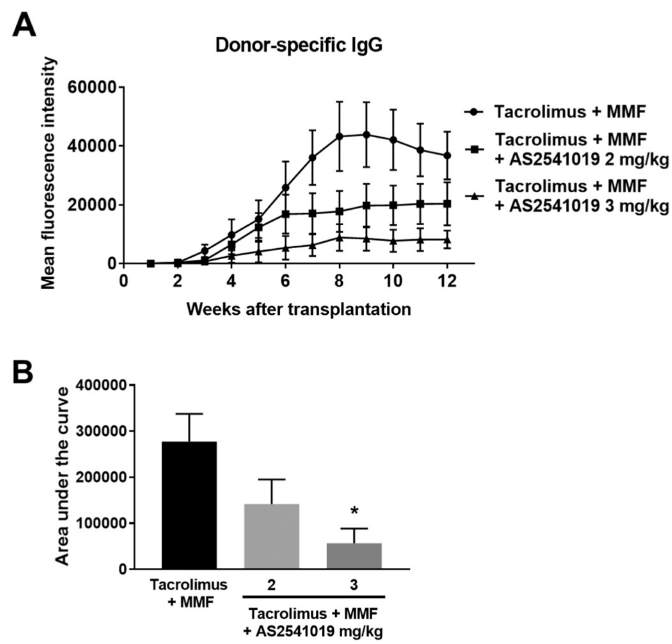

Fig. 6. Effect of AS2541019 on DSA production in a cynomolgus monkey renal transplantation model. Renal transplantation was performed on day 0 of the experiment, and each concomitant dose of AS2541019 was administered with tacrolimus 1 mg/kg and MMF 20 mg/kg. All compounds were orally administered once daily. Peripheral blood was collected from recipients and plasma samples were used for DSA detection ($n = 4$ for each group). (A) Changes in donor-specific IgG levels were evaluated by detecting peripheral antibodies bound to donor PBMCs using flow cytometry. (B) Area under the curve for donor-specific IgG production was evaluated and the effect of AS2541019 administration was compared with tacrolimus and MMF combination therapy group. MFI means the difference from the value of control sample from pre-transplantation. In case that MFI was lower than that of control, the value was defined as 0. Values are expressed as the mean ± S.E.M. Differences between AS2541019 treatment groups and the tacrolimus and MMF combination therapy group were analyzed by Dunnett's multiple comparisons test. $^*P < 0.05$. MMF, mycophenolate mofetil.

transplant model [22]. However, administration of tacrolimus 1 mg/kg and MMF 20 mg/kg did not eliminate DSA production, indicating that this combination therapy is reflective of current immunosuppressive treatments. To assess the inhibitory effect of AS2541019 on residual DSA production following combination immunosuppressive treatment, each dose of AS2541019 was concomitantly administered with tacrolimus and MMF, and the treatment was continued from the day of transplantation to the end of the study. Doses of AS2541019 were determined from our results in the anti-tetanus toxoid antibody production model. Concomitant administration of AS2541019 at doses of 2 and 3 mg/kg with tacrolimus and MMF inhibited donor-specific IgG production (Fig. 6A). Further, concomitant AS2541019 administration reduced the AUC for donor-specific IgG in a dose-dependent manner, and the effect was statistically significant at 3 mg/kg of AS2541019 compared to the tacrolimus and MMF combination therapy group (Fig. 6B). These results indicate that concomitant administration of

AS2541019 inhibits the DSA production that remains following treatment with tacrolimus and MMF combination therapy in a cynomolgus monkey renal transplant model. We expected that transplanted kidneys would be strongly rejected after 91 days of transplantation because the median survival time following treatment with tacrolimus 1 mg/kg and MMF 20 mg/kg was 21 days in a monkey renal transplant model [22]. As expected, pathological examination demonstrated the presence of coagulative necrosis on rejected kidneys, which hindered the ability to conduct immunological assessment to show potential AMR development.

4. Discussion

DSA-induced AMR is a major unsolved problem that induces chronic allograft rejection [23,24]. Therefore, we focused on inhibitory effects of AS2541019 on DSA production in this study. We previously reported that AS2541019 is a novel PI3Kδ selective inhibitor that prevents antibody production by inhibiting B cell immunity [17]. In the present study, concomitant administration of AS2541019 with tacrolimus and MMF exerted inhibitory effects on DSA production in an ACI-to-Lewis rat cardiac allotransplant model. In cynomolgus monkeys, AS2541019 inhibited the proliferation and MHC class II expression of B cells. Oral administration of AS2541019 downregulated MHC class II expression on peripheral B cells and inhibited anti-tetanus toxoid antibody production. In cynomolgus monkey renal transplant model, concomitant administration of AS2541019 with tacrolimus and MMF inhibited DSA production. Reports from clinical studies indicate that induction of AMR by DSA is a key mechanism for shortening long-term graft survival [5]. Our findings therefore suggest that inhibiting DSA production using AS2541019 may prolong graft survival by preventing AMR development.

While T cell-mediated acute rejection is well controlled by current immunosuppressants in clinical settings, DSA production derived from insufficient B cell suppression can lead to AMR development and result in a shortened graft survival time [25]. In our rat cardiac allotransplant model, combination therapy comprising tacrolimus 0.02 mg/kg and MMF 15 mg/kg markedly prolonged graft survival, with the median survival time extending beyond the experimental period (Table 1). However, treatment with tacrolimus and MMF did not eliminate DSA production (Fig. 1). Given that acute graft rejection is generally induced by T cell-dependent mechanisms in rat cardiac allotransplant models [26], these doses of tacrolimus and MMF were expected to sufficiently inhibit T cell activation in preventing acute rejection; however, their inhibitory effect on B cells may have been insufficient to suppress DSA production. These findings indicate that combination therapy comprising tacrolimus and MMF reflects the situation with current clinical therapies, which sufficiently inhibit T cells to prevent acute rejection but do not adequately suppress DSA. Therefore, the result that concomitant administration of AS2541019 with tacrolimus and MMF efficiently inhibited DSA production (Fig. 1) indicates that AS2541019 can effectively eliminate the DSA production remaining following clinical immunosuppressive treatment. Further, in the memory-recall antibody production study, while MMF decreased the number of B cells (Fig. 2C), AS2541019 inhibited the class switching to IgG in B cells (Fig. 2B) without changing B cell number. The results indicate that AS2541019 inhibits B cell activation in different manner from MMF. Both AS2541019 and MMF comparably inhibited memory-recall antibody production, however using drugs with different mechanisms may have advantages in terms of inhibiting B cells more effectively.

In addition to the rat transplantation experiments, we examined the clinical usefulness of the inhibitory effect of AS2541019 on DSA production in a non-human primate allotransplant model, given that the immune system of primates markedly differs from that of rodents. A previous study in a cynomolgus monkey renal transplant model showed that combination therapy with tacrolimus 1 mg/kg and MMF 20 mg/kg prolonged graft survival [22]. However, the present study showed that these doses of tacrolimus and MMF likewise did not eliminate DSA production (Fig. 6). Our results showed that concomitant administration of AS2541019 with tacrolimus and MMF did inhibit DSA production (Fig. 6). We therefore demonstrated that AS2541019 inhibited the DSA production remaining following administration of standard immunosuppressive therapy in rat and cynomolgus monkey allotransplant models. Considering that AS2541019 also inhibits human B cell activation [17], these results in allotransplant models suggest that AS2541019 may inhibit DSA production in clinical settings. Further, our finding that 3 mg/kg AS2541019 almost completely inhibited anti-tetanus toxoid antibody production validates its inhibitory effects on DSA production in cynomolgus monkey renal transplantation. We therefore propose that the inhibitory effects of AS2541019 on DSA production can be predicted using an anti-tetanus antibody production model, which can be conducted using a relatively simple and short experiment.

The graft survival rate in recipients with high preexisting DSA is significantly lower than that in recipients with low preexisting DSA [27], indicating that preexisting DSA is a risk factor for reducing graft survival time. To prevent preexisting DSA-derived acute AMR, desensitization therapies including PP, IVIG and rituximab injections are performed before transplantation and provide a significant survival benefit [28]. However, recent analysis revealed that AMR appeared in 20% of desensitized recipients and that the graft survival time was shortened in cases with recall DSA production [29], indicating the desensitization therapies are not sufficient for complete inhibition of DSA production. Further, when memory B cells exist in recipients, recall DSA production via memory B cell activation occurs more frequently, leading to AMR development [30]. For these reasons, recall DSA production is a key risk factor for AMR, and preventing recall DSA production may be one strategy for avoiding AMR development. Our study demonstrated that AS2541019 administration inhibited memory-recall antibody production in a rat DNP-KLH immunization model (Fig. 2). This study showed that AS2541019 inhibited antibody production induced by two immunizations, indicating that AS2541019 administration has the potential to inhibit recall DSA production following transplantation. Therefore, we propose that AS2541019 treatment may improve the long-term graft survival rate by inhibiting recall DSA production.

Preventing B cell function has another advantage in organ transplantation besides inhibiting DSA production. Some reports suggest that the generation and maintenance of T cell memory is dependent on antigen presentation from B cells. One study showed that memory $CD4^+$ T cells from B cell-deficient mice produced lower levels of interleukin 2 against a T cell-dependent antigen [31], indicating that B cells are associated with memory $CD4^+$ T cell function. Another study showed that there were fewer memory $CD4^+$ T cells in mice genetically deficient MHC class II on B cells compared to wild type, suggesting that MHC class II-expressing B cells were important for memory $CD4^+$ T cell differentiation [20]. In our studies, AS2541019 inhibited MHC class II expression on B cells from non-human primates (Fig. 3), rats and humans [17], suggesting that AS2541019 may inhibit memory $CD4^+$ T cell differentiation by inhibiting MHC class II expression on B cells. Memory T cell function is important for the maintenance of donor-specific immunity because memory T cells can be rapidly recruited to initiate early responses to donor antigens [32–34]. Further, in a mouse renal transplantation study, injection of donor-specific memory $CD4^+$ T cells induced more frequent DSA production and AMR development [35]. Taken together, these findings indicate that, through inhibiting MHC class II expression on B cells, AS2541019 may inhibit memory immune responses including memory $CD4^+$ T cell differentiation and subsequent DSA production.

Given that controlling DSA production is one of the effective methods for preventing AMR development, several medical treatments are targeted at controlling DSA levels. Non-immunosuppressive treatments, including PP and IVIG are performed to reduce DSA [8,36,37].

However, these treatments are partially effective only by removing the existing DSA and have no effect on production of DSA due to activation of immune cells. One study reported that IVIG had minimal effects on de novo DSA production, and did not prevent de novo DSA-induced AMR development in kidney-transplanted recipients [38]. Therefore, non-immunosuppressive treatments cannot inhibit de novo DSA production and are not sufficient therapies for inducing long-term graft survival. Among currently available drugs, the anti-CD20 monoclonal antibody rituximab depletes CD20-expressing B cells and prevents AMR development [39,40]. While plasma cells that develop from B cells produce antibodies, rituximab does not deplete antibody-producing plasma cells because CD20 is not expressed on plasma cells [41]. Therefore, while rituximab is effective for preventing DSA production when used before transplantation, it has no significant benefit when administered after AMR development [42]. Further, retrospective analysis revealed that rituximab-treated patients have a high risk of infectious diseases [43]. Therefore, while rituximab is effective for preventing de novo DSA production, it is not an ideal treatment because of its adverse effects. Among small molecule drugs, the proteasome inhibitor bortezomib is used for off-label to prevent DSA production [44,45]. Bortezomib, which was originally used to treat multiple myeloma, a type of cancer formed by malignant plasma cells [46], depletes antibody-producing plasma cells by inhibiting proteasome activation. However, bortezomib use is associated with toxicities, including pneumonia [47]. Further, a study using mice showed that bortezomib administration significantly decreased body weight [48]. In contrast, AS2541019 administration did not induce body weight loss (Table 1) or any other severe adverse effects in a rat cardiac allotransplant model, indicating that AS2541019 is a relatively safe compound compared to bortezomib. Although the safety profile in human has not been confirmed, our studies indicate that AS2541019 is effective for inhibiting de novo DSA production. Because plasma cells do not express B cell receptors on their surface however, this PI3Kδ inhibitor may not be effective for depleting antibody-producing plasma cells. In contrast, bortezomib can reduce DSA levels via depletion of plasma cells [49], therefore differential use of AS2541019 for inhibiting de novo DSA production and bortezomib for reducing existing DSA levels after transplantation with close attention to adverse effects may be an effective approach for preventing AMR development.

In conclusion, a PI3Kδ selective inhibitor AS2541019, prevented DSA production in rat cardiac and cynomolgus monkey renal allotransplant models. Previous study using rodent xenograft transplantation model revealed that inhibiting DSA production exerted the graft survival prolongation [17], indicating inhibiting DSA production may prevent AMR development and lead to the long-term graft survival. AS2541019 also inhibits human B cell activation [17], we therefore speculate that our finding that AS2541019 inhibits DSA production in allotransplantation is translatable to humans. Given that DSA-derived AMR is a major problem leading to reduced graft survival time associated with current therapies for organ transplantation, AS2541019 has a potential to be clinically effective therapeutic agent for prolonging long-term graft survival time by preventing DSA production.

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