Research Paper

Drug repurposing: Ibrutinib exhibits immunosuppressive potential in organ transplantation

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Abstract

Long-term administration of classic immunosuppressants can induce severe adverse effects. The development of novel immunosuppressants confronts great challenges and opportunities. Ibrutinib, an approved drug for B-cell lineages and chronic graft versus host disease (cGVHD), exhibits immunosuppressive efficacy in autoimmune diseases. Ibrutinib's potential as an immunosuppressant in organ transplantation has not been investigated to date. In a xeno-artery patch model *ex vivo*, ibrutinib inhibited the proliferation of PBMCs (POD 14-42), mainly CD3+CD4+ and CD3+CD8+ T cells *ex vivo*. The secretion of cytokines (IL-6, IL-2 and IFN-γ) was suppressed in response to ibrutinib. In allo-skin transplantation models, ibrutinib delayed the rejection of grafted skins. Ibrutinib decreased the amount of T/B cells and lymphocyte infiltration. Altogether, ibrutinib exhibited immunosuppressive potential through cytokine regulation and T cell inhibition *ex vivo* and *in vitro*. Repositioning of ibrutinib as an immunosuppressant will greatly facilitate novel immunosuppressant development.

Key words: Ibrutinib, Immunosuppressant, Immune rejection, Allo-transplantation, Xeno-transplantation

Introduction

Immune rejection after organ transplantation usually results from the innate immune system[1] (the complement system[2]) and T-cell mediated immune rejection[3]. Except for surgical manipulation, long-term administration of immunosuppressants is necessary to alleviate immune rejection (especially T-cell mediated immune rejection)[4]. However, numerous adverse effects (such as nephrotoxicity, malignancies, and autoimmune imbalance) are not negligible[5-7]. It is urgent to develop novel immunosuppressive therapies with high efficacy and favorable safety profiles. Small molecule immuno-suppressants targeting key biological molecules (such as sphingosin-1-phosphate receptor (S1P)[8-10], mTORs[11, 12], kinases[13-19], and HDACs[20]) have drawn much attention in drug discovery.

Kinases are promising drug targets in the treatment of malignancies[21, 22]. However, the study of kinase inhibitors in autoimmune disease and other immune disorders remains rare. To date, several kinase inhibitors (Supplementary Table 1) have attracted attention in the field of organ transplantation[14, 16, 17]. These kinase inhibitors have exhibited potent immunosuppressive effects in organ transplantation. Tec kinases ITK and RLK are important regulators of the development of CD4+ and CD8+ T cells, which were demonstrated in *ITK*- and *RLK/ITK*-deficient mice[23].

Ibrutinib is an irreversible inhibitor of Bruton's tyrosine kinase (Btk)[24, 25] and IL-2 inducible T cell kinase (Itk)[26, 27]. The FDA has approved Ibrutinib for the treatment of several B-cell lineages and

cGVHD. The potency of ibrutinib in immune disorders, such as autoimmune diseases[24] and graft-versus-host disease[28], is now drawing much attention. However, the immune-regulating potential of ibrutinib in organ transplantation has not been reported. As an approved clinical drug, the safety profile and pharmacokinetic effects of ibrutinib have been confirmed. Moreover, in previous study[29], ibrutinib allowed for recovery of humoral immune function in patients with chronic lymphocytic leukemia (CLL). The innocuity to normal immune system gave it superiority over traditional immunosuppressants. Repositioning ibrutinib as an immunosuppressant will be of great value to drug discovery by saving costs and time[30].

In this article, we present a study of ibrutinib as a potential immunosuppressant in allo- and xeno-transplantation. A xeno-artery patch model has been employed to evaluate the anti-immune response effects of ibrutinib *ex vivo*. An allo-skin transplantation model from C57BL/6 to BALB/c mice has been established to study the potential of ibrutinib *in vivo*. In this study, we evaluated the immunosuppressive effects of ibrutinib by T/B cell count, cytokine detection, histological analysis and other tests. We found that ibrutinib exhibited potent inhibitory effects on T cell proliferation and cytokine secretion in the xeno-artery patch model *ex vivo*. In allo-skin transplantation model, ibrutinib delayed and abated the graft rejection via inhibiting T cells and B cells.

Materials and Methods

Reagents and cell culture

Ficoll-Paque PLUS (Cat. No. 17-1440-03) was purchased from GE Healthcare. Cell Counting Kit-8 (CCK8) was purchased from Dojindo Laboratories (Kumamoto, Japan). CellTrace™ CFSE Cell Proliferation Kit (Cat. No. C34554) was purchased from ThermoFischer Scientific. Phytohemagglu-tinin-M (PHA-M) (Cat. No. 11082132001) was purchased from Roche. BD™ Cytometric Bead Array NHP Th1/Th2 Cytokine Kit (Cat. No. 557800) was purchased from BD pharmingen. PE/Cy7 anti-mouse CD4 (Cat. No. 100421), PE anti-mouse CD3 (Cat. No. 100205), FITC anti-mouse CD20 (Cat. No. 150407), APC anti-mouse CD8α (Cat. No. 100711) were purchased from Biolegend. PE-Cy7 anti-human CD4 (Cat. No. 557852), PE anti-human CD8 (Cat. No. 555367), FITC anti-human CD3ε (Cat. No. 556611), APC anti-human CD20 (Cat. No. 560853) were purchased from BD pharmingen. FITC-conjugated goat-derived anti-human IgM (μ chain-specific) (Cat. No. 62-7511) and IgG (γ chain-specific) polyclonal antibody (Cat. No. 62-8411), fetal bovine serum (Cat.

No. 10099141), Penicillin-Streptomycin-Glutamine (100×) (P/S, Cat. No. 10378016), and RPMI-1640 medium (Cat. No.11875119) were purchased from ThermoFischer Scientific. Optimal Cutting Temperature (OCT) compound was purchased from Agar Scientific (Cat. No. AGR1180).

Peripheral blood mononuclear cells (PBMCs) were harvested from cynomolgus monkeys after artery patch. Isolated PBMCs were cultured with RPMI-1640 containing 10% (vol/vol) FBS, 1% (vol/vol) P/S at 37 °C with 5% CO_2. Mouse spleen cells were harvested and cultured in RPMI-1640 containing 10% (vol/vol) FBS, 1% (vol/vol) P/S at 37 °C with 5% CO_2.

Animals

C57BL/6 (8-10 weeks, male, 20-30 g) and BALB/c (6-10 weeks, female, 20-25 g) mice were purchased from Guangdong Medical Lab Animal Center. Female Bama minipigs (age 2~4 months), the donors of artery patch grafts, were purchased from BGI Ark Biotechnology (Shenzhen, China). Male cynomolgus monkeys (M15001: nine years old, 7.5 kg; M15003: nine years old, 9.0 kg; M16003: fourteen years old, 4.0 kg), the recipients of pig artery patch grafts, were purchased from Guangdong Landao Biotechnology (Guangzhou, China).

Artery patch transplantation

Pig-to-monkey artery patch xenotransplantation was performed at Guangdong Landao Biotechnology under full inhalational anesthesia as previously described[31]. Three independent pig-to-monkey artery patch xenotransplantations were performed and named as M15001, M15003, and M16003. The animal experiments were approved by the Institutional Review Board on Bioethics and Biosafety of Beijing Genomics Institute (BGI-IRB) (following IACUC-approved protocols published by the Yerkes Primate Center, Atlanta, GA, USA). All animal experiments were performed in accordance with the Ministry of Health guidelines for the care and use of laboratory animals (GB 14925-2001), and the procedures were approved by the Laboratory Animal Ethics Committee of the Sun Yat-sen University. All experiments were performed in accordance with relevant guidelines and regulations.

IgG/IgM binding

The washed PBMCs were suspended in staining buffer (PBS containing 1% bovine serum albumin (Invitrogen) and 0.1% NaN_3) for IgM/IgG binding assays. Serum from cynomolgus monkeys after xeno-artery patch was collected at designated time. Binding of serum from cynomolgus monkeys after xeno-artery patch to PBMCs of wild type Bama pig

was measured by flow cytometry using the relative geometric mean (rGM), as previously described[32]. Briefly, PBMCs (1×10^5 / tube) were incubated with 5 µl pooled monkey serum for 0.5 h at 4 °C. After incubation, cells were washed with staining buffer to remove unbinding monkey serum and were blocked with 10% goat serum (Sigma) for 20 min at 4 °C. After further washing with staining buffer, FITC-conjugated goat-derived anti-human IgM (mµ chain-specific) or IgG (γ chain-specific) polyclonal antibody (concentration 1:100 for pPBMCs; Invitrogen) was added, and the cells were incubated for 30 min at 4 °C. After washing with staining buffer, 200 µl fixation buffer was added, and the cells were allowed to sit at 4 °C for 30 min before adding 100 µl staining buffer. Flow cytometry was carried out using BD Aria II (BD, San Jose, CA).

Cell viability

Peripheral blood mononuclear cells (PBMCs) (1×10^6, 100 µl) stimulated with PHA (5 µg/ml) were seeded into 96-well plates (round bottom) and treated with ibrutinib (final conc. 1 µM and 5 µM) for 0, 1 and 5.5 days. 10 µl CCK8 was added to the supernatant for 2 hours. Two hours later, the absorbance values of wells were measured with OD450, which was read using a multiscan GO spectrophotometer (Thermo Scientific, Waltham, MA, USA). The cell viability was calculated by the following formula. Cell viability % = (OD450 $_{sample}$- OD450 $_{medium}$)/(OD450 $_{DMSO}$- OD450 $_{medium}$) × 100.

Proliferation

Adjust the cell density of peripheral blood mononuclear cells (PBMCs) to 2×10^6 / ml in 1640 medium supplemented with 10% FBS. Remove supernatant from the cell pellets. Add CellTrace™ CFSE (1:1000 dilution) staining solution and gently re-suspend the cells. Incubate at 37 °C for 20 minutes, protected from light. Add complete 1640 culture medium and mix. Incubate at 37 °C for another 5 minutes. Remove the supernatant and adjust the cell density to 1×10^6 / ml. Resuspend the cell pellets in fresh, pre-warmed complete 1640 culture medium and stimulate with PHA (5 µg/ml). Add DMSO and ibrutinib (final conc. 1 µM) for 5.5 days' incubation. Collect cell pellets and re-suspend in FACS buffer for flow cytometry.

T/B cell count

The washed PBMCs were suspended in staining buffer (PBS containing 1% bovine serum albumin (Invitrogen) and 0.1% NaN$_3$) for T/B cell count. Briefly, PBMCs (1×10^5 / tube) were suspended in 100 µl staining buffer. PE-Cy7 anti-human CD4 (5 µl per test), PE anti-human CD8 (2.5 µl per test), FITC anti-human CD3ε (10 µl per test) and APC anti-human CD20 (10 µl per test) were added, and the cells were incubated for 30 min at 4 °C protected from light. After washing with FACS buffer (0.5% BSA in PBS), 200 µl FACS buffer was added, and the cells allowed to sit at 4°C until running flow cytometry. Flow cytometry was carried out using BD Aria II (BD, San Jose, CA).

The washed spleen cells were suspended in staining buffer (PBS containing 1% bovine serum albumin (Invitrogen) and 0.1% NaN$_3$) for T/B cell count. Briefly, spleen cells (1×10^6 / tube) were suspended in 100 µl staining buffer. PE/Cy7 anti-mouse CD4 (12.5 µl per test), PE anti-mouse CD3 (12.5 µl per test), FITC anti-mouse CD20 (10 µl per test) and APC anti-mouse CD8α (12.5 µl per test) were added, and the cells were incubated for 30 min at 4 °C protected from light. After washing with FACS buffer (0.5% BSA in PBS), 200 µl FACS buffer was added, and the cells allowed to sit at 4 °C until running flow cytometry. Flow cytometry was carried out using BD Aria II (BD, San Jose, CA).

Skin transplantation

Animals were maintained under specific pathogen-free conditions. Skin from C57BL/6 mice was transplanted to BALB/c recipients as previously described [33]. Ibrutinib (30 mg/kg·d) was administrated orally daily starting from two days before skin transplantation (no dosage at the day of operation). For skin transplantation, 80% of necrosis, ulceration, progressive shrinkage and desquamation were considered to be rejected. Skin status was evaluated daily according to the standard of rejection. Photographs, skin grafts, and recipient mouse spleens were harvested at day 1, 3, 6, 10, 15, 21, 28 after operation. At least three mice were recorded per group (three for ibrutinib, three for control) at designated time (day 1, 3, 6, 10, 15, 21, 28).

All animal experiments were performed in accordance with the Ministry of Health guidelines for the care and use of laboratory animals (GB 14925-2001), and all the procedures were approved by the Laboratory Animal Ethics Committee of the Sun Yat-sen University.

Graft survival

Graft viability was analyzed by observation and photo documentation of the transplanted skin at designated time. During the rejection process, the blood supplied into the graft was progressively restricted, which could be observed as an increasing area of necrosis within the graft. Transplants were classified as vivid if the necrotic part was less than 80%.

Histological analyses

The skin grafts were removed at designated time (POD 1, 3, 6, 10, 15, 21, 28), rinsed in cold saline, placed in Optimal Cutting Temperature (OCT) compound and immediately frozen in liquid nitrogen for histological analysis. The fixed skin grafts were embedded with paraffin, and sectioned. Hematoxylin and eosin (H&E) staining was performed as described previously[33].

Statistical analysis

The data collected were analyzed using Graphpad Prism for the independent Student's t-tests. Experimental data were presented as the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ns = not significant vs without ibrutinib. All experiments were replicated at least three times. At least three mice for ibrutinib-treated group and control group separately at seven time points.
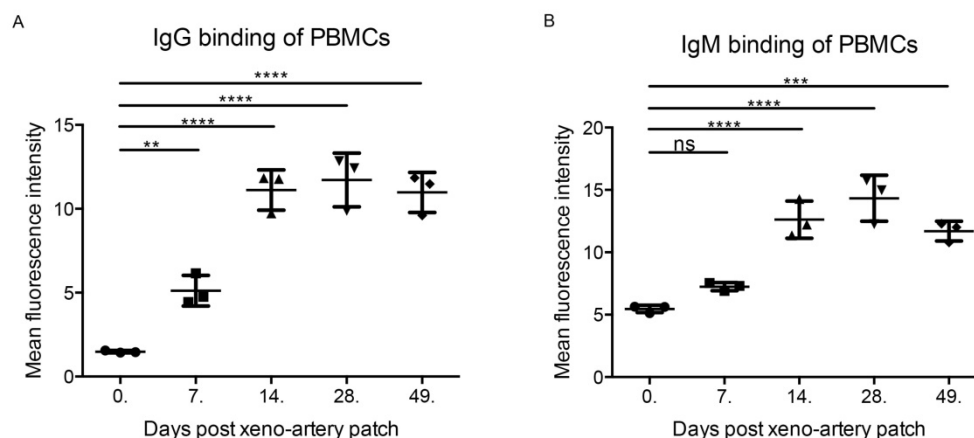
Results

Ibrutinib inhibited proliferation of PBMCs (POD14) with slight cell cytotoxicity.

The recipient cynomolgus monkey exhibited a strong immune response after an artery patch during POD14 to 42, which was validated by IgG and IgM binding (Figure 1). PBMCs (POD14) were employed as the evaluation system *ex vivo*. After incubating PBMCs (POD14) with ibrutinib (final concentration: 1 µM and 5 µM) for 24 hours and 5.5 days separately, the cell viability was detected by CCK8. A 24 hours' incubation with ibrutinib was intended to evaluate the cytotoxicity of ibrutinib on PBMCs. After 24 hours' incubation with ibrutinib, the cell viability decreased slightly at the final concentration of both 1 µM and 5 µM (Figure 2A). In the following experiments, a final concentration of 1 µM was employed as the treating concentration. When PBMCs
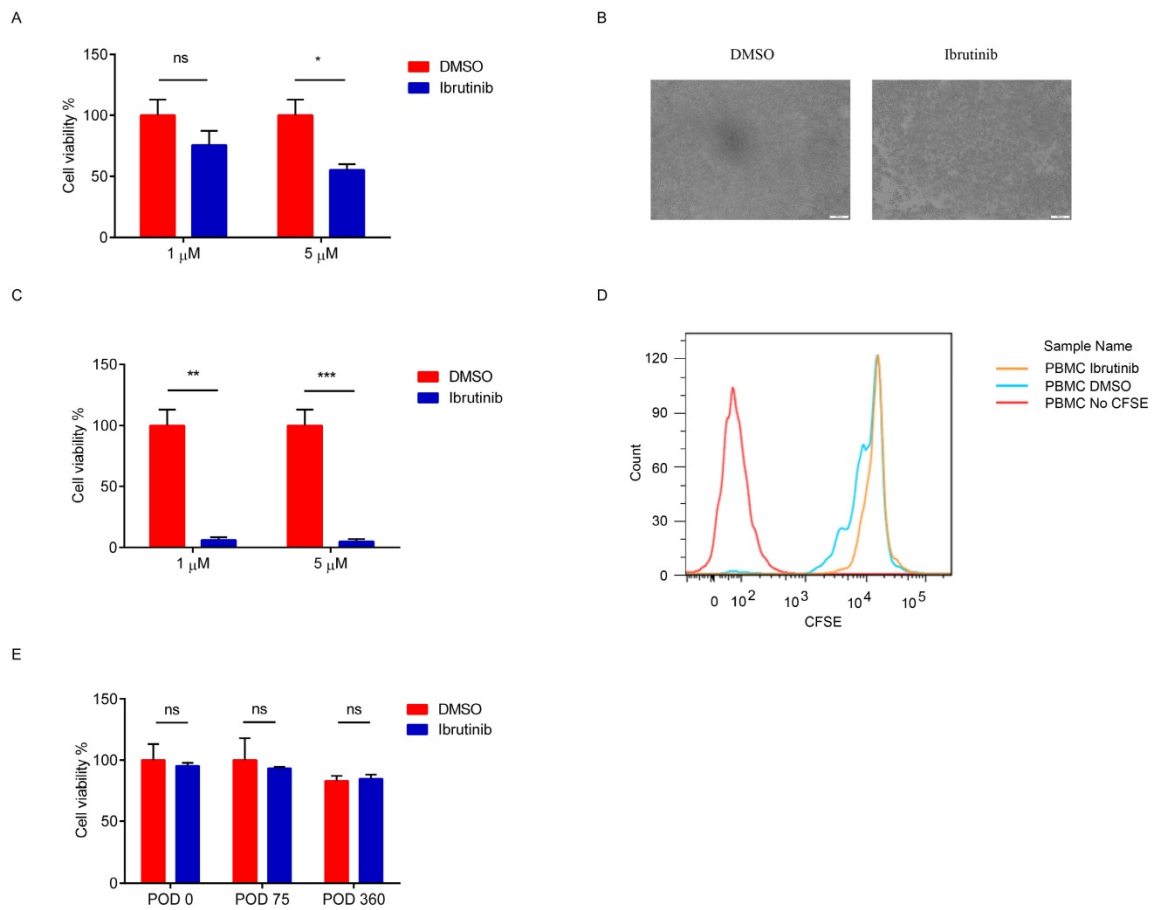
(POD 14) were exposed to ibrutinib for 5.5 days, which was a general evaluating assay for proliferation of PBMCs, the cell density was greatly decreased (Figure 2B). The cell viability assay showed that ibrutinib significantly decreased the cell viability of PBMCs (POD 14) after 5.5 days' incubation (Figure 2C), showing that ibrutinib inhibited the proliferation of PBMCs (POD 14). CFSE-labeled PBMCs (POD 14) were further analyzed by FACS to evaluate effects on proliferation of PBMCs. FACS analysis showed that the proliferation of CFSE-labeled PBMCs (POD 14) was almost halted after incubation with ibrutinib (1 µM) for 5.5 days (Figure 2D). IgG/IgM binding of PBMCs gradually decreased to base level after POD 49, implying the immune response gradually eliminated. We chose PBMCs at POD 0, 75 and 360 to evaluate the effects of ibrutinib on PBMCs with normal immune response. After incubation with ibrutinib (final conc. 1 µM) for 5.5 days, the cell viability of PBMCs was slightly influenced (Figure 2E). The different effects implied that ibrutinib (final conc. 1 µM) inhibited proliferation of PBMCs with strong immune response, but slightly influenced PBMCs with weak immune response. This difference made ibrutinib a priority over traditional immunosuppressants in minimizing adverse effects when long-term administrated. Adjusting therapeutic agents dosing could artificially control therapeutic effects and adverse effects.

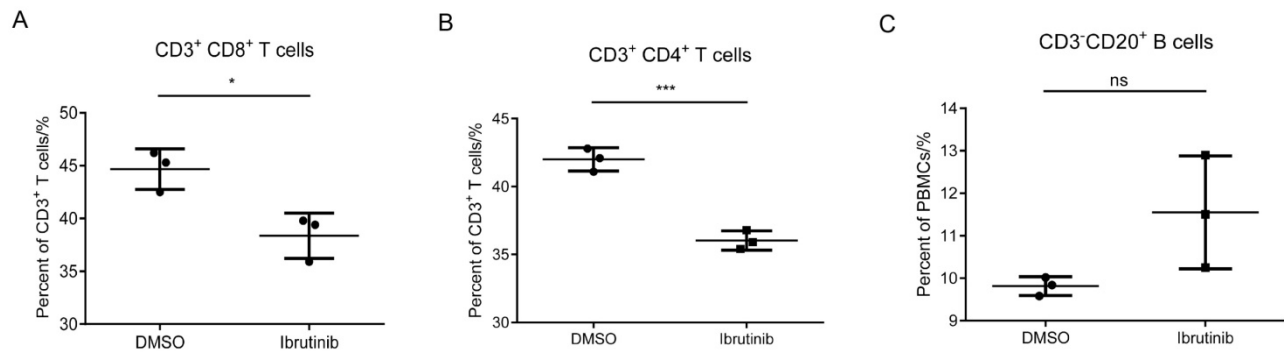### Ibrutinib mainly inhibited the proliferation of T/B cells.

In the previous assays, we found that ibrutinib efficiently inhibited the proliferation of PBMCs (POD 14), while exhibiting minor cytotoxicity and influence on PBMCs (> POD 49). In this part, a T/B cell count assay was employed to investigate the influence of ibrutinib (final conc. 1 µM) on subpopulations of PBMCs (POD 14). The results showed that ibrutinib

Figure 1. IgG and IgM binding of PBMCs. (A) IgG binding of PBMCs after xeno-artery patch. (B) IgG binding of PBMCs after xeno-artery patch. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.001, ns: not significant; n=3).

Figure 2. Effects of ibrutinib on PBMCs. (A) Cell viability of PBMCs (POD 14) after treatment with ibrutinib (final conc. 1 μM) for 24 hours. (B) Image of PBMCs (POD14) after treatment with ibrutinib (final conc. 1 μM). (C) Cell viability of PBMCs (POD14) after treatment with ibrutinib (final conc. 1 μM) for 5.5 days. (D) Inhibition of PBMCs (POD14) proliferation after treatment with ibrutinib (final conc. 1 μM) for 5.5 days. (E) Cell viability of PBMCs (POD 0, 75, 360) after treatment with ibrutinib (final conc. 1 μM) for 5.5 days. (*p<0.05, **p<0.01, ***p<0.001, ns = not significant vs without ibrutinib; n=3).

Figure 3. Effect of ibrutinib on T/B cells. Statistics of three independent T/B cell counts. (*p<0.05, **p<0.01, ***p<0.001, ns = not significant vs without ibrutinib; n=3).

significantly decreased the $CD3^+CD8^+$ and $CD3^+CD4^+$ T cell populations, but slightly increased the $CD3^-CD20^+$ B cell population (Figure 3). The influence of ibrutinib on T cell subpopulations was much more significant than B cells, implying that ibrutinib mainly interfered with T-cell-mediated rejection.

Ibrutinib inhibited the secretion of immune rejection related cytokines.

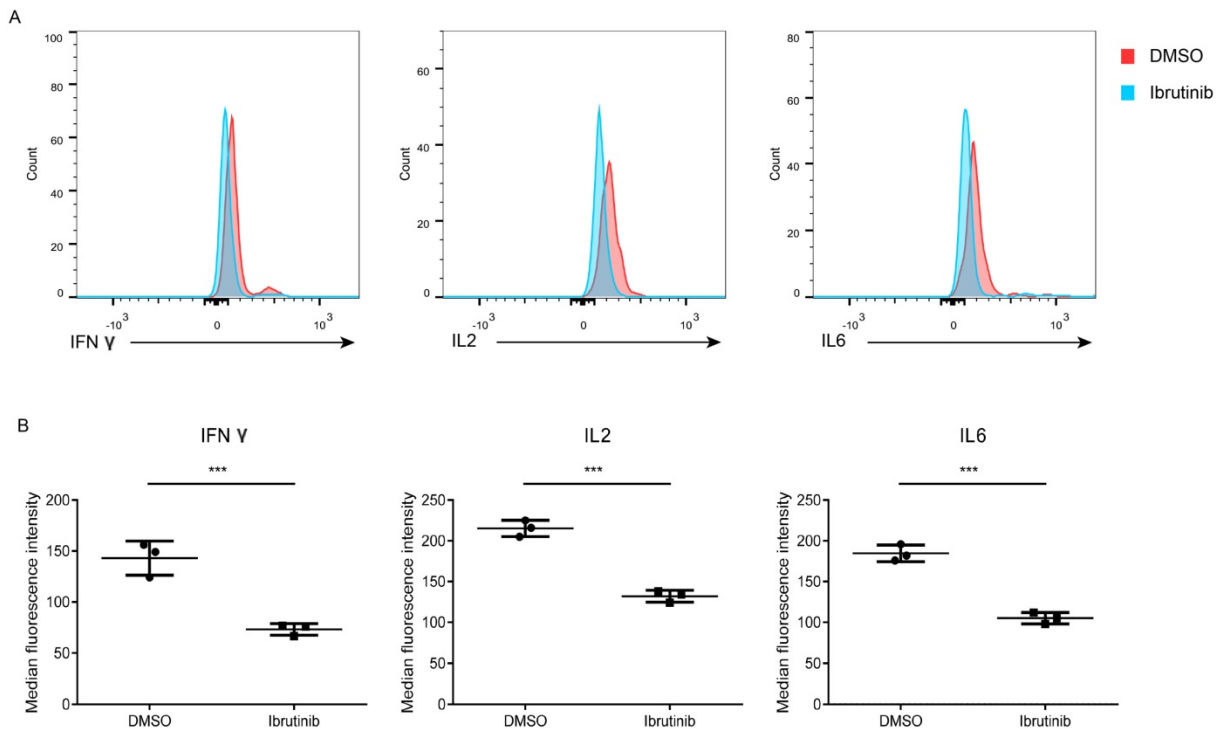Cytokines are the key mediators of immune balance, the expression and secretion of which are under strict spatial and temporal control. Cytokines storm may be responsible to the immune rejection after organ transplantation. A cytometric bead array was used to detect cytokines secreted in the supernatant of PBMCs (POD 14) after treated with ibrutinib (final conc. 1 μM). The results revealed that cytokines (IFN-γ, IL-2 and IL-6) were sensitive to ibrutinib treatment (Figure 4). However, TNF-α, IL-4, and IL-5 displayed no obvious change in both groups (Supplementary Figure 1). Influence of ibrutinib on PBMCs (POD 14) was divergent depending on the

cytokine types. Ibrutinib may exhibit immunosuppressive potential via modulating the secretion of cytokines (IFN-γ, IL-2 and IL-6).
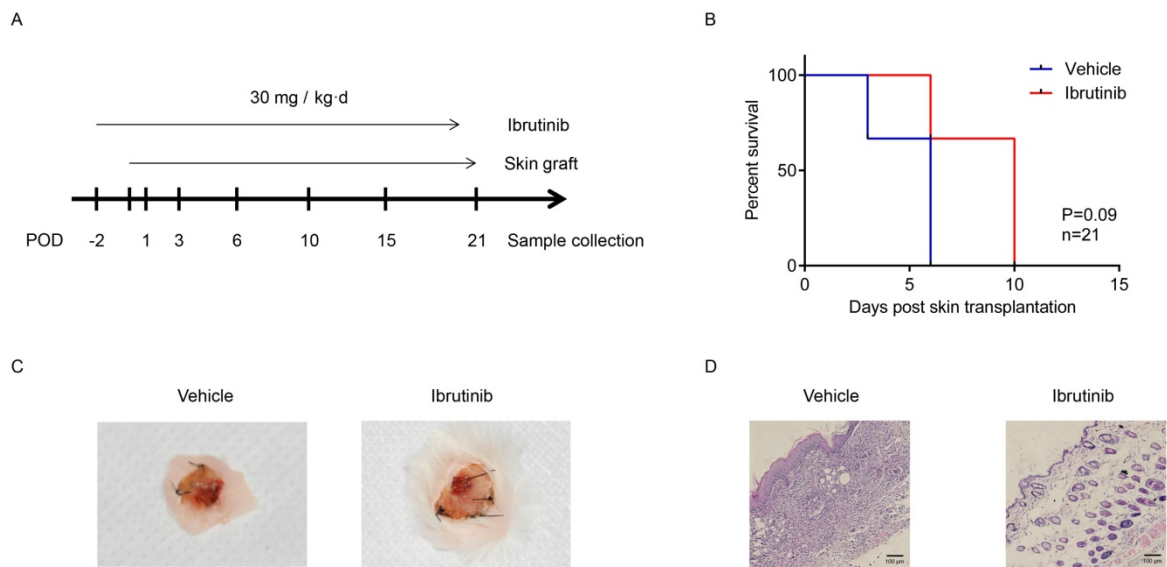
Ibrutinib delayed and alleviated immune rejection in allo-skin transplantation.

Murine skin transplantation is a routine model for evaluating immunosuppressant candidates *in vivo*. In this study, a C57BL/6 to BALB/c full-thick skin transplantation model was established to evaluate the potential of ibrutinib as an immunosuppressant. A dosage of 30 mg/kg·d of ibrutinib and vehicle were orally administered to recipient BALB/c mice daily starting from two days before the operation. The status of the grafted skin and recipient mice were monitored and recorded daily. Recipient mouse spleens and grafted skin were harvested at designated time (Figure 5A). The graft survival indicated that
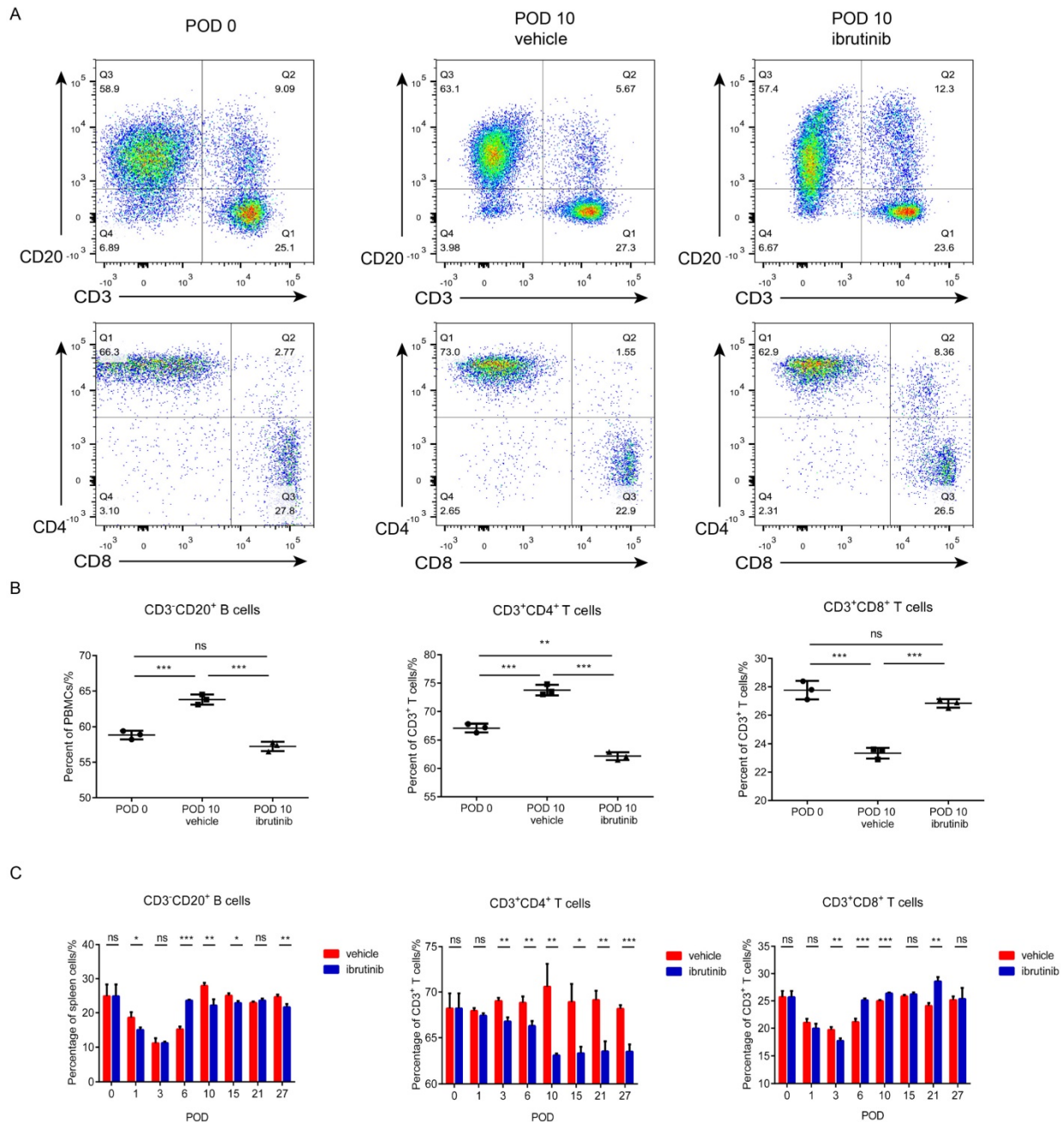

Figure 4. Influence of ibrutinib on cytokines secretion. (A) Representative result of cytokines (IFN-γ, IL-2 and IL-6) secreted from PBMCs (POD 14) after treatment with ibrutinib (final conc. 1 μM). (B) Statistics of MFI of cytokines (IFN-γ, IL-2 and IL-6) secreted from PBMCs (POD 14) after treatment with ibrutinib (final conc. 1 μM). Datas are representative of at least three independent experiments (mean±SEM). (*p < 0.05, **p < 0.01, ***p < 0.001 by Student's *t* test.)

Figure 5. Effects of ibrutinib on allo-skin transplantation. (A) Main sketch of allo-skin transplantation. (B) Graft survival of the grafted skin between the ibrutinib-treated group and the control. (C) The appearance of grafted skins. (D) H&E staining of the grafted skin at POD 10. At least three mice for ibrutinib-treated group and control group separately at seven time points. (*p < 0.05, **p < 0.01, ***p < 0.001 by Student's *t* test.)

ibrutinib improved the status of grafted skin and postponed the rejection compared with the vehicle group, which was summarized in Figure 5B. Approximate 15% of the grafted skin was rejected in the ibrutinib-treated group, while 50% was rejected in the vehicle group at POD 10 (Figure 5C). T/B cells of the recipient spleens harvested at the designated time were detected (Figure 6). The T/B cell count demonstrated that the immune response was intense during the interval of POD 7 to 21 (Figure 6C) in coincidence with the appearance. At POD 10,

$CD3^-CD20^+$ B cells and $CD3^+CD4^+$ T cells were decreased in the ibrutinib-treated group compared with vehicle (Figure 6A and 6B). H&E staining of the grafted skin at POD 10 revealed that the histology of the skin grafts in the ibrutinib-treated group exhibited more intact tissue alignment and less lymphocytic inflammatory infiltrate (Figure 5D). In conclusion, ibrutinib delayed and alleviated the immune rejection in allo-skin transplantation via interefering with $CD3^-CD20^+$ B cells and $CD3^+CD4^+$ T cells.

Figure 6. Effect of ibrutinib on T/B cells in allo-skin transplantation. (A) Representative figure of T/B cells on POD 0 and POD 10 (vehicle and ibrutinib). (B) Statistics of T/B cells on POD 0 and POD 10 (vehicle and ibrutinib). (C) Statistics of T/B cells on POD 0, 1, 3, 6, 10, 15, 21, 27 (vehicle and ibrutinib). At least three mice for ibrutinib-treated group and control group separately at seven time points. (*p < 0.05, **p < 0.01, ***p < 0.001 by Student's *t* test.)

Discussion

In organ transplantation, after the acute immune rejection, immunosuppressants are necessary for maintenance therapy to alleviate immune rejection and increase long-term survival. Chemical immunosuppressants have the advantages of being convenient, inexpensive, and easily optimized. However, traditional chemical immunosuppressants (such as anti-proliferative agents, steroids and calcineurin inhibitors) cause serious issues either poor immunosuppressive effects or severe adverse effects (such as high risk of infection, malignancies, nephrotoxicity, hepatotoxicity, and other sequelae). The development of novel immunosuppressants with high efficacy and a favorable safety profile is urgent and challenging. Ibrutinib, an approved drug for B-cell lymphomas and cGVHD, has been recently reported to be an irreversible inhibitor of ITK and exhibited potential therapeutic effects in autoimmune diseases and graft-versus-host disease. In the present study, we evaluated the potential of ibrutinib as an immunosuppressant in allo- and xeno-transplantation. The repositioning of ibrutinib as an immunosuppressant would be of great significance to drug development.

The artery patch model of wild type or genetically modified pigs to cynomolgus monkeys is a convenient and reliable xenotransplantation model. The physiological status of the recipient monkey is good enough for further evaluation without any immunosuppressants. Besides, the grafts can activate the immune system and induce anti-pig antibodies and cell-mediated immune rejection. David Cooper has firstly monitored xeno-immune rejection in xeno-artery patch model [34]. In the artery patch model of Bama wild-type pig to cynomolgus monkey, IgG/IgM binding of recipient PBMCs demonstrated that the immune response was relatively strong for 14-42 days after the artery patch. Comparing the effects of ibrutinib on PBMCs with the levels of immune response, ibrutinib inhibited PBMCs with a strong immune response, but showed minor effects on normal PBMCs. This finding may reflect the specialty of ibrutinib over traditional immunosup-pressants. T-cell mediated rejection is the major barrier to graft long-term survival [35, 36] and participates in antibody-mediated rejection (ABMR) [37]. T-cell mediated rejection is treatable under the control of effective immunosuppressants, such as T-cell costimulatory blockades [38] and T cell inhibitors [39]. The potential biological targets of ibrutinib in PBMCs might be ITK and BTK, which are the key mediators of T/B cells. The T/B cell count assay indicated that ibrutinib induced a decrease in $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells *ex vivo*, but slightly increased $CD3^-CD20^+$ B cells. These results indicate that ibrutinib may have a predominant effect on the T-cell mediated immune response, implying the potential of ibrutinib as a maintenance therapy agent.

Cytokines have been identified as strong regulators and potential biomarkers of immune responses (immune rejection, tolerance and effects of immunosuppressants) after organ transplantation [40]. The systemic regulation of cytokines plays a central role in the maintenance of immune homeostasis. IFN-γ [41] and TNF-α [42, 43] are typical Th1-cytokines, which are responsible for immune rejection [44]. IL-6 reportedly stimulates the inflammatory and autoimmune processes in many immune disorder diseases and has become a potent therapeutic target [45]. Th17 cells and IL-6 are considered to contribute to the mechanisms of rejection after organ transplantation [46, 47]. In this study, ibrutinib decreased the secretion of IFN-γ and IL-6. The secretion of IL-6 and IFN-γ might be regulated under a comprehensive network, implying that except for the direct cytokines, other regulators in the by-pathway are also mediated by ibrutinib. The cytokine analysis demonstrated that IL-6, IFN-γ and IL-2 are the main effectors sensitive to ibrutinib.

Full-thickness skin transplantation is considered to be a reliable and well-established animal model to evaluate the potential of immunosuppressant candidates. In this model, T-cell mediated rejection and graft survival are easily evaluated. In the previous *ex vivo* study, ibrutinib was found to suppress the proliferation of T cells and secretion of cytokines. Ibrutinib delayed the immune rejection of grafted skin *in vivo* and prolonged graft survival by decreasing $CD3^+CD4^+$ T cells and $CD3^-CD20^+$ B cells. However, ibrutinib delayed the immune rejection but not eliminated it, implying that the immuno-suppressive effects of ibrutinib were not strong enough in the allo-skin transplantation model. Compared with solid organ transplantation, the immune response of recipient mice after skin transplantation was too mild to adequately evaluate the potential of immunosuppressant candidates. Considering the different targets and potency of ibrutinib and other classic immunosuppressants, it is difficult to determine the exact agents for comparison of immunosuppressive potential in allo-skin transplantation model. The effects of ibrutinib *ex vivo* and *in vitro* demonstrated that ibrutinib has an immunosuppressive potential via interfering with T-cell mediated rejection and cytokine regulation. A more suitable solid organ transplantation model with typical and prominent immune rejection is needed to comprehensively evaluate the potential of ibrutinib as an efficient immunosuppressant.

It was obvious that ibrutinib decreased the amount of CD3$^+$CD4$^+$ T cells in both PBMCs after xeno-artery patch and spleen cells after skin transplantation. Cytokine analysis showed that ibrutinib inhibited the secretion of IL-2, IFN-γ and IL-6 while IL-4, IL-5 and TNF-α were basically not influenced by ibrutinib. The cytokine analysis further demonstrated the inhibitory effect of ibrutinib on helper T cells. Ibrutinib had more obvious effects on Th1-type cytokines than Th2-type cytokines, which was not coincide with the conclusion by Dubovsky [26]. Dubovsky demonstrated ibrutinib drive a Th1-selective pressure in naïve CD4$^+$ T lymphocytes by inhibiting ITK. But in our system, ibrutinib inhibited the secretion of both Th1 and Th2 type cytokines in PBMCs after xeno-artery patch, which exhibited strong immune responses. Ibrutinib was cytokine responsive but not Th-type responsive. Besides, ibrutinib had different effects on CD8$^+$ T cells in PBMCs after xeno-artery patch and spleen cells after skin transplantation. Berg [23] and Schwartzberg [48] have demonstrated that Tec kinases (ITK and RLK) had an important role in the development of conventional versus innate CD4$^+$ and CD8$^+$ T cells in *Itk$^{-/-}$* and *Itk$^{-/-}$Rlk$^{-/-}$* mice. However, the precise role of ITK and RLK in the differentiation of T cells is still obscure. Ibrutinib could inhibit both ITK and RLK but with different inhibitory effects. The roles (amount and activity) of ITK and RLK in PBMCs after xeno-artery patch and spleen cells after skin transplantation were not experimentally proved or reported. And the responses of ITK and RLK to ibrutinib at the working dosage were hard to determine and compare in these two different experimental models. Thus, quantitative fluorescent probes like PCI33380 [24], probe 1 [49], and Ibrutinib-SiR-COOH [50] may provide useful information about target occupation and metabolism which will help explain the influence of ibrutinib by ITK and RLK inhibition and the possible drug dosage and interval. The effects of ITK and RLK on the development of CD8$^+$ T cell lineages, the subtype of CD8$^+$ T cells in these two different experimental models, the interaction of CD4$^+$ T cells, CD8$^+$ T cells and even other T/B cells, and the different immune responses made it quite difficult to explain the effects of ibrutinib on CD8$^+$ T cells. The separation and enrichment of subtypes of T/B cells or differentiation *in vitro* may be good choices to look into the precise mechanism of ibrutinib in T/B cell related immune responses.

Resembled to many drug discovery stories, therapeutic effects *in vitro* do no equal or even represent the effects *in vivo*. Whether ibrutinib is a potential immunosuppressant in organ transplan-

tation still need comprehensive evaluation *in vivo*. Thus an ideal experimental model, reference immunosuppressants, administration dosage, combined therapies with known immunosuppressants (such as cyclosporine, CNIs, mTORs), and evaluation platform are necessities for novel immunosuppressants development. Kinase inhibitors for the treatment of cancers have been development successfully. Kinase inhibitors targeting T cells or B cells are potential lead compounds for immunosuppressants development. Repurposing of known kinase inhibitors with therapeutic potential to immunosuppressants would be of great value to facilitate the development of novel immunosuppressants by reducing risks and costs.

In this study, ibrutinib exhibited potential immunosuppression effects *ex vivo* and *in vivo* via suppressing T-cell mediated rejection and mediating the cytokine network. The immunosuppressive potency of ibrutinib was not ideal in the allo-skin transplantation model as expected. Ibrutinib was shown to delay immune rejection by interfering with T cells, especially CD3$^+$CD4$^+$ T cells. However, how ibrutinib inhibited the proliferation of T cells remained unknown. Ibrutinib had a negative influence on cytokines (IFN-γ, IL-2, and IL-6), mainly the Th1-type cytokines. The regulation of cytokine expression and secretion is a complex process and difficult to interpret, especially when multiple cytokines are involved.

Conclusion

The immunosuppressants applied clinically have been faced with severe safety issues. In this study, we found that ibrutinib, an approved drug for several B-cell lymphomas, exhibited anti-immune rejection potential by T-cell and cytokine mediation. Although the immunosuppressive effect of ibrutinib in allo-skin transplantation model was not as expected, the potential in T-cell mediated rejection and improvement in graft survival implied that ibrutinib is a promising candidate for immunosuppression in xeno- and allo- transplantation. Furthermore, the alternative application of ibrutinib to other diseases is a convenient avenue to drug development. Bypassing tedious safety evaluating processes would greatly facilitate and accelerate drug development. Ibrutinib offers a promising platform for immunosuppressants development. Structural optimization of ibrutinib and the elucidation of its mechanisms would greatly facilitate the development of novel immunosuppressants. Kinase inhibitors are potent therapeutic agents for many diseases. Repurposing of known kinase inhibitors targeting T/B cells to immunosuppressants would greatly help

the development of novel immunosuppressants.

Abbreviations

Btk: Bruton's tyrosine kinase; IFN-γ: Interferon gamma; IL-2: Interleukin-2; IL-4: Interleukin-4; IL-6: Interleukin-6; ITK: IL-2 inducible T cell kinase; RLK (TXK): Resting lymphocyte kinase; JAK: Janus kinases; PBMCs: Peripheral blood mononuclear cells; PKC: Protein kinase C; POD: Post operation days; SYK: Spleen Tyrosine Kinase; Th cells: helper T cells; TNF-α: Tumor necrosis factor alpha.

Supplementary Material

Supplementary figure and table.
http://www.medsci.org/v15p1118s1.pdf

Competing Interests

The authors have declared that no competing interest exists.

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