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RESEARCH

Targeting interleukin-2-inducible T cell kinase ameliorates immune-mediated aplastic anemia

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Abstract

Allogeneic stem cell transplant and immunosuppressive therapy (IST) are the current standard treatments for patients with aplastic anemia (AA). However, IST also carries significant risks and side effects, and up to 30–50% of patients experienced refractory or relapsed disease following IST. Treating AA remains challenging and novel efficient therapies are in critical need. The IL-2 inducible T-cell kinase (ITK) plays a crucial role in the T cell response and functions as a regulator of T cell activity. While ITK inhibition has shown promise in various immune-related disorders, its potential role in the pathophysiology of AA has not been thoroughly investigated. We observed elevated level of phosphorylated ITK in T cells from AA patients and AA mouse models. Moreover, we found that both treatment with an ITK inhibitor or conditional depletion of *Itk* in donor mice alleviated bone marrow hypoplasia, improved cytopenia, and extended survival rates. Notably, ITK inhibition orchestrates T cell quantity and function by reducing T cell infiltration and suppressing the secretion of key inflammatory cytokines in AA mice. Our data suggest that ITK inhibitor could potentially offer a new therapeutic strategy for AA.

Keywords Aplastic anemia · IL-2-induced T cell kinase · T lymphocytes · Targeted therapy

Introduction

Aplastic anemia (AA) is a condition characterized by bone marrow failure (BMF), leading to pancytopenia and marrow hypocellularity. The pathogenesis involves a complex interplay where immune cells directly or indirectly attack hematopoietic stem and progenitor cells (HSPCs) [1, 2]. $CD8^+$ T cells cause direct cytotoxic damage, while $CD4^+$ T cells, expressing negative regulatory factors like IFN-γ and TNF-α, and deficient regulatory T cells (Tregs), contribute to immune dysregulation [3–8]. Recent single-cell transcriptomics studies have further elucidated enhanced crosstalk between HSPCs and T cells in AA patients [9].

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Immunosuppressive therapy (IST), including anti-thymocyte globulin (ATG) combined with cyclosporine, achieves initial response in 60–70% of AA patients [1]. However, relapses and long-term cyclosporine use in one-third of patients underscore persistent immune dysregulation and chronic toxicity concerns associated with conventional IST [9–12]. Therefore, there is a great need for novel and efficient therapies for AA patients.

Animal models of AA have facilitated the exploration of pathogenesis and new immunosuppressive agents. For instance, rapamycin inhibits mTOR and improves BMF in AA mice by suppressing T cell growth and metabolism. Correction of the Th1/Th2 imbalance via NOTCH signaling modulation and All-trans-retinoic acid (ATRA) treatment prolongs survival in AA mice. JAK 1/2 inhibition also shows promise in suppressing T cell effector functions and preventing mortality in AA mice [13–15]. Despite success in these models, novel effective inhibitors for AA patients remain elusive.

IL-2 induced T-cell kinase (ITK), a key regulator in TCR signaling [16, 17], modulates T cell development, differentiation, and effector functions[18–23]. Dysregulated ITK is implicated in various T cell-mediated diseases, including autoimmune diseases [24], inflammatory diseases [25–27], neoplastic disease [28] and GVHD (Graft-versus-Host Disease) /HVGR (Host-versus-Graft Reaction) [29, 30]. While ITK inhibition has shown therapeutic potential in models like allergic asthma and inflammatory bowel disease [31, 32], its role in AA pathophysiology has not been explored.

Ibrutinib is a clinically available inhibitor of ITK. Although primarily approved for B-cell malignancies via suppressing Bruton's tyrosine kinase (BTK), it has also been shown to irreversibly target ITK [33]. Clinical trials for chronic lymphocytic leukemia (CLL) indicate that ibrutinib reduces $CD8^+$ T cell proliferation and activation while significantly enhancing T cell repertoire diversity in patients undergoing long-term treatment [34, 35]. Additionally, murine models demonstrate that ibrutinib decreases the number of $CD8^+$ effector T cells and their activation markers, impairing their proliferation and effector functions [36]. Moreover, ibrutinib enhances Th1 responses by inhibiting ITK-regulated TCR signaling in $CD4^+$ T cells and affects the number and percentage of regulatory T cells. Furthermore, in mouse models of allergic asthma, ibrutinib suppresses Th2/Th17 immune responses and alleviates asthma symptoms [31, 37]. Given the multifaceted effects of ibrutinib on T cell function through ITK inhibition demonstrated in various studies, we further explored its potential application in the context of AA.

In this study, we investigated ITK activation in both AA mouse model and AA patients and found the therapeutic potential of targeting ITK to mitigate T cell dysfunction and improve BMF in AA mice, laying the groundwork for clinical translation of new IST strategies in AA patients.

Materials and methods

Animals

C57BL/6(B6, H-$2D^b$) mice were obtained from the animal facility of the State Key Laboratory of Experimental Hematology, and procedures were approved by the Institutional Animal Care and Use Committee at animal facility of the State Key Laboratory of Experimental Hematology (Tianjin, China). CD45.1 congenic B6(H-$2D^b$) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). F1 progeny (CB6F1, H-$2D^d$) were obtained from Beijing Vital River Laboratory Animal Technology Company (Beijing, China) by crossing BALB/c females with B6 males. $Itk^{fl/+}$ mice were obtained from GemPharmatech (Nanjing, China). All mice were bred and maintained in a pathogen-free facility. Offspring between the age of 8–12 weeks were used in experiments. In animal experiments, there were at least 4–5 mice in each group, and experiments were repeated for at least three times.

Generating conditional *Itk* KO mice

Itk conditional floxed mice were generated by crossing $Itk^{fl/fl}$ mice to *Mx1*-Cre mice. PolyIC was given to $Itk^{fl/fl}$, *Mx1*-Cre;$Itk^{fl/+}$ and *Mx1*-Cre;$Itk^{fl/fl}$ mice at a dose of 10ug/g body weight every other day via 4–5 i.p. injections to conditionally delete *Itk*. Mice were rested for 4 weeks after the last injection before being used.

AA model induction and treatment of AA mice

CB6F1 mice were total body irradiated (TBI) with 4 Gy as recipients. 3×10^6 inguinal, cervical, axillary, and lateral axillary lymph node cells from B6 mice were intravenously injected into F1 recipients 4–6 h after irradiation. Mice were bled and euthanized 13–14 days later for analyses. PCI-32765 was obtained from MedChemExpress (NJ, USA), dissolved in DMSO at 500 mg/ml for storage at -20℃, diluted to 5 mg/ml with phosphate buffer saline (PBS) before use, and administrated through gavage once daily for 0-12d at 25 mg/kg or 50 mg/kg body weight.

Colony forming assay

Isolated BM cells were seeded in M3434 medium at a concentration of 5×10^4 cells/550 µl in duplicate wells. After 11 days of incubation at 37 °C in a humidified chamber, colonies were counted and identified following the manufacturer's protocol (STEMCELL Technologies).

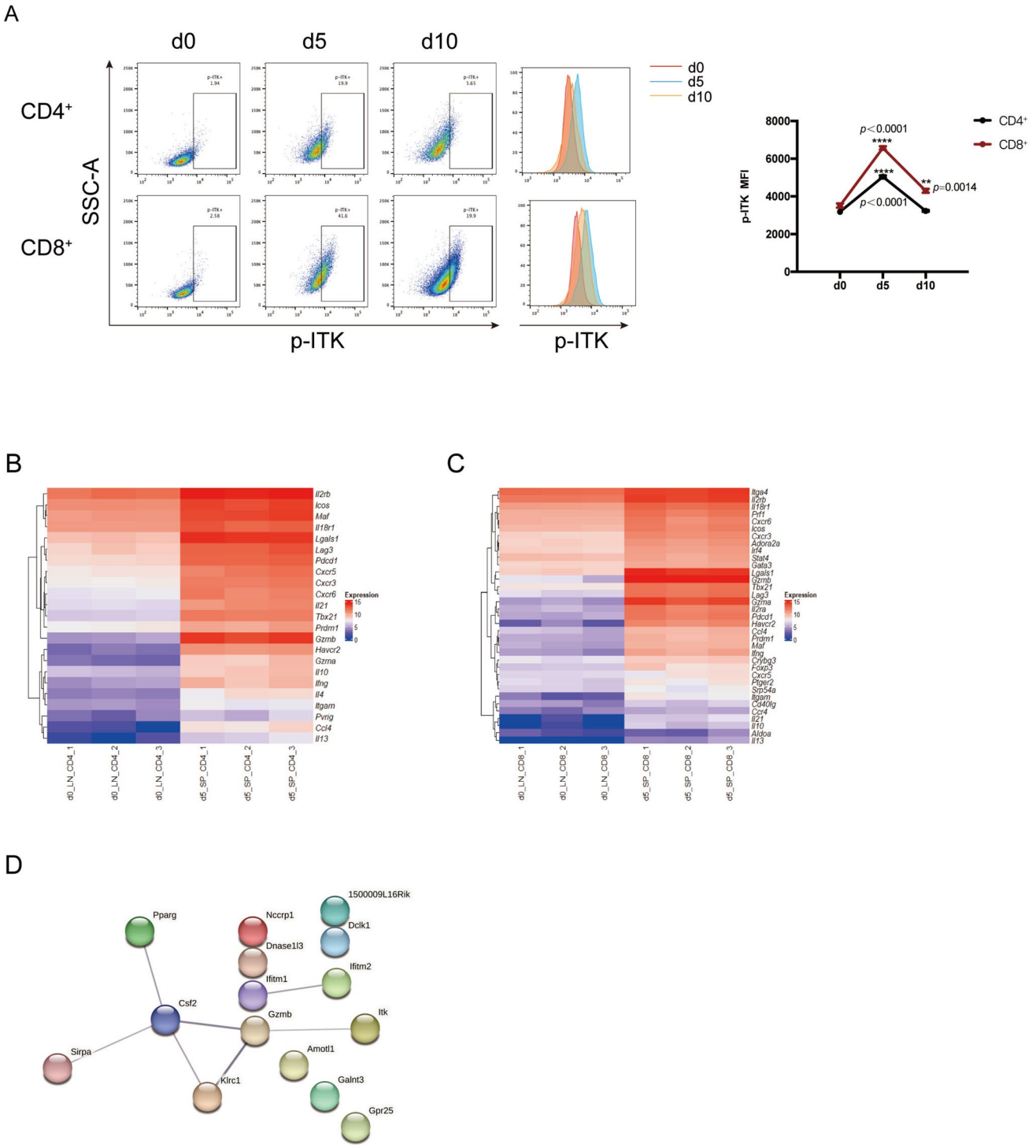
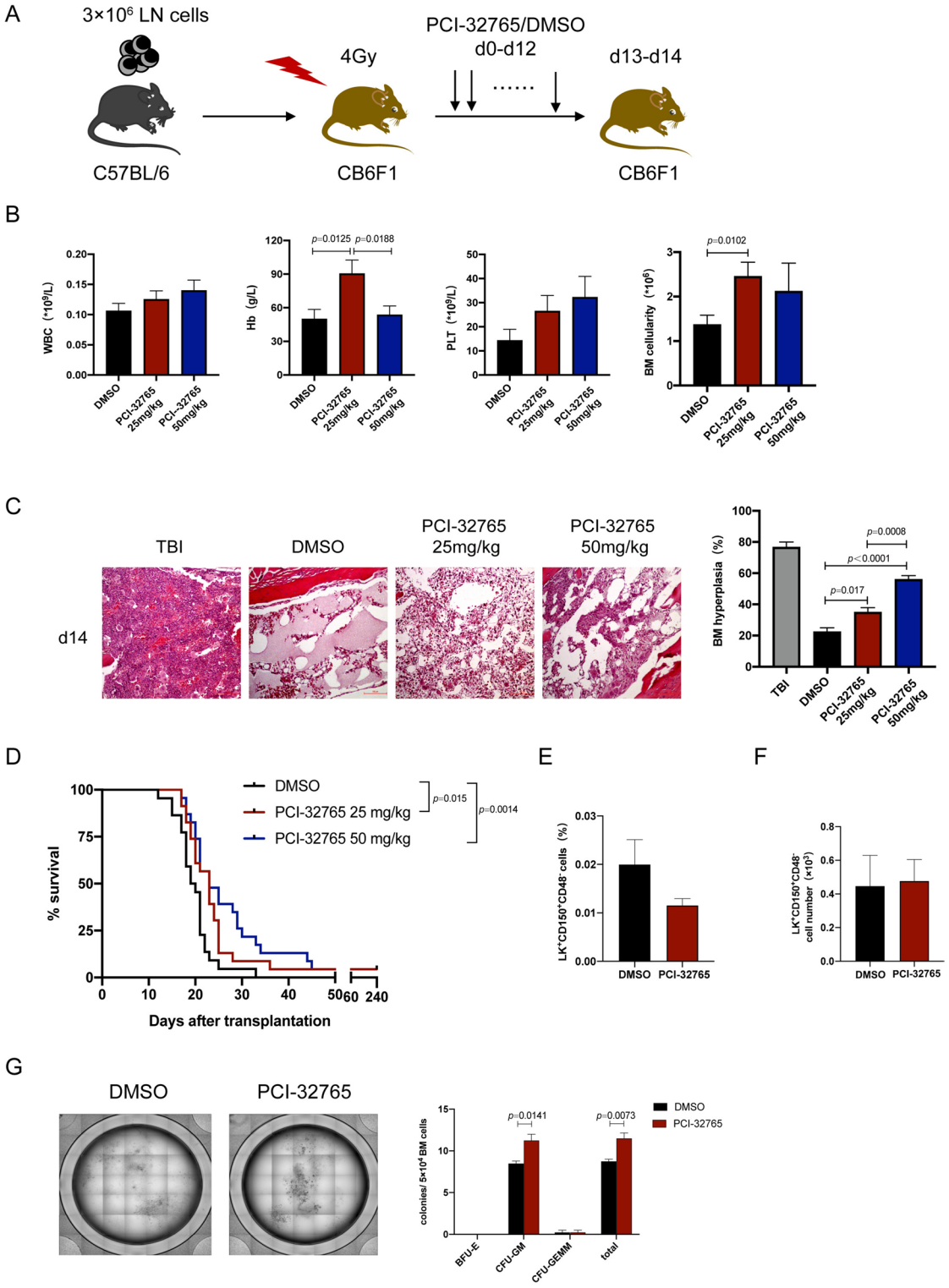


Fig. 1 ITK phosphorylation is increased with T cell transcriptional activation during immune-mediated aplastic anemia. **A** Flow cytometry analysis (left) and statistical analysis (right) of p-ITK expression level of donor T cells in AA mice spleen 5 days (d5) after transplantation and BM 10 days (d10) after transplantation respectively compared with pre-transplantation (d0). **B** Transcriptome analysis of spleen-infiltrating $CD4^+$ donor T cells 5 days after transplantation compared with pre-transplantation using RNA-Seq. **C** Transcriptome analysis of spleen-infiltrating $CD8^+$ donor T cells 5 days after transplantation compared with pre-transplantation using RNA-Seq. **D** The relationship between *Itk* and the common genes among the top 100 genes respectively highly expressed in $CD4^+$ and $CD8^+$ T cells after transplantation



Flow cytometry analysis

For intracellular cytokine staining, cells were cultured in RPMI 1640 medium with 50 ng/ml PMA, 1 μg/ml Ionomycin and GolgiPlug for 4–6 h in a 37 ℃ humidified CO_2 incubator, and then fixed and permeabilized using the Fixation/Permeabilization solution Kit (554714, BD Biosciences, San Diego, CA, USA) and stained with

◂**Fig. 2 ITKi treatment prolongs survival of AA mice by ameliorating BM hypoplasia and preserving the function of residual HSCs. A** CB6F1 mice received 4 Gy TBI and infusion of 3×10^6 B6 LN cells, leading to BMF. Recipient mice received PCI-32765 daily oral gavage for 13 days, while the control group received DMSO. Mice were euthanized on d13 or d14 for analyses. **B** White blood cell (WBC) counts, hemoglobin (Hb), platelets (PLT) counts and BM nucleated cell number in TBI, BMF (DMSO), and PCI-32765 treated mice. **C** Hematoxylin and eosin-stained BM (left) and the degree of BM hyperplasia (right) in TBI, BMF (DMSO) and PCI-32765 treated mice. **D** Kaplan–Meier survival curve for TBI, BMF (DMSO) and PCI-32765 treated mice. **E** Frequencies of BM $Lin^-c\text{-}kit^+CD150^+CD48^-$ HSCs in TBI, BMF (DMSO) and PCI-32765 25 mg/kg treated mice. **F** Absolute numbers of BM $Lin^-c\text{-}kit^+CD150^+CD48^-$ HSCs in TBI, BMF (DMSO) and PCI-32765 25 mg/kg treated mice. **G** Colonies under high-content imaging(left) and colony number of BM cells (right) from TBI, BMF (DMSO) and PCI-32765 25 mg/kg treated mice in colony forming unit assay

intracellular markers. Human samples were cultured with CD3/CD28 T-Activator (11456D, Thermo Fisher Scientific, Waltham, MA, USA) for 12 h before test. Fix Buffer I (557870, BD Biosciences, San Diego, CA, USA) and Perm Buffer III (558050, BD Biosciences, San Diego, CA, USA) were used for human T cell p-ITK detection after activated by CD3/CD28 T-Activator in vitro. IntraSure™ Kit (641776, BD Biosciences, San Diego, CA, USA) were used for Ki-67 detection.

Bulk RNA sequence

$CD4^+$ and $CD8^+$ cells were sorted by flow cytometry, and total RNA was extracted using Trizol according to the manufacturer's instructions. The integrity of the RNA was assessed using an Agilent 2100 bioanalyzer. Then the NEBNext® Ultra™ RNA Library Prep Kit from Illumina was utilized to construct libraries from the total RNA. Finally, the libraries were sequenced on an Illumina sequencing platform.

Patient's samples

Peripheral blood was collected from patients and healthy donors from Institute of Hematology & Blood Diseases Hospital after informed consent in accordance with the Declaration of Helsinki protocol. All patients received a diagnosis of AA according to the International Study of Aplastic Anemia and to the criteria of Camitta [38, 39]. None of the patients had received specific therapy at the time of sampling.

Statistical analysis

Data from cell counts, flow cytometry and qPCR between different groups were compared by unpaired *t*-tests using Graphpad Prism 8.0(GraphPad Software, San Diego, CA, USA). Data were shown as mean ± SEM. Quasi-Likelihood F-Test was used for differential expression testing in RNA-Seq data. The differences in survival between different animal groups were evaluated by a log rank test. $P<0.05$ was considered statistically significant.

Results

Increased ITK phosphorylation and transcriptional activation in T cells during immune-mediated aplastic anemia in mice

In the AA mouse model, T cells exhibited immune activation post-transplantation, particularly in the early stage in the spleen[40]. Despite modest changes in ITK expression (Figure S1A), phosphorylated ITK (p-ITK), the activated form, significantly increased in $CD4^+$ and $CD8^+$ donor T cells at 5 days post-transplantation, declining by day 10 but remaining elevated in $CD8^+$ T cells compared to pre-transplant levels (Fig. 1A).

To further understand the transcriptome changes, RNA sequencing was performed with $CD4^+$ and $CD8^+$ donor T cells at day 0 and day 5. Principal component analysis showed that the biological characteristics of T cells underwent significant changes during this process (Figure S1B) and the heatmap showed upregulated expression of genes involved in activation, migration, and effector genes (e.g., *Il2rb*, *Cxcr3*, *Gzmb*) in $CD4^+$ and $CD8^+$ T cells at day 5 post-transplantation in AA mice spleen compared to pre-transplantation (Fig. 1B and 1C). Furthermore, protein interaction analysis identified Gzmb, a key cytotoxic molecule, as a prominent ITK-interacting protein among the 15 common genes in top 100 upregulated genes of both $CD4^+$ and $CD8^+$ T cells post-transplantation (Fig. 1D). These findings suggest a link between ITK activation and the functional activation of T cell transcriptomes during immune-mediated AA.

Inhibiting ITK activation attenuates lethal BMF in AA mice

Given the role of ITK in various T cell-mediated diseases and the observed increase in ITK phosphorylation in AA mice, we wondered whether an ITK inhibitor (ITKi) could ameliorate BMF. We established an AA mouse model by transplanting LN cells from C57BL/6J donors into MHC-mismatched CB6F1 recipients, and administered PCI-32765, an ITK inhibitor, at 25 mg/kg or 50 mg/kg from day 0 to day 12 post-transplantation (Fig. 2A). BMF with severe pancytopenia developed in mice treated with DMSO by days 13–14 post-transfusion. Treatment with PCI-32765 at 25 mg/kg preserved BM cellularity and improved hemoglobin levels compared to DMSO controls (Fig. 2B). Although higher

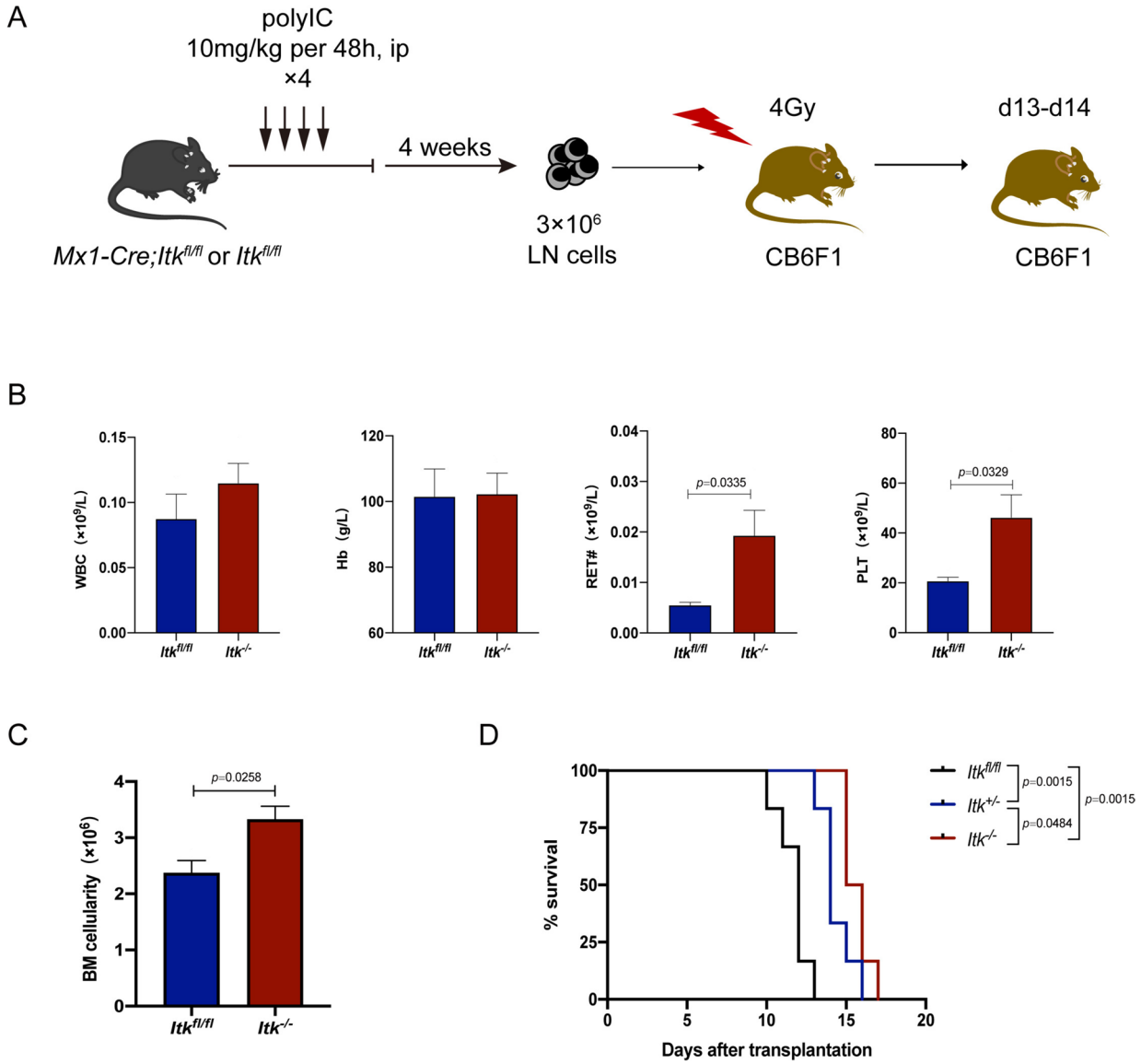


Fig. 3 Conditionally deleting *Itk* ameliorates bone marrow failure and prolongs survival of AA mice. A *Mx1-cre*;$Itk^{fl/fl}$ and $Itk^{fl/fl}$ mice were administered polyIC (10 mg/kg body weight) via intraperitoneal injection every other day for 7 days. Mice were rested for 4 weeks and then the LN cells were used for induction of aplastic anemia models. **B** White blood cell (WBC) counts, hemoglobin (Hb), reticulocytes(Ret) counts and platelets (PLT) counts in TBI, $Itk^{fl/fl}$, and $Itk^{-/-}$ mice. **C** BM nucleated cell number in TBI, $Itk^{fl/fl}$, and $Itk^{-/-}$ mice. **D** Kaplan–Meier survival curve for TBI, $Itk^{fl/fl}$, and $Itk^{-/-}$ mice

doses (50 mg/kg) showed marginal improvements (Fig. 2B), bone marrow biopsies indicated amelioration of empty BM spaces in both treatment groups(Fig. 2C). Notably, hematopoiesis in some mice treated with PCI-32765 was substantially restored by 8 months post-transplantation (data not shown), correlating with prolonged survival compared to DMSO controls (Fig. 2D).

To further elucidate the effect of PCI-32765 on hematopoietic stem and progenitor cells (HSPCs) in AA mouse models, we analyzed the frequency and cellularity of $Lin^{-}c\text{-}kit^{+}CD150^{+}CD48^{-}$ hematopoietic stem cells (HSCs) in BM. Although no significant differences were observed between PCI-32765 treated and control groups (Fig. 2E and F), Colony-forming assays revealed increased numbers of Colony Forming Unit-Granulocyte Macrophage(CFU-GM) and total colony (Fig. 2G). These observations indicate that treatment with ITKi in AA mice reduced bone marrow hypoplasia, improved cytopenia, prolonged survival rates and enhanced residual HSCs, with particularly notable effects at a dose of 25 mg/kg.

Conditional deletion of *Itk* improves disease outcomes in AA mice

PCI-32765 is an irreversible inhibitor targeting both ITK and BTK, which are predominantly expressed in T and B cells respectively. Acalabrutinib, a highly selective inhibitor of BTK, was found to have no impact on T cell activation even at the concentration of 10 μM in vitro (Figure S2A and B). This result confirm the effect of PCI-32765 on AA mouse is not directly targeting T cells through BTK. To further exclude BTK involvement, we used *Itk* conditional knockout ($Itk^{-/-}$) mice as donors to induce AA.

Considering ITK signaling is essential for T cell development, we chose to induce the conditional deletion of *Itk* in mature T cells. *Itk* deletion was induced by treating adult $Mx1\text{-}cre;Itk^{fl/fl}$ mice with polyIC (Fig. 3A), resulting in reduced *Itk* expression at both DNA and RNA levels compared to controls (Figure S3A and B). However, results showed us conditional deletion of *Itk* in adult mice still affected T cell development in some other ways (Figure S4A–M).

Particularly noteworthy is observation that transfusing AA model mice with $Itk^{-/-}$ donor cells mitigated disease severity, evident from improved peripheral blood counts, BM cellularity, and prolonged survival compared to controls (Fig. 3B-D). Despite the incomplete rescue of BMF and survival in AA mice following *Itk* knockout, which may be partially associated with other impacts of polyIC on the immune system of the knockout mice, survival rates positively correlated with the degree of *Itk* deletion (Fig. 3D), further emphasizing the crucial role of *Itk* in T cell-mediated BMF.

ITK inhibition ameliorate T cell infiltration and effector molecule expression in bone marrow

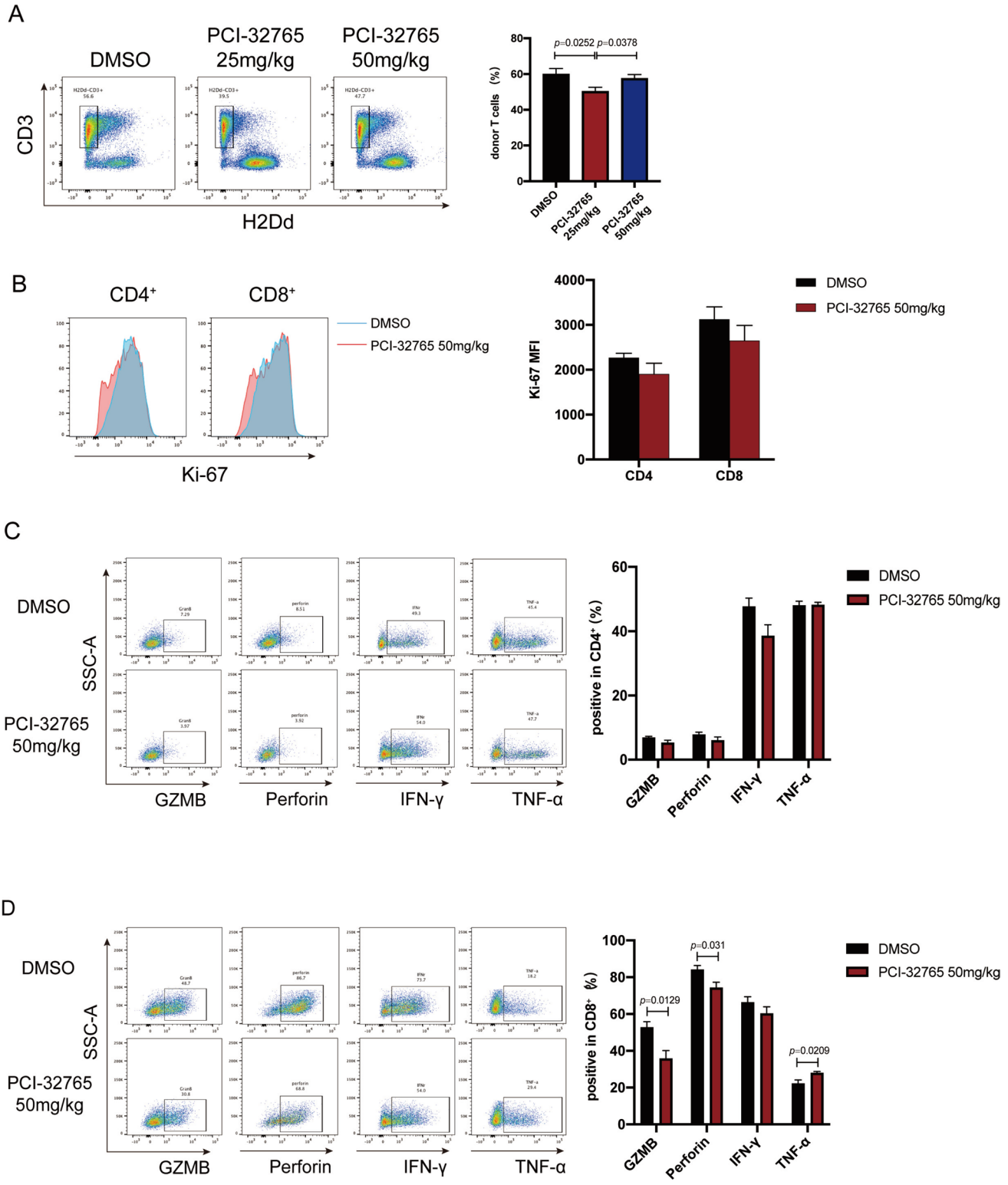
To evaluate the mechanism of ITKi on T cell function in AA condition, we examined the effect of ITKi on T cell infiltration and proliferation. We found that PCI-32765 treatment reduced $H2Dd^{-}CD3^{+}$ donor T cell infiltration at 14 days post-transfusion compared to controls, particularly in the 25 mg/kg group (Fig. 4A). We also explored whether the proliferation of T cells was affected by ITK inhibitor in AA mice, and found a slight decline of Ki-67 expression in ITKi treated group (Fig. 4B). Then intracellular staining was performed to detect effector molecules expression in T cells in AA mice. The result showed that about 50% $CD4^{+}$ T cells expressed IFN-γ and TNF-α and less than 10% cells expressed granzyme B (GZMB) and perforin, as expected. The treatment of PCI-32765 did not alter their expression in $CD4^{+}$ T cells (Fig. 4C). But when it comes to $CD8^{+}$ T cells, the PCI-32765 group showed significant decline of GZMB and perforin expression, despite the increasing TNF-α after PCI-32765 treatment (Fig. 4D). This evidence reveals that ITKi diminished T cell infiltration and proliferation in AA mice, with a significant reduction in GZMB and perforin in CD8 + T cells.

Activated ITK signaling in AA patient T cells can be functionally blocked by ITKi

Previous reports showed that ITK was enhanced in T cells of patients with inflammatory skin diseases or systemic lupus erythematosus (SLE). To determine whether ITK signaling is also involved in human autoimmune BMF, we evaluated p-ITK in peripheral blood of newly diagnosed AA patients. In comparison to healthy controls, elevated p-ITK levels in both $CD4^{+}$ and $CD8^{+}$ T cells from newly diagnosed AA patients were observed (Fig. 5A–D). In vitro activation further elevated the p-ITK levels in AA patient T cells (Fig. 5A–D), suggesting persistent ITK activation in AA pathogenesis. More interestingly, PCI-32765 treatment suppressed IFN-γ in $CD4^{+}$ and $CD8^{+}$ T cells from AA patients, with greater suppression of GZMB and TNF-α in $CD8^{+}$ T cells under both PCI-32765 and PRN694(Fig. 5E and F), consistent with the findings observed in the AA mouse model. Overall, increased p-ITK levels and reduced critical inflammatory cytokines by ITKi in AA patients further highlight the role of ITK signaling in the immune condition. However, these results warrant further validation through more precise subgroup analyses based on factors such as disease duration and comorbidities, which may influence the outcomes.

Discussion

HSPCs damage caused by autoreactive T cells is the core pathogenesis of acquired aplastic anemia, so that IST mainly including CsA and ATG/ALG brings a great success in AA treatment. AA mouse model was successfully constructed in 2004 by sublethal TBI combined with MHC-mismatched LN cells infusion [41]. While this model shares some minor features with GVHD, it specifically recapitulates key characteristics of human acquired aplastic anemia (AA), including pancytopenia, marrow hypocellularity, and immune-mediated bone marrow destruction. Notably, aside from mild weight loss, it does not exhibit the multi-organ damage typically associated with GVHD. Moreover, despite the inability of AA mice to mimic the onset of AA across different age groups in patients, and the impact of TBI on



the mice's immune system as well as their overall health, which may further affect their response to immunosuppressive drugs and thus interfere with experimental results, the restoration of bone marrow function in AA mice treated with cyclosporine A (CsA) and anti-thymocyte globulin (ATG) highlights the model's utility for studying the molecular pathogenesis of AA and evaluating new immunosuppressive therapies.

◂**Fig. 4 ITK inhibition suppresses T cell infiltration in BM and the expression of effector molecules. A** Flow cytometry analysis (left) and statistical analysis (right) of donor T cells infiltrated in AA mice bone marrow 14 days after transplantation. The percentage of BM-infiltrating donor T cells was compared between BMF (DMSO), and AA mice treated with different dose of PCI-32765. **B** Flow cytometry analysis (left) and statistical analysis (right) of Ki-67 expression level of donor $CD4^+$ and $CD8^+$ T cells in AA mice spleen 5 days after transplantation between BMF (DMSO) and PCI-32765 treated mice. **C** Flow cytometry analyses (left) and statistical analysis (right) of GZMB, perforin, IFN-γ and TNF-α expression level of donor $CD4^+$ T cells in AA mice spleen 5 days after transplantation between BMF (DMSO) and PCI-32765 treated mice. **D** Flow cytometry analysis (left) and statistical analysis (right) of GZMB, perforin, IFN-γ and TNF-α expression level of donor $CD8^+$ T cells in AA mice spleen 5 days after transplantation between BMF (DMSO) and PCI-32765 treated mice

As a key molecule downstream of the TCR signaling pathway, ITK participates in actin cytoskeleton remodeling, adhesion, and transcriptional regulation in T cells [42], which is partially dependent on its kinase activities. Studies have shown that abnormal expression of ITK is related to the occurrence of various of T cell mediated diseases. The increasing phosphorylation of ITK was detected in peripheral blood T cells of SLE patients [24]. Similar results were found in tumor tissues of patients with vascular immunoblastic T-cell lymphoma [28], which indicated poorer prognosis. In addition, genomic research showed us genetic polymorphism of ITK was also related to the attack of asthma and specific reactive dermatitis [25, 27]. In our study, higher phosphorylation of ITK was observed in peripheral blood T cells of AA patients than healthy donors, no matter whether activated by CD3/CD28 activator or not in vitro. The excessive activation of ITK in AA patients indicates that ITK might be a potential target for the IST in AA.

Given that AA mouse model is an ideal in vivo model to test the efficacy of immunosuppressants, we first treated AA mice with an ITK inhibitor PCI-32765, also known as ibrutinib. Consistent with other treatment, like rapamycin, CsA, JAK1/2 inhibitor, et al., PCI-32765 did improve the bone marrow failure and survival of AA mice. Constructing AA model using *Itk* knockout B6 mice as donor showed similar results, conforming the role of ITK in T cell mediated bone marrow failure. But another ITK inhibitor PRN694 didn't get desired results in AA model (data not shown). The failure of PRN694 may related with the severe drug side effect because obvious weight decline was observed in treatment group. Additionally, a study revealed that the JAK/STAT pathway is activated in T cells of patients with AA [43], suggesting that targeting both ITK and STAT1 may enhance treatment efficacy in AA, and positioning ITK inhibitors as a potential therapeutic avenue for STAT1 gain-of-function AA.

The study of dynamic changes of donor derived T cells in AA mouse model showed that T cells at 5 days after infusion were in greatly active state both in proliferation and differentiation [40]. In our study, higher expression of genes relative to migration, activation, and effector function in the early stage after infusion illustrated the activation status of T cells in AA model at transcriptome level. During this process, p-ITK increased significantly, but inhibiting ITK didn't affect the proliferation and differentiation of T cells like other immunosuppressants in AA model. Research in 2009 of Neal S. Young group showed us that mice lack of perforin as donor could also cause moderate to high degree of BMF, indicating that perforin-mediated cell death plays a minor role in this process [44]. Protein interaction analysis in this study shows us that, in the top 100 genes respectively upregulated in $CD4^+$ and $CD8^+$ T cells, GranB interacts most strongly with ITK among the 15 common genes. The decline of GranB in T cells both in AA mice and AA patients responding to ITK inhibition furtherly demonstrated that the efficacy of ITKi in alleviating BMF may achieved by inhibiting GranB-mediated T cell effector function. Previous studies have shown that Treg and Th17 also play important roles in AA. Therefore, changes of Treg and Th17 cells in AA mice and AA patients after ITKi treatment should be further investigated.

It is worth noting that ITKi did not inhibit the secretion of TNF-α in human $CD4^+$ and AA mice $CD8^+$ T cells but promote its secretion in our study. Previous study has shown that TNF-α regulates T cell metabolic reprogramming in an ITK-dependent manner in RA patients [45] and it reminds us ITK inhibition may lead to a negative feedback by increasing TNF-α when T cell under activation conditions. Considering that TNF-α plays an important role in the occurrence of AA disease in mice [46], this may explain why inhibition of ITK has limited effect on the alleviation of BMF and simultaneous inhibition of TNF-α and ITK may furtherly improve disease status in AA.

ITK is important for the cytotoxic function of T cells because the degranulation capacity would be affected in ITK deleted condition. But recent research found inhibiting ITK could reduce GVHD by affecting the ability of T cells migrating to target organs but preserved its cytotoxic function in GVL effects [30]. It indicates that the effect of inhibiting ITK is also depending on the condition that the T cells was on.

However, the complexity of TCR-Itk signaling in T cell function and the multiple factors involved in T cell-mediated AA necessitate further investigation into the effectiveness of ITKi for treating AA. Furthermore, allogeneic T-cell driven mouse models may not fully capture the complexity of patients with AA. Recently, dysregulation of B cells

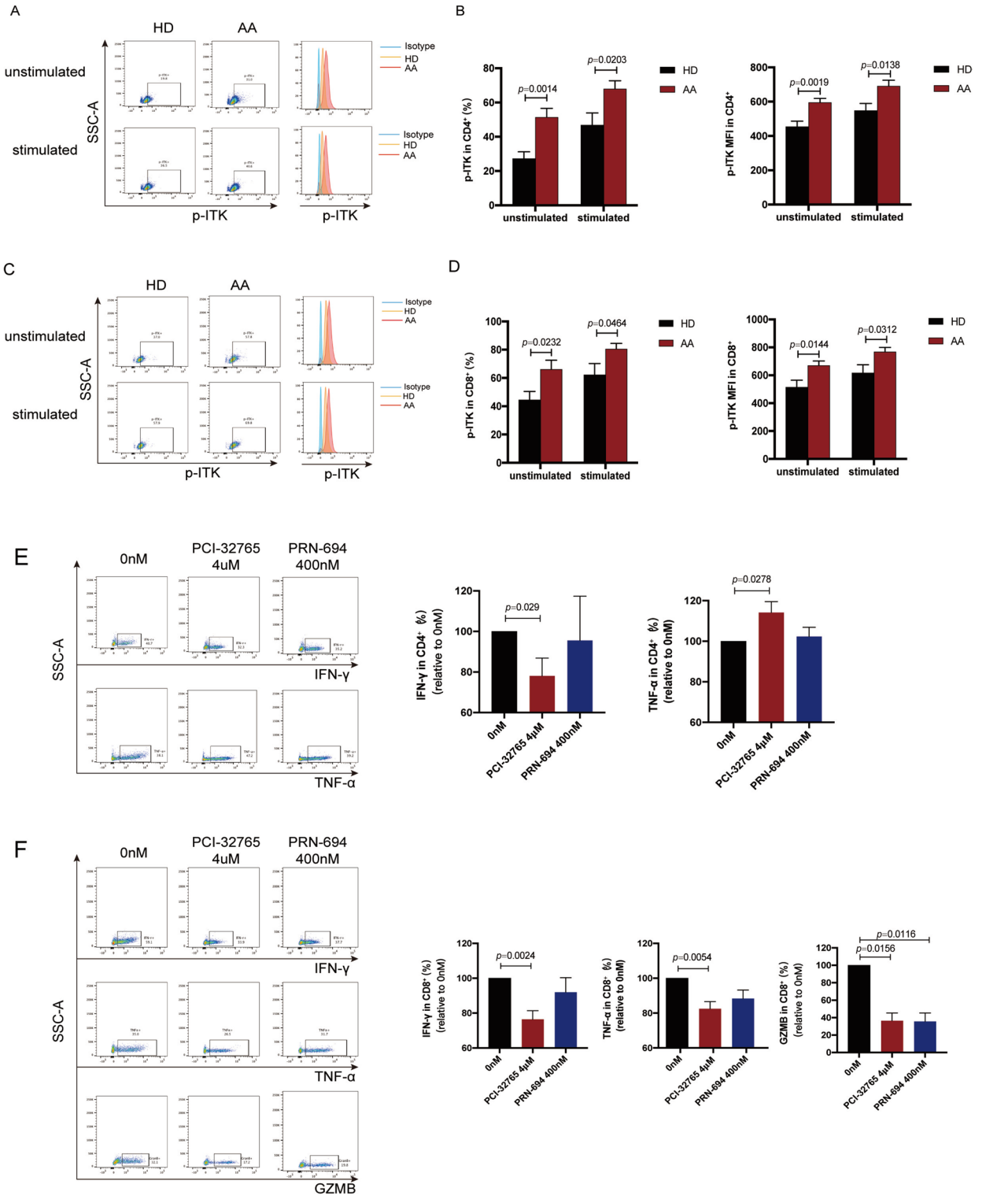


Fig. 5 ITK phosphorylation is increased in T cells of AA patients and its function can be inhibited by ITKi. A-B Flow cytometry and statistical analysis of p-ITK expression level in AA patients and healthy donors (HD) $CD4^+$ T cells before activation (unstimulated) and after 5 minutes of activation by CD3/CD28 beads (stimulated) in vitro respectively. **C-D** Flow cytometry statistical analysis of p-ITK expression level in AA patients and healthy donors (HD) $CD8^+$ T cells before activation (unstimulated) and after 5 min of activation by CD3/CD28 beads (stimulated) in vitro respectively. **E** IFN-γ and TNF-α expression level in $CD4^+$ T cells of AA patients inhibited by PCI-32765 or PRN694 relative to DMSO (0 nM). **F** GranB, IFN-γ and TNF-α expression level in CD8.$^+$ T cells of AA patients inhibited by PCI-32765 or PRN694 relative to DMSO (0 nM)

and myeloid subsets has also been observed in AA. In particular, there is a significant increase in $CCR6^+$ B cells in AA, which may exacerbate immune injury by recruiting pro-inflammatory immune cells[47]. Interestingly, B cells may also be depleted if AA patients received ibrutinib. It remains to be determined whether this suppression enhances therapeutic efficacy in AA patients compared to the mouse model or leads to more complex effects that require further investigation. Future research should focus on developing more diverse human models. Additionally, large-scale, multicenter clinical trials are essential to assess the potential of ITK inhibitors as targets for combination therapy in AA.

In conclusion, ITK activation is highly upregulated in aplastic anemia; blocking ITK kinase activity could alleviated bone marrow hypoplasia, ameliorated cytopenia and prolong survival in murine AA model. ITK inhibitor orchestrates T cell quantity and effector function by mitigating T cell infiltration and prohibiting the secretion of pivotal inflammatory cytokines. Our data imply that ibrutinib, as an ITK inhibitor, may largely increase the accessibility of new target treatment strategy for immune aplastic anemia into clinical practice, providing promise and novel insight to immune bone marrow failure and other autoimmune diseases.

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Author Contributions WL, YL, LZ and RL designed and performed most of the experiments, wrote and revised the paper. QL, JM, CQ, HL, KH, QL, and YS, LT, HP, ZG, WL, and JZ assisted with experiments and data analysis. FY, LT, TX, SG, HP, ZG, JZ, FL, and MG contributed to the research design and paper discussion. JS, YC and WY conceived the project, supervised the research, and revised the paper, and gave the final approval of manuscript.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interests The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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