

Effect of iNKT Cell Deficiency on Pulmonary Fibrosis Formation in Mice

Efecto de la Deficiencia de Células iNKT en la Formación de Fibrosis Pulmonar en Ratones

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SUMMARY: This study investigates the role of invariant natural killer T (iNKT) cells in pulmonary fibrosis using C57BL/6 wild-type (WT) and Ja18^{-/-} knockout (KO) mice. KO mice exhibited accelerated fibrosis progression, marked by significant weight loss, elevated lung wet weight, and increased hydroxyproline content compared to WT mice. Histopathological analysis revealed earlier inflammatory cell infiltration (day 14 vs. later in WT) and pronounced collagen deposition in KO mice, alongside upregulated fibrosis-related biomarkers (TGF- β 1 and α -SMA) via Western blot. Flow cytometry demonstrated complete iNKT cell absence in KO mice and reduced total macrophages, with skewed M1/M2 macrophage polarization. These findings suggest iNKT cell deficiency exacerbates pulmonary fibrosis by disrupting macrophage homeostasis. The study proposes that iNKT cells modulate fibrosis through macrophage regulation, potentially influencing pro-fibrotic cytokine production and collagen synthesis. This highlights iNKT cells as critical regulators in idiopathic pulmonary fibrosis (IPF) pathogenesis and positions iNKT-based immunotherapy as a novel therapeutic strategy to restore immune balance and mitigate fibrotic progression. Further research should explore mechanistic links between iNKT cells, macrophage subsets, and downstream fibrotic pathways to validate these targets for clinical translation in IPF treatment.

KEY WORDS: Pulmonary fibrosis; Natural killer T-Cells; Transforming growth factor Beta1; Immunotherapy.

INTRODUCTION

The most common disease within interstitial lung diseases (ILD) is idiopathic pulmonary fibrosis (IPF), which is a chronic fibrotic process of lung tissue associated with remodeling due to irregular deposition of collagen and extracellular matrix (Mei *et al.*, 2021). This ultimately progresses to respiratory failure, with a high mortality rate (Spagnolo *et al.*, 2021). The etiology of IPF remains unclear, and it typically affects individuals between 70 and 75 years old, with both intrinsic and extrinsic risk factors potentially involved in disease progression (Bartold *et al.*, 2024). However, the exact triggering mechanisms are still undefined, and the 5-year survival rate for IPF patients is comparable to that of cancer patients (Song *et al.*, 2021). Currently, clinical treatment options for IPF are limited; nintedanib and pirfenidone are the only drugs approved by the FDA for IPF treatment (Alsomali *et al.*, 2023). Despite this, the prognosis for IPF remains poor, as these drugs only slow disease progression (Finnerty *et al.*, 2021). Thus, finding new therapeutic approaches is urgently needed. iNKT cell immunotherapy has shown certain therapeutic effects in various cancers, systemic sclerosis, kidney fibrosis, and

liver fibrosis, but there is a lack of evidence in pulmonary fibrosis-related studies (Li *et al.*, 2021; Liu *et al.*, 2022; Gu *et al.*, 2022; Yasuma *et al.*, 2024).

At present, many studies focus on the regulation of the immune microenvironment during disease development (Lao *et al.*, 2022). Natural killer T (NKT) cells have a specific subset, known as invariant natural killer T (iNKT) cells (Pellicci *et al.*, 2020), which play a key role in chronic inflammation and fibrosis in lung and liver diseases. Activation of iNKT cells is considered an important upstream event in chronic lung inflammation and fibrosis formation (Park *et al.*, 2009; Byrne *et al.*, 2024). iNKT cells are regarded as a type of innate T lymphocyte population, characterized by both innate and adaptive immune functions, bridging these two immune responses. They can initiate or suppress immune reactions based on the environment (Gapin, 2010). iNKT cells reside within tissues and play a critical role in the homeostasis and immunity of lung tissue. They patrol the vasculature and stromal tissue of the lungs until an infection in the lung tissue triggers danger signals, causing

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iNKT cells to migrate to damaged areas with glycolipid antigens and induce early host defense mechanisms (Wang *et al.*, 2024). This study investigates the role of invariant natural killer T (iNKT) cells in pulmonary fibrosis using C57BL/6 wild-type (WT) and Ja18^{-/-} knockout (KO) mice.

MATERIAL AND METHOD

Animal Model Establishment

Experimental Animals: C57BL/6J wild-type mice and Ja18^{-/-} knockout mice, aged 6-8 weeks, weight 22 ± 2 g, male, sourced from HuaFuKang Biotechnology Co., Ltd. **Grouping and Modeling:** Mice were randomly divided into three groups:

Control group (WT-NS): Received 20 μ l of normal saline via intratracheal instillation.

Pulmonary fibrosis groups (WT-IPF, KO-IPF): Received 20 μ l of 3 mg/kg Bleomycin Sulfate (from Hebei RuiPaTe Biotechnology Co., Ltd.) via intratracheal instillation (Finnerty *et al.*, 2021).

Histological Analysis

Tissue Processing: Mouse lung tissues were dehydrated using an automatic tissue dehydrator, embedded in paraffin, and sectioned into 4 μ m thick slices using a fully automated microtome. **H&E Staining:** After baking, deparaffinization, and rehydration, sections were stained with Hematoxylin and Eosin (H&E) (Vector; H-3502). Following dehydration, sections were mounted with neutral balsam (Beyotime; C0173-100 ml). **Masson Staining:** Lung tissue sections underwent Masson staining (Solarbio; 637552862540SD) after baking, deparaffinization, and rehydration (Liu *et al.*, 2022).

Spectrophotometric Assay of Lung Tissue Hydroxyproline Content

Hydroxyproline content in lung tissue was determined using a spectrophotometric assay kit (NJJC BIO; A030-2-1). Briefly, 30-100 mg of lung tissue was alkaline-hydrolyzed in a 95°C water bath for 20 min, and the pH was adjusted to 6.0-6.8. Activated charcoal was added, and the mixture was centrifuged. Then, 1 ml of sample supernatant, double-distilled water, and standard solution (5 mg/ml) were mixed with Reagent 1, Reagent 2, and Reagent 3, respectively, according to the manufacturer's instructions. After centrifugation, an aliquot of the supernatant was taken for measurement. The absorbance at 550 nm was read using a microplate reader. The

hydroxyproline concentration in the lung tissue was calculated using the formula: $(A - \text{sample} - A - \text{blank}) / (A - \text{standard} - A - \text{blank}) * C - \text{standard} * V / M - \text{sample}$ (Alsomali *et al.*, 2023).

Flow Cytometry assay

iNKT Cell Frequency and Subset Analysis: Single-cell suspensions were prepared from fresh mouse lung tissue by grinding. Red blood cells were lysed at room temperature for 7 min. Cells were then stained for flow cytometry using monoclonal antibodies against Anti-TCR β (BioLegend; 351706) and α -GalCer/CD1d tetramer (BioLegend; 140508). After surface staining, cells were fixed with 4 % paraformaldehyde for 45 min. Subsequently, intracellular staining was performed using Anti-T-bet (BioLegend; 644817) and Anti-PLZF (BioLegend; 625602) antibodies at 4 °C in the dark for 30 min before acquisition on the flow cytometer. **Macrophage Immunophenotyping:** Lung single-cell suspensions were prepared as above. Cells were stained with Anti-F4/80 (BioLegend; Alexa Fluor® 647 anti-mouse F4/80 Recombinant), Anti-CD11b (BioLegend; PE/Cyanine7 anti-mouse/human CD11b), and Anti-CD11c (BioLegend; APC anti-mouse CD11c) monoclonal antibodies. After incubation in the dark for 30 min, cells were washed twice with PBS and resuspended in 500 μ L PBS. Following fixation and permeabilization using a commercial kit (Biosharp; BL1133A), intracellular staining was performed with Anti-CD206 (BioLegend; Alexa Fluor® 700 anti-mouse CD206 (MMR)) by incubating in the dark for 30 min. Cells were washed twice, resuspended in 500 μ L PBS, and acquired on the flow cytometer (Li *et al.*, 2021).

Western Blot assay

Protein Extraction from Mouse Lung Tissue: Mouse lung tissues were pulverized under liquid nitrogen and thoroughly lysed with RIPA lysis buffer (Beyotime; S3062-100 ml) for 20 min. After centrifugation, the supernatant was transferred to a new EP tube. 5X loading buffer (HUAYUN; HY P0015) was added, and samples were denatured using a heating block. **Electrophoresis and Detection:** Proteins were separated by SDS-PAGE, transferred to membranes, and blocked. Membranes were incubated overnight at 4 °C with specific primary antibodies: β -Actin (CST; 19245), α -SMA (Affinity Biosciences; BF9212-50ul), TGF- β (CST; 79424S). This was followed by incubation with HRP-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies (ZSBIo). Protein bands were visualized using an enhanced chemiluminescence detection system in a gel imaging system. Band intensities were quantified using ImageJ 1.52 software (Yasuma *et al.*, 2024).

Statistical Analysis

FlowJo software was used for the analysis of FACS data. Statistical analysis was conducted using GraphPad Prism software. For a single comparison between two groups, unpaired t-tests were used. Data are presented as the mean $\pm$ SEM or mean $\pm$ SD. Spearman correlation analysis was used to evaluate the correlation.

RESULTS

Establishment of Mouse Model: Mouse Body Weight Curve, Lung Coefficient, and Hydroxyproline Measurement

iNKT cell deficiency in TraiJ18 knockout mice has a significant effect on pulmonary fibrosis model formation. We observed the status and body weight changes in mice over 28 days post-modeling. No significant changes in status or weight were seen in the first two days, but by day 4, mice in the IPF modeling group began eating less and showed a noticeable weight decrease compared to the saline (NS) control group (Fig. 1A). Compared to WT mice, the TraiJ18 knockout mice showed a more pronounced weight loss in the pulmonary fibrosis model (Fig. 1D), as well as reduced food intake and activity, with a dull, yellowish coat. Lung wet weight and hydroxyproline content in lung tissue were measured post-euthanasia on days 7, 14, 21, and 28, showing significant increases in both lung fibrosis model

mice and KO mice compared to WT mice (Figs. 1B, C, E, F). The pulmonary fibrosis model was established by intratracheal administration of bleomycin in mice, which had minimal impact on other organs but did affect the mice's quality of life.

Comparison of Various Factors in Pulmonary Fibrosis Formation in WT and KO Mice: WB, Pathology, and Masson Staining

HE staining of mouse lung tissue shows increased consolidation and thickening of the alveolar septa, with increased inflammatory cell infiltration and fibroblast proliferation over time. In the KO-IPF group, consolidation occurred earlier than in the WT-IPF group (Fig. 1A). Masson staining revealed that pulmonary fibrosis began forming by day 14, with higher collagen content in KO mice (Fig. 2C). Differences in collagen content between groups were more pronounced on days 21 and 28 (Fig. 2D). Western blot analysis of α -SMA and TGF- β 1 protein expression in the lung tissue of both groups confirmed that KO mice had significantly higher levels of these proteins compared to WT mice, especially in the lung tissue of mice 28 days post-modeling (Figs. 2B, E, F). These findings suggest that iNKT cell deficiency significantly influences pulmonary fibrosis formation in mice.

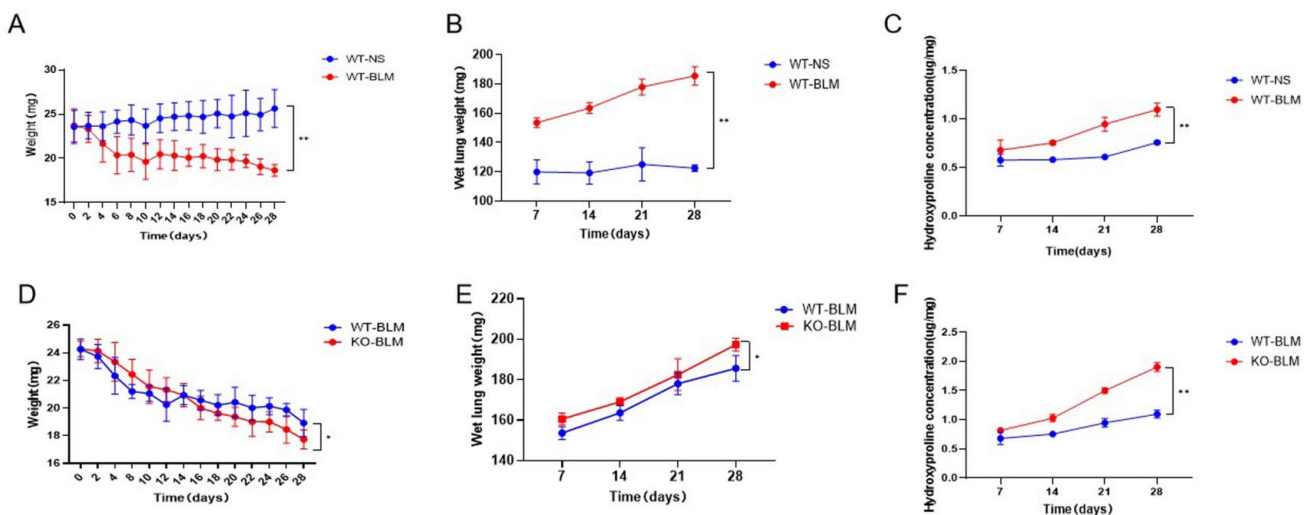


Fig. 1. Figures A and D show the 28-day body weight changes in mice. Figure A compares the NS and IPF groups in WT mice, while Figure D compares the IPF groups in WT and KO mice. Figures B and E illustrate changes in lung wet weight post-euthanasia on days 7, 14, 21, and 28, with Figure B comparing the NS and IPF groups in WT mice and Figure E comparing WT and KO mice in the IPF group. Figures C and F show changes in hydroxyproline content in lung tissue post-euthanasia on days 7, 14, 21, and 28, with Figure C comparing WT mice in the NS and IPF groups and Figure F comparing WT and KO mice in the IPF group.

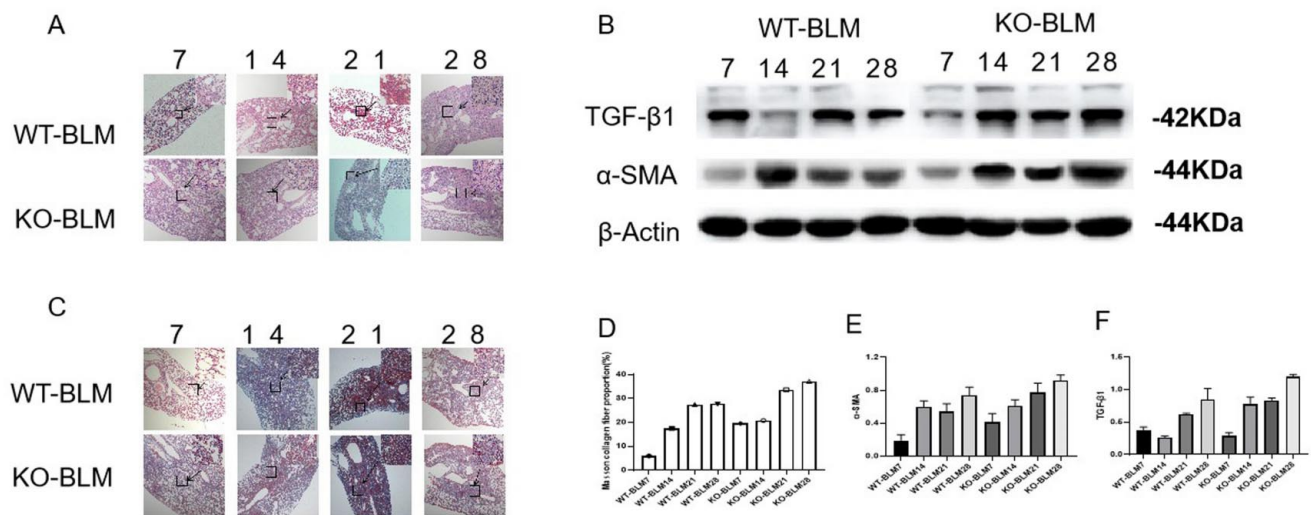


Fig. 2. Figure A shows HE-stained pathological images of mouse lung tissue on days 7, 14, 21, and 28, all taken with a 5X objective lens. Figure B shows the expression levels of α-SMA and TGF-β1 proteins in lung tissue from the WT-IPF and KO-IPF groups on days 7, 14, 21, and 28. Figure C shows Masson trichrome-stained images of mouse lung tissue on days 7, 14, 21, and 28, all taken with a 5X objective lens. Figure D shows a quantification of collagen fibers in Masson-stained lung tissue. Figures E and F show bar graphs quantifying α-SMA and TGF-β1 protein in lung tissue, respectively.

Analysis of iNKT and Its Subtype Expression, and Macrophage and Its Subtype Expression in KO Mice

Following modeling, total macrophages in mouse lung tissue show reactive proliferation on days 7, 14, and 21, but decrease by day 28 (Fig. 3). KO mice have a lower proportion of total macrophages compared to WT mice, possibly due to iNKT cell deficiency in KO mice, which affects macrophage regulation. KO mice also show an overall lower proportion of iNKT cells than WT mice, with no significant change on days 7, 14, and 21. The

proportion of M1 and M2 macrophages is significantly lower in KO mice than in WT mice. However, after pulmonary fibrosis forms by day 28, the M1/M2 ratio is higher in KO mice than in WT mice. The iNKT1/iNKT2 cell ratio is lower in KO mice than in WT mice but becomes higher in KO mice after day 28, correlating positively with the M1/M2 macrophage ratio. This suggests that the absence of iNKT cells in KO mice leads to a lack of regulation over macrophages, resulting in marked changes in the M1/M2 macrophage ratio during pulmonary fibrosis formation.

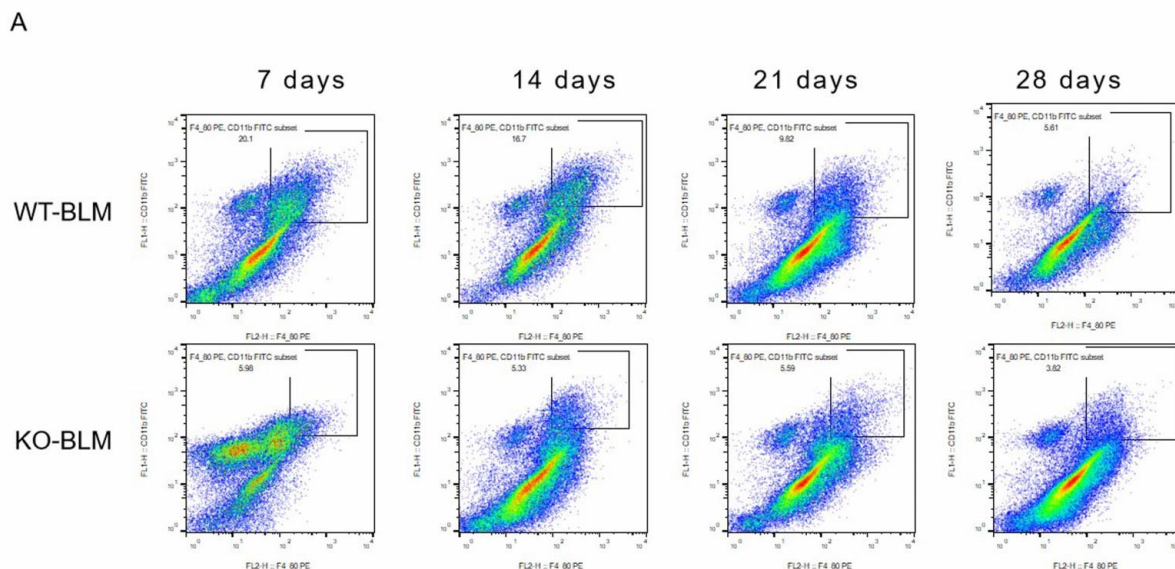


Fig. 3A. Shows the total proportion of macrophages in lung tissue of WT and KO model mice on days 7, 14, 21, and 28 via flow cytometry.

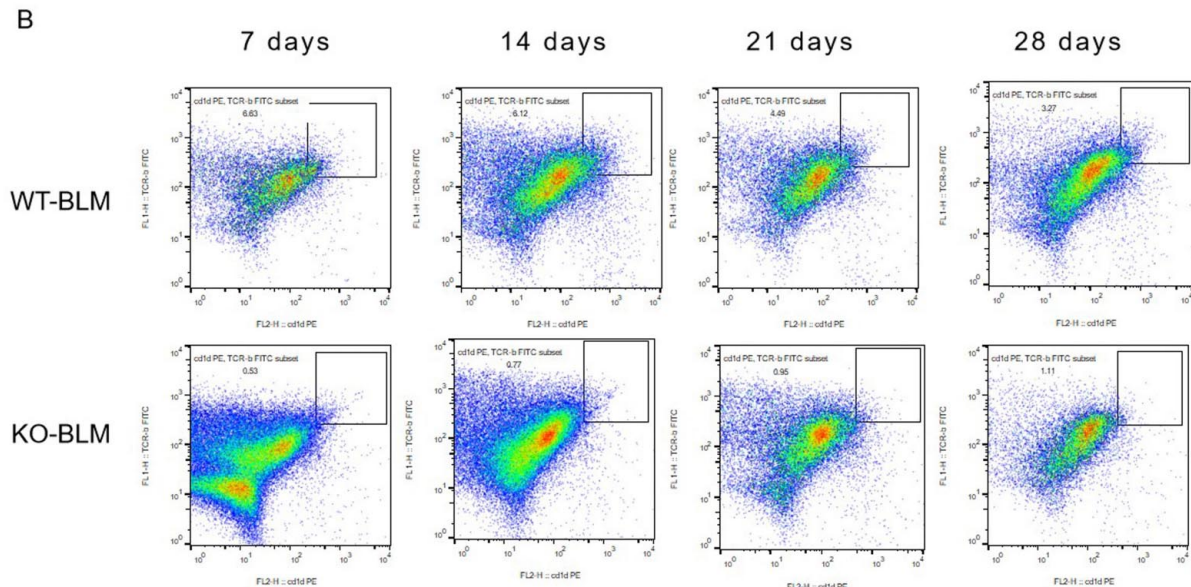


Fig. 3B. Shows the total proportion of iNKT cells in lung tissue of WT and KO model mice on days 7, 14, 21, and 28.

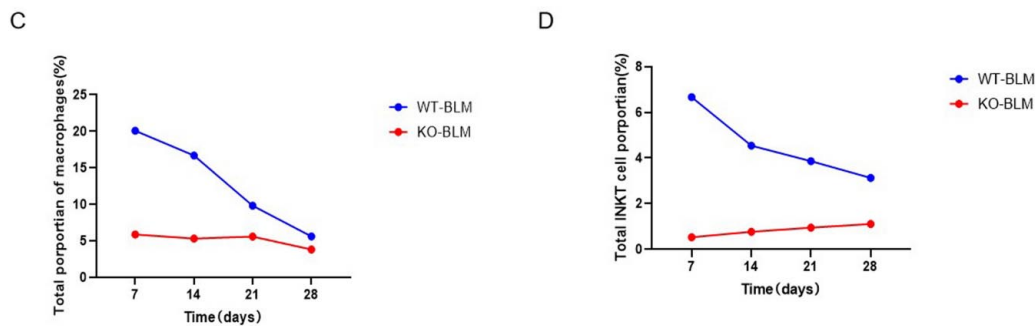


Fig. 3C and 3D. Show the trend of total macrophages and iNKT cells in lung tissue of WT and KO model mice on days 7, 14, 21, and 28.

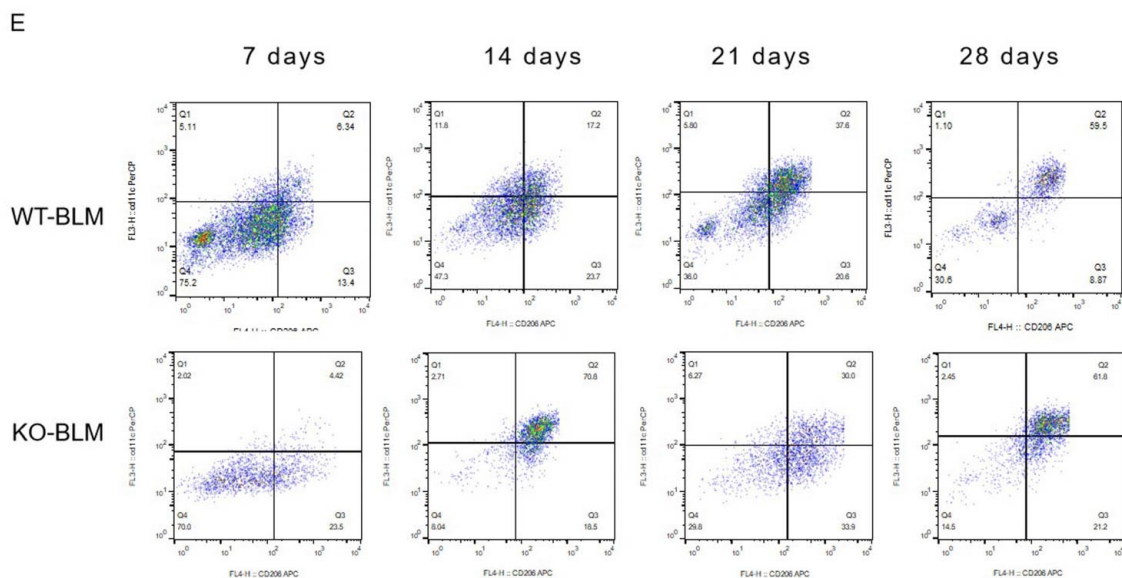


Fig 3E. Shows the distribution of M1 and M2 macrophages in lung tissue of WT and KO model mice on days 7, 14, 21, and 28.

F

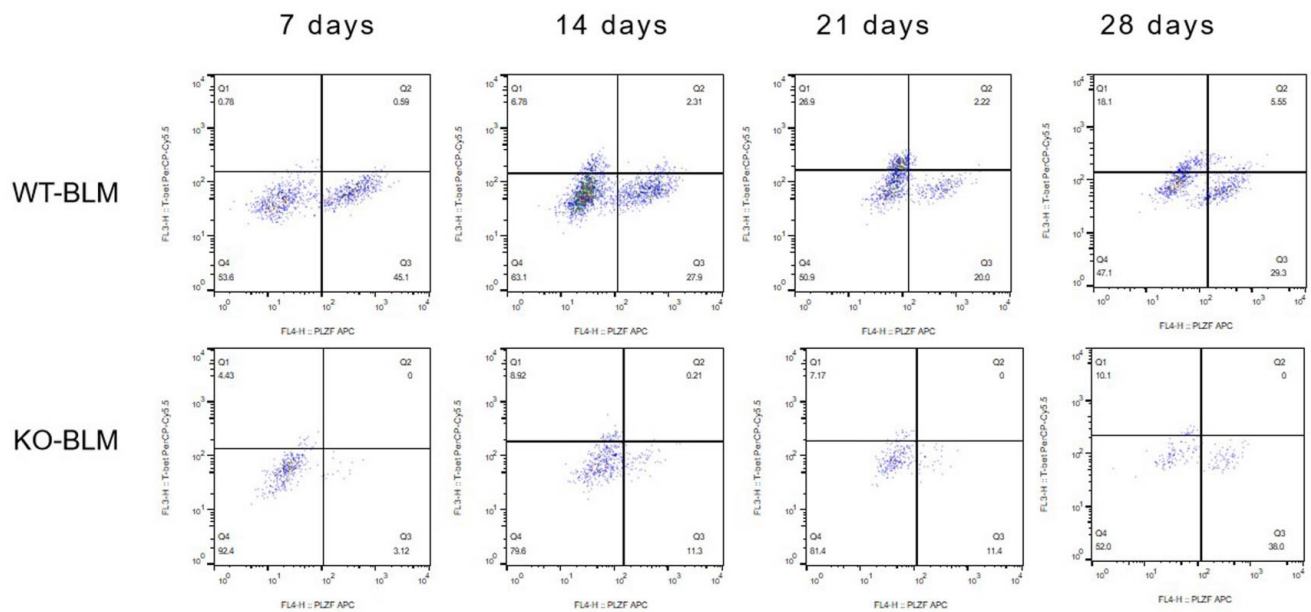
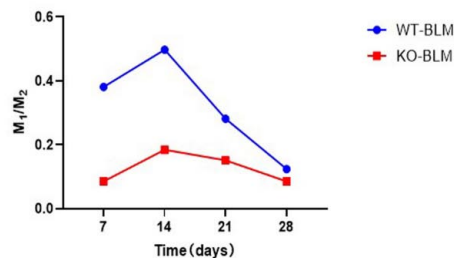


Fig. 3F. Shows the distribution of iNKT1 and iNKT2 cells in lung tissue of WT and KO model mice on days 7, 14, 21, and 28.

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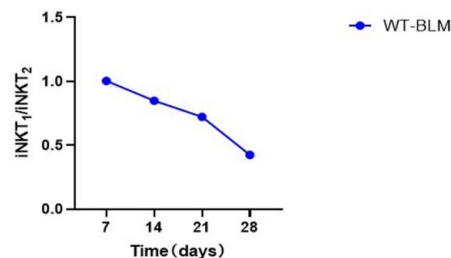


Fig. 3G and 3H. Display trend graphs for the M1/M2 and iNKT1/iNKT2 ratios in WT and KO model mice on days 7, 14, 21, and 28.

DISCUSSION

iNKT cell immunotherapy has emerged as a new therapeutic approach with substantial potential for intervention. Further research is still needed to fully understand the specific mechanisms through which iNKT cells affect fibrosis formation and their interaction with other immune cells. Through this study, we provide evidence that immune modulation via iNKT cell activation may benefit pulmonary fibrosis regulation. To date, few studies have examined the role of iNKT cells in lung diseases. However, many animal studies have investigated fibrosis in other organs, demonstrating the importance of NKT cells in controlling collagen deposition. Administering NKT cell agonists in animal models has shown beneficial effects on pulmonary fibrosis (PF) in mice (Chen *et al.*, 2020; Zhang

& Zhang, 2020; Jandl *et al.*, 2022). Nevertheless, it remains unknown whether iNKT cell activation is suitable for treating pulmonary fibrosis.

In our experiment, we intratracheally administered bleomycin to wild-type C57BL/6 mice and Ja18 *-/-* gene knockout C57BL/6 mice. This modeling method is commonly used for inducing pulmonary fibrosis in mice, and most studies on animal models of pulmonary fibrosis adopt this approach. However, some studies have found that high concentrations of bleomycin cause severe acute damage to lung tissue, and even wild-type B6 mice cannot tolerate the acute pulmonary edema induced by drug stimulation (Liu *et al.*, 2017; Ayilya *et al.*, 2023). Therefore, administering

different doses of bleomycin under the same experimental conditions could significantly affect the mortality rate of mice. In our study, some iNKT-deficient mice died during the early stage of fibrosis induction before day 7 post-intratracheal injection, with a sharp and continuous decline in body weight visible on day 4, likely due to acute pulmonary edema and lung damage rather than fibrosis-induced death. This suggests that the bleomycin dose needs careful control during modeling, as too high a dose may cause early death due to acute lung injury, whereas too low a dose might make fibrosis formation difficult, prolonging the experiment and risking failure.

We used iNKT-deficient mice to evaluate the effect of iNKT deficiency on pulmonary fibrosis by observing fibrosis status post-bleomycin modeling. We found that fibrosis was more severe in iNKT-deficient mice compared to wild-type mice, consistent with findings by Bergantini *et al.* (2019). Additionally, iNKT-deficient mice had significantly higher lung wet weight and hydroxyproline content than wild-type mice, with more pronounced histopathological differences, as seen in fibrosis model validation studies (Hao *et al.*, 2023; Shao *et al.*, 2024). Studies have shown that CD1d-recognized NKT cell adoptive transfer in iNKT-deficient fibrosis model mice can inhibit TGF- β 1 production (Kim *et al.*, 2005). This result aligns with our findings, where we observed increased α -SMA and TGF- β 1 levels in lung tissue in iNKT-deficient mice following fibrosis formation. This suggests that iNKT cells influence TGF- β 1 production and fibrosis formation. Studies have shown that iNKT cells affect the secretion of growth factors such as Pdgf, Igf-1, Tgf β 1, Vegf, and Timp1 in liver fibrosis, impacting liver fibrosis formation (Cheng *et al.*, 2023). The mechanism of pulmonary fibrosis may be similar, necessitating further research.

iNKT cells are regulatory immune cells central to immune responses, and macrophage regulation is crucial in pulmonary fibrosis formation. To investigate the relationship between iNKT cells and macrophages and their subtype distribution, we conducted flow cytometry, which revealed that macrophage levels were lower in iNKT-deficient mice compared to WT mice. In the WT model, the M1/M2 macrophage ratio showed a decreasing trend, but there was no significant change in this ratio in the iNKT-deficient model, suggesting that iNKT cell levels may influence macrophage production and polarization. Research indicates that iNKT cells primarily exist in two subtypes, iNKT1 and iNKT2 cells, with opposing roles in controlling inflammation, where iNKT1 cells mainly produce pro-inflammatory factors (Nilsson *et al.*, 2020). iNKT cells can activate M2 macrophages via IL4 signaling, promoting the transformation of fibroblasts into

myofibroblasts and influencing pulmonary fibrosis (Liu *et al.*, 2021; Calamita *et al.*, 2024). iNKT cell regulation of macrophages may play a significant role in pulmonary fibrosis formation. In our study, we found that iNKT cell deficiency exacerbates pulmonary fibrosis in mice, and the underlying mechanism may be related to the impact of iNKT cell deficiency on macrophage regulation, warranting further investigation.

CONCLUSION

This study demonstrates that iNKT cell deficiency exacerbates pulmonary fibrosis progression in Ja18/- knockout mice, as evidenced by accelerated weight loss, elevated lung hydroxyproline content, and pronounced collagen deposition compared to wild-type mice. Histopathological and biomarker analyses (TGF- β 1, α -SMA) confirmed enhanced fibrotic activity in iNKT-deficient mice, while flow cytometry revealed disrupted macrophage homeostasis, characterized by reduced total macrophages and skewed M1/M2 polarization. These findings underscore iNKT cells as critical modulators of pulmonary fibrosis, likely through their regulatory influence on macrophage dynamics and pro-fibrotic cytokine production. The absence of iNKT cells appears to impair immune balance, promoting unchecked collagen synthesis and tissue remodeling. This positions iNKT-based immunotherapy as a promising therapeutic strategy to restore macrophage equilibrium and mitigate fibrotic pathways in IPF. Future research should elucidate precise mechanistic links between iNKT subsets, macrophage polarization, and downstream fibrotic signaling to advance clinical translation. Targeting iNKT-macrophage crosstalk may offer novel avenues for halting or reversing IPF progression.

Conflict of interest. The authors declare that there is no conflict of interest.

Statement of Informed Consent and Ethical Approval.

This study followed ethical standards for the use of animal subjects, ensuring that animal welfare was prioritized by minimizing any pain, discomfort, or distress. Ethical approval was obtained from the Hebei University for conducting the animal research.

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RESUMEN: Este estudio investiga el papel de las células T asesinas naturales invariantes (iNKT) en la fibrosis

pulmonar utilizando ratones C57BL/6 de tipo salvaje (WT) y ratones knockout Ja18^{-/-} (KO). Los ratones KO mostraron una progresión acelerada de la fibrosis, caracterizada por una pérdida de peso significativa, un mayor peso húmedo pulmonar y un mayor contenido de hidroxiprolina en comparación con los ratones WT. El análisis histopatológico reveló una infiltración más temprana de células inflamatorias (día 14 frente a un día posterior en los ratones WT) y una marcada deposición de colágeno en los ratones KO, junto con una mayor expresión de biomarcadores relacionados con la fibrosis (TGF- β 1 y α -SMA) detectada mediante Western blot. La citometría de flujo demostró la ausencia total de células iNKT en ratones KO y una reducción en el número total de macrófagos, con una polarización M1/M2 alterada. Estos hallazgos sugieren que la deficiencia de células iNKT exacerba la fibrosis pulmonar al alterar la homeostasis de los macrófagos. El estudio propone que las células iNKT modulan la fibrosis mediante la regulación de los macrófagos, influyendo potencialmente en la producción de citocinas profibróticas y la síntesis de colágeno. Esto resalta el papel fundamental de las células iNKT como reguladoras en la patogénesis de la fibrosis pulmonar idiopática (FPI) y posiciona la inmunoterapia basada en iNKT como una estrategia terapéutica novedosa para restaurar el equilibrio inmunitario y mitigar la progresión fibrótica. Se necesitan más investigaciones para explorar los vínculos mecanísticos entre las células iNKT, los subtipos de macrófagos y las vías fibróticas posteriores, con el fin de validar estas dianas para su aplicación clínica en el tratamiento de la FPI.

PALABRAS CLAVE: Fibrosis pulmonar; Células T; Factor de crecimiento transformante beta 1; Inmunoterapia.

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