Crystalline silica-induced recruitment and immuno-imbalance of CD4 + Tissue Resident Memory T cells promote the progression of silicosis.

Chao Li[1], Yichuan You[1], Xiulin Wu[1], Yangyang He[1], Haoyang Yuan[1], Yinghui Chen[1], Sisi Wang[1], Hui Min[1], and Jie Chen[1]

[1]China Medical University

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Abstract

Background: Occupational crystalline silica (CS) particle exposure leads to silicosis. The burden of CS-associated disease remains high, and treatment options are limited due to vague mechanisms. CD4 $^{+}$ tissue-resident memory T cells (T $_{RM}$) accumulate in the lung responded to CS particles, mediating the pathogenesis of silicosis. Methods: Based on silicosis murine model by single intratracheal instillation of CS suspension, we further employ adoptive transfer, FTY720 treatment, and parabiosis murine model to explore their source. After defining T $_{RM}$ cell subsets by CD103 and CD69, we intervene CD103 $^{+}$subset and block IL-7 signaling to alleviate silicosis. Results: The CD4 $^{+}$ T $_{RM}$ cells are derived from peripheral lymphocyte recruitment and *in situ* expansion. Specifically, T $_{RM}$-Treg cells depend more on circulating T cell replenishment. The cell retention markers CD103 and CD69 can divide the T $_{RM}$ cells into effector and regulatory subsets. However, targeting CD103 $^{+}$ T $_{RM}$-Treg cells do not mitigate disease phenotype since the T $_{RM}$ subsets exerted immunosuppressive but not pro-fibrotic roles. We further dissect that IL-7 signaling promotes the progression of silicosis by tuning the maintenance of T $_{RM}$-effector T cells. Conclusion: Our findings highlight the distinct role of CD4 $^{+}$ T $_{RM}$ cells in mediating CS-induced fibrosis and provide potential therapeutic strategies for silicosis.

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Yichuan You[1, 2#], Xiulin Wu[1, 2#], Yangyang He[1, 2], Haoyang Yuan[1, 2], Yinghui Chen[1, 2], Sisi Wang[1, 2], Hui Min[3], Jie Chen[1, 2*], Chao Li[1, 2, 4*].

1 Key Laboratory of Environmental Stress and Chronic Disease Control & Prevention (China Medical University), Ministry of Education, No.77 Puhe Road, Shenyang North New Area, Shenyang, 110122, Liaoning, PR China

2 Department of Occupational and Environmental Health, School of Public Health, China Medical University, No.77 Puhe Road, Shenyang North New Area, Shenyang, 110122, Liaoning, PR China

3 Department of Immunology, College of Basic Medical Sciences, China Medical University, No.77 Puhe Road, Shenyang North New Area, Shenyang, 110122, Liaoning, PR China

4 Lead contact.

# These authors contribute equally to this work.

* Corresponding author.

mail: lichao@cmu.edu.cn (C. Li); jchen@cmu.edu.cn (J. Chen)

Abstract

Background: Occupational crystalline silica (CS) particle exposure leads to silicosis. The burden of CS-associated disease remains high, and treatment options are limited due to vague mechanisms. $CD4^+$ tissue-resident memory T cells (T_{RM}) accumulate in the lung responded to CS particles, mediating the pathogenesis of silicosis.

Methods: Based on silicosis murine model by single intratracheal instillation of CS suspension, we further employ adoptive transfer, FTY720 treatment, and parabiosis murine model to explore their source. After defining T_{RM} cell subsets by CD103 and CD69, we intervene $CD103^+$subset and block IL-7 signaling to alleviate silicosis.

Results: The $CD4^+$ T_{RM} cells are derived from peripheral lymphocyte recruitment and *in situ*expansion. Specifically, T_{RM}-Treg cells depend more on circulating T cell replenishment. The cell retention markers CD103 and CD69 can divide the T_{RM} cells into effector and regulatory subsets. However, targeting $CD103^+T_{RM}$-Treg cells do not mitigate disease phenotype since the T_{RM} subsets exerted immunosuppressive but not pro-fibrotic roles. We further dissect that IL-7 signaling promotes the progression of silicosis by tuning the maintenance of T_{RM}-effector T cells.

Conclusion: Our findings highlight the distinct role of $CD4^+$ T_{RM} cells in mediating CS-induced fibrosis and provide potential therapeutic strategies for silicosis.

Keywords: crystalline silica; fibrosis; inflammation; silicosis; tissue-resident memory T cells

Introduction

Crystalline silica (CS) is a typical inorganic particle in natural and industrial settings. Environmental exposure to respirable CS leads to pneumoconiosis, characterized by chronic inflammation and progressive pulmonary fibrosis [1,2]. Though redoubled efforts were made to minimize CS exposure, stubbornly high morbidity and mortality of silicosis emphasized the hazardous burden of CS-related diseases [3,4]. The new emerging industries like sandblasting denim jeans and manufacturing of artificial stone benchtops reignited the emergence of silicosis around the world[5]. While silicosis is a preventable disease, unfortunately, patients continue to suffer from this progressive disease[2]. Interventions against silicosis progression are in high demand.

The inhaled CS particles deposited in the lung interstitium trigger inflammatory cascades involving innate and adaptive immune responses[6]. Although some early events, such as macrophages response to CS particles, in silicosis are clear, the following steps leading to fibrosis are less well-understood[7,8,9]. Different from the simple exposure-response relationship, adaptive immune response characterized as disorders of T lymphocytes orchestrate chronic inflammation and fibrogenesis[10]. $CD4^+$ helper T (Th) cells have been identified as vital players in fibrotic disorders, including silicosis [11]. The $CD4^+$ Th cells can be divided into pathogenic effector T cells (including Th1, Th2, Th17 cells) and immunosuppressive regulatory T cells (Tregs), whose fate was governed by transcriptional factors such as T-bet, GATA-3, ROR-γt, and FOXP3, respectively[12]. They altered tissue local microenvironment that orchestrates the remodeling of lung tissue[13]. Th cell polarization and functions of effector cytokines in silicosis were explored[14]. However, these research findings were largely based on the evidence of peripheral T cells. Whether these cells were circulating or residing in the lung tissue remained to be investigated.

T cell-mediated adaptive immune response characterized as immunological memory. Memory T cells (T_M) expressed memory-T-cell-associated molecule, CD44, and can be divided into distinctive subsets. Central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) were defined as two major subsets until tissue-resident memory T cells (T_{RM}) were discovered [15]. T_{CM} patrols the blood and secondary lymphoid organs, which is mediated by chemokine receptor CCR7 and CD62L, while T_{EM} lacks these molecules but expresses homing molecules, allowing them access to peripheral tissues [16]. The T_{CM} and T_{EM} cells are abundant in blood and lymphoid tissue, while the newly defined, T_{RM} cells, which are distinct from circulating memory cells that preferentially localize in epithelial barrier tissues are required for protection at mucosal sites,

including pulmonary respiratory tract, gastrointestinal tract, reproductive tract, and skin, in mice and humans [17,18].

T cells migrate to the damaged tissue, mature, and maintain in non-lymphoid tissues, exerting enhanced effector functions compared to their lymphoid tissue counterparts. They rapidly respond to the invading pathogen within peripheral tissues, providing first-time and robust protections upon cognate antigen stimulation [19]. Though have primarily been described as their protective functions, particularly in the context of pathogen infections, T_{RM}cells, especially $CD4^+$ T_{RM} cells, have also been reported to be pathogenic in chronic inflammatory settings, including atopic dermatitis, psoriasis, allergy asthma, inflammatory bowel disease (IBD) [16,20]. Although recent works explored the participation of $CD4^+$ T_{RM}cells in some pulmonary diseases, our knowledge about the contribution of T_{RM} cells in silicosis is limited[21,22,23].

In the pathogenesis of silicosis, the inhaled CS particles can be engulfed by macrophages but cannot be cleared, which results in the death of macrophages and the re-release of CS particles, which leads to antigens repeatedly, locally re-initiated chronic inflammation and fibroblast activation [24]. Additionally, our previous study demonstrated the cytokine-producing $CD4^+$ T cells in the silicotic lung manifested T_M cell characterization [25]. Given the situation of repeated CS stimulation and the immunologic memory function of T_{RM} cells, we hypothesized that $CD4^+$ T_{RM} cells are involved in the pathogenesis of silicosis.

To this end, we utilized murine model of silicosis delineating the effects of CS particles on $CD4^+$ T_{RM}cells and further explored its pathogenic role in the progression of silicosis. Our results proved the source and function of $CD4^+T_{RM}$ cells in the pathogenesis of silicosis. Significantly, targeting the maintenance of functional distinct $CD4^+T_{RM}$ cells in the lung exerted protective roles against silicosis.

Methods

Mice. C57BL/6JGpt mice (Strain NO. N000013), CD45.1 congenic mice (C57BL/6JGpt-$Ptprc^{em1Cin(p.K302E)}$/Gpt, straining No. T054816), $Rag1^{-/-}$ mice (C57BL/6JGpt-$Rag1^{em1Cd3259}$/Gpt, Strain NO. T004753) involved in this study were purchased from GemPharmatech (Nanjing, China). $FOXP3^{YFP}$ mice (B6.129(Cg)-*Foxp3* $^{tm4(YFP/icre)\ Ayr}$/J, Strain #016959) were purchased from Jackson laboratory. All mice were bred and maintained under specific pathogen-free conditions in the animal care facility at China Medical University. Sex- and age-matched mice were used. All mice were used at the age of 7–8 weeks. Littermate mice were selected if possible.

Crystalline Silica particles. Crystalline silica (CS) particles were purchased from the U.S. Silica Company (Frederick, MD, USA). The characteristics of crystalline silica particles are described in detail[55]. Briefly, the size distribution of CS particles is as follows: 97% $<$ 5 μm in diameter, 80% $<$ 3 μm in diameter, median diameter of 1.4 μm. The particles were suspended in sterile saline after drying. The suspension was autoclaved and sonicated for 10 min prior to use.

The murine model of silicosis establishment. The mouse model of silicosis was established according to our previously published method[55,56,57]. In brief, the mouse was treated with 3.0 mg CS in 50 μL saline solution by intratracheal (*i.t.*) instillation after anesthetization with pentobarbital sodium (30 mg/kg,*i.p.* injection, Sigma). An equal amount of sterile saline was applied to the control groups. 56 days post CS instillation was regarded as the stage of fibrogenesis.

Single-cell suspension preparation. Isolation of lymphocytes from mouse lung and spleen was previously described[25]. In short, the lung was minced and digested with a digestion solution containing type I collagenase (2 mg/mL), DNase I (100U/mL), and complete media (DMEM plus 4% BSA). The suspension was incubated on a rocker at 37°C. Collagen-digested lungs were dispersed. Red blood cells were lysed using ACK lysis buffer. Leukocytes were enriched from the cell digestion using Percoll gradients (80%/40%) (Cytiva). Spleen cells were prepared by pressing the tissues through 70 μm cell strainers using the end of a sterile plunger of a 5 mL syringe. Single-cell suspension was prepared for subsequent flow cytometry staining or cell sorting.

Flow cytometry analysis. Flow cytometry (FC) was performed according to the guidance [58]. Live/Dead

cell viability dye Aqua (Thermo Fisher) was used to exclude the influence of dead cells. Fluorescently labeled antibodies to cell surface antigens were applied and incubated at 4°C for 30 min. For the intracellular transcriptional factor staining, cells were first stained for cell-surface markers, and fixed with 4% paraformaldehyde, permeabilized with the FOXP3 transcription factor staining buffer set (eBioscience), then stained with indicated antibodies to transcriptional factors at 4°C overnight. For the cytokine staining, leukocyte stimulation cocktail containing PMA, ionomycin, brefeldin A, and monensin (Invitrogen) was used to stimulate cells for 4 h before staining. The antibodies detecting transcriptional factors or cytokines were incubated at 4°C overnight. FC analysis was performed on BD FACS Celesta (BD Bioscience, San Jose, CA, USA). FACS data were analyzed with Flowjo 10.6 software. The gating strategy is shown in Fig S1A. All involved anti-mouse antibodies are listed in Table S1.

Intravascular immune cell labeling. To discriminate tissue-resident cells from circulating cells, we carried out a well-established intravenous (*i.v.*) staining approach[26], illuminated in Fig 1A. Mice were *i.v.*injected with 1.5 μg of CD45-APC-Cy7 antibody (30-F11, Biolegend) diluted in 150 μL sterile saline, 4 min before sampling.

Magnetic-activated cell sorting and adoptive transfer.$CD4^+$ αβT cells were purified from the tissues of lung and spleen by the magnetic-activated cell sorting (MACS) method with a $CD4^+$ T cells Isolation Kit (Miltenyi Biotec, catalog no. 130-104-454). The purity of the isolated cells was greater than 95%. The MACS-purified $CD4^+$ T cells or PBS were*i.v.* transferred into littermate $Rag1^{-/-}$ mice (3×10^5 cells per mouse) after that CS was instilled. One week after the CS treatment, the transferred mice were analyzed. The donor and recipient mice were sex matched.

Flow cytometric cell sorting and adoptive transfer. $FOXP3^{YFP+}$ mice were treated by CS particles. The activated $CD103^+$ Tregs ($CD4^+CD44^+FOXP3^{YFP+}CD103^+$) were sorted from the silicotic $FOXP3^{YFP+}$ mice by flow cytometric cell sorting (FACS Aria II instrument, BD Biosciences). The gating strategy shown in Fig 5E. The sorted Tregs (1×10^4 cells each mouse) or PBS were *i.v.*transferred into littermate C57BL/6J mice that were treated with CS 4 weeks before transfer. Two weeks later, the reconstituted mice were analyzed. The donor and recipient mice were sex matched.

Parabiosis model. The experiment was adopted as the published protocols[59]. Briefly, sex- and age-matched congenic mice (CD45.1/2 and CD45.1/1) were prepared. Their dorsal and ventral skin were approximated with sutures after parabiosis surgery. The conjoined mice recovered for 14 days to establish new circulation. Then, CD45.1/1 mice were CS administered and sacrificed 7 days later.

RNA extraction and quantitative PCR (qPCR) analysis. RNA was extracted from lung tissue by RNA isolater Total RNA Extraction Reagent (R401, Vazyme Biotech), then reversely transcribed into cDNA by HisScript III RT SuperMix (R323, Vazyme Biotech). The ChamQ Universal SYBR qPCR Master Mix (Q711, Vazyme Biotech) was used for the amplification of RNA samples from each group and gene expression were analyzed via real-time PCR assay (7500 software, Applied-biosystem). GAPDH was used as the internal control for determining $2^{-\Delta\Delta^{\sim}T}$ values. All primers were synthesized by Sangon Biotech and sequences were listed in Table S2.

FTY720 treatment. FTY720, an agonist of sphingosine-1-phosphate receptor 1 (S1PR1), was used to block lymphoid cell migration. FTY720 (20 μg in vehicle every time) or vehicle was *i.p.* administered daily (Fig 3A). Littermate mice were used in each independent experiment. The mice were divided into three groups: (A) CS-treated group, in which animals received CS instillation and vehicle; (B) 8 weeks FTY720 treatment (0 week to 8 week), in which the mice received CS instillation combined with FTY720 i.p. every day from 0 week to 8 week; (C) 28 days FTY720 treatment (4 week to 8 week), in which the mice received CS instillation combined with FTY720 i.p. every day from 4 week to 8 week.

*Neutralizing antibody treatments. Anti-CD103 treatment:*Anti-mouse CD103 (M290, BE0026) and isotype-matched antibody were purchased from Bio X cell. C57BL/6J mice were divided into two groups (Fig S2C): Isotype group, in which mice received CS instillation and FTY720 treatment (D28 to D56 every day) together with isotype antibody (100 μg *i.v.* D36 to D56 every other day). Anti-mouse CD103 group, in which

the mice received CS instillation and FTY720 treatment (D28 to D56 every day) together with anti-mouse CD103 antibody (100 μg*i.v.* D36 to D56 every other day). *Anti-IL-7 treatments:*Anti-mouse IL-7 antibody (M25, BE0048) and isotype-matched antibody were purchased from Bio X cell. Briefly, C57BL/6J mice were divided into anti-IL-7 group and Isotype group, in which mice received CS instillation and isotype or anti-mouse IL-7 antibody (30 μg,*i.t.* , twice a week from D36 to D56).

Lung histological analysis. The lung tissues of mice were fixed with 4% paraformaldehyde (PFA) and embedded in paraffin. Slices (4 μm thick) were cut, mounted on slides, and stained. Hematoxylin & Eosin (H&E) were carried out to assess the degree of inflammation and pathological changes. Collagen fiber content was quantified using Masson's trichrome staining. The staining was performed according to the manufacturer's protocol. Stained lung sections were photographed in a microscope (Leica M205 FA, Wetzlar, Germany). The inflammation score was done by a semi-quantitative analysis based on a previously published method [25]. Briefly, lung inflammation was graded into four stages and scored as follows: normal lung, 1 point; light inflammation, 20% of lung area, 2 points; medium inflammation, 20 to 50% of lung area, 3 points; severe inflammation, 50% of lung area, severe structure distortion, 4 points. The fibrotic areas were presented as a percentage, which were measured by Image J.

Statistical analysis. It was assumed that sampling was from a normally distributed population. Statistical analysis was performed using GraphPad Prism 9.0 software. All data were presented as the Mean ± SD. Unpaired/paired two-sided Student's t -test (2 groups) or one-way analysis of variance (ANOVA) followed by Tukey's test (more than 2 groups) was used to evaluate the statistical differences between groups. For all analyses, P value less than 0.05 was considered to indicate significance.

Study approval. The experimental protocols were approved by the Institutional Animal Care and Use Committees (IACUC) of China Medical University and all animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

3. Results

3.1 CS particles stimulated $CD4^+T_{RM}$ cell emergence and expansion along with silicosis progression.

First, we utilized the *in vivo* labeling method distinguishing tissue-resident cells commonly used in multivascular tissues (Fig 1A)[26]. We observed a remarkable appearance of $CD4^+$ T_{RM} cells ($CD44^+CD45$ i.v.$^-$) in the silicotic lung (Fig 1B), whereas few $CD4^+$ T_{RM}cells were found in naive mice. Unlike the circulating $CD4^+$ T_{EM} cells ($CD44^+CD45$ i.v.$^+$), $CD4^+T_{RM}$ cells surged continuously with the progression of silicosis (Fig 1B and Fig S1B), suggesting a causal relation between $CD4^+$ T_{RM} cells and silicosis progression. Furthermore, the elevated expressions of cell retention marker, CD69, CD103, and CXCR6 further confirmed their lung retention ability (Fig 1C). With the expansion of $CD4^+T_{RM}$ cells, there was an increasing number of $CD69^+CD103^+$ and $CD69^+CD103^-$subsets (Fig 1D), implying their pathogenic role in silicosis. Comparatively, the phenotype of circulating $CD4^+T_{EM}$ cells was analogic in saline and CS-treated mice, which differed from T_{RM} cells (Fig 1E). Notably, CD69 and CD103 expressions on splenic $CD4^+T_{EM}$ cells were not affected by CS injury in the lung (Fig 1F), indicating CS led to a tissue-local response. Collectively, these data demonstrated CS stimulated the emergence and expansion of pulmonary $CD4^+$ T_{RM} cells that were tightly related to silicosis progression.

3.2 Pulmonary $CD4^+$ T_{RM} cells mediated severe lung inflammatory response to CS particles.

In view of the immunologic memory role of T_{RM} cells, we next sought to explore the response of $CD4^+T_{RM}$ cells to CS particles by T-cell transfer studies. $CD4^+$ T cells were sorted from the CS-treated lung containing certain T_{RM} cells, the spleen under CS treatment only involving T_{EM} cells, or the spleen of saline-treated mice, including naïve T cells, respectively. The sorted cells were adoptively transferred into $Rag1^{-/-}$ mice that lack T cells and then treated with CS (Fig 2A). H&E staining revealed that CS-induced the severest inflammation in the mice lung transferred $CD4^+$ T cells sorted from CS-treated lung. In contrast, the mice who transferred $CD4^+$ cells from the saline-treated spleen exerted relatively mild responses (Fig 2B and C).

These phenotypes were further confirmed by the transcripts of cytokine and transcription factors associated with adaptive immunity, including *Ifng* , *Tnfa* ,*Il17a* , and *Tbx21* , but not *Il6* , related to innate immunity (Fig 2D). We further adopted flow analysis to the lungs. Significantly, more pulmonary resident T cells were observed in the mice transferred with CS-treated pulmonary $CD4^+$ T cells ($CD4^+T_{RM}$) than those of naive counterparts (saline-treated splenic T cell) (Fig 2E), highlighting a rapid reaction and expansion of the $CD4^+$ T_{RM} cells to CS particles. In supporting the notion, a higher Ki67, cell proliferating marker, was observed in the T_{RM} cells from the mice transferred $CD4^+$ T_{RM} (Fig 2F). While lung resident T cells in the mice transferred $CD4^+T_{EM}$ cells (CS-treated splenic T cell) resembled those transferred with certain $CD4^+$ T_{RM}cells, implying a potential of T_{EM} cells converting into T_{RM} cells (Fig 2E). Besides, we discovered the ratio of $CD103^-$ to $CD103^+$ in $CD69^+T_{RM}$was affected by the distinct cellular sources that more $CD69^+CD103^-$ subsets resided in the $CD4^+$ T_{RM} transferred mice (Fig 2G), implying the $CD69^+CD103^-T_{RM}$ cells mediated proinflammatory effects to the invaded CS particles. Unexpectedly, there were fewer T_{RM}-Tregs in the mice transferred with CS-treated pulmonary $CD4^+$ T than those transferred with saline-treated splenic $CD4^+$ T cells (Fig 2H). Further characterization indicated the T_{RM}-Tregs from lung-transferred CS-treated pulmonary $CD4^+$ T cells exerted a decreased ratio of $KLRG1^+CD103^+$ subsets (Fig 2I), suggesting an impaired Treg immunosuppressive function [27]. Moreover, these Tregs expressed lower CD25 and ICOS (Fig 2J), confirming the deficiency of suppressive functions [28,29]. Collectively, these results demonstrated CS particles stimulating T_{RM} cell expansion mediated severe inflammatory responses in the lung, which was related to T_{RM}'s rapid reaction to invaded CS particles, as well as the impaired Treg immunosuppressive function.

3.3 CS-induced T_{RM} cells, especially T_{RM}-Treg, depended on replenishment from circulating T cells.

We next sought to study the origin of $CD4^+T_{RM}$ cells in silicotic lung. By applying FTY720, we blocked leukocyte egress from the peripheral lymphoid tissue, minimizing the recruitment of circulating leucocytes [22]. Particularly, the treatments at different time points within silicosis progression were employed to elucidate the origin and maintenance of $CD4^+$ T_{RM} cells (Fig 3A). H&E staining demonstrated that FTY720 treatment exerted protective effects on silicosis. However, though half-time intervention (4W-8W) reduced inflammatory cell recruitment, we could not get an equal attenuated phenotype compared to full-time blockage (0W-8W) (Fig 3B and C), suggesting that the T_{RM} cells are derived from peripheral circulating cells. Specifically, full-time FTY720 treatment resulted in a significant vanish of $CD4^+T_{RM}$ cells in number, albeit with surged percentages, while half-time FTY720 intervening reduced T_{RM} cells but not as much as those of full-time treatment (Fig 3D), implying that CS-induced pulmonary T_{RM} cells actively expanded i*n situ* . In supporting the notion, high levels of Ki-67 were observed in the T_{RM} cells but not impaired by FTY720 treatments (Fig 3E). Notably, we noticed affected ratios of $CD103^-$ to $CD103^+$ in $CD69^+T_{RM}$ by the FTY720 intervention. Full-time FTY720 treatment resulted in a high portion of $CD103^-CD69^+$ T_{RM}subsets (Fig 3F), which was analog to the phenotypes of previous transfer experiments. We further tested the FTY720's effects on the emergence of Tregs and found that full-time treatment decreased the portion of $FOXP3^+$ Tregs in T_{RM} cells (Fig 3G), reminding us that T_{RM}-Tregs were prone to be replenished by circulation.

To further confirm the hypothesis, we aimed to perform a parabiosis model to illuminate the cellular source of the T_{RM} cells in the silicotic lung. To do this, naïve CD45.1/2 mice were cojoined with naive congenic mice (CD45.1/1) by parabiosis surgery (Fig 3H). The blood circulation between parabionts was established 14 days later, indicated by an equal portion of CD45.1 and CD45.2 lymphocytes in the blood (Fig 3I), after which the CD45.1/1 congenic mice were exposed to CS particles. Another seven days later, we checked the component of circulating T_{EM} cells and the pulmonary T_{RM} in the CS-exposed mice. We found that the composition of CD45.2 lymphocytes in circulating effector cells was equal to the proportion in the blood (Fig 3J). Notably, we found that there were $CD45.2^+$ T cells in the $CD4^+$ T_{RM} cells of CS-treated CD45.1/1 mice lung (Fig 3J), suggesting circulating T cells contributed to T_{RM} cells formation. Significantly, when scrutinizing the origin of T_{RM} subsets, we found that $CD45.2^+$ cells accounted for a higher proportion in Tregs than those of Teff cells, further proving our hypothesis that Tregs depended more on circulating T cell replenishment (Fig 3K). Additionally, we observed that T_{RM}-Tregs expressed higher CD103 than the Teff cells, while within T_{RM}-Tregs, the newly recruited Tregs (CD45.2) expressed high adhesive molecule

CD103, but not the T_{RM}-Teff cells (Fig 3L). These results remind us that the pattern of CD103 and CD69 seems related to the constitutions of $CD4^+T_{RM}$ subsets in the lung. Collectively, these data proved that CS-induced pulmonary $CD4^+$ T_{RM} cells came in two ways: recruited from circulation and proliferating *in situ* . While the T_{RM}-Tregs were prone to be replenished by the circulation.

3.4 Differential CD69 and CD103 expressing patterns defined $CD4^+$ T_{RM} cells into relatively functional distinct lineages.

Now that the cell retention markers CD103 and CD69 expressions were related to the constitution of T_{RM} subsets, we next aim to scrutinize the phenotype. By differential CD69 and CD103 patterns, $CD4^+$ T_{RM} cells were divided into 4 subsets and checked transcriptional factor expressions (Fig 4A). Interestingly, the $CD69^+CD103^-$ and $CD69^-CD103^-$ subpopulations expressed higher T-bet, indicating pro-inflammatory Th1 cells were enriched (Fig 4B). The $CD69^+CD103^+$subset exerted the highest GATA-3 expression (Fig 4C). The comparison of ROR-γt manifested that the $CD69^+CD103^-$ subpopulation expressed the highest level (Fig 4D). In sharp contrast, a higher portion of $FOXP3^+$ was observed in $CD69^+CD103^+$ and $CD69^-CD103^+$ subsets, complementing the subsets of high T-bet expression (Fig 4E). Collectively, we demonstrated cell retention markers could depict T_{RM}into relatively distinct subsets. Tregs were enriched in $CD103^+$ subsets. In line with this, Tregs' functional markers, CD25 (IL-2Rα), PD-1, ST2 (IL-33R), ICOS (CD278), and CD39 were highly expressed within the subsets (Fig 4F).

To corroborate the findings, we further divided $CD4^+T_{RM}$ cells into Tregs and Teff cells and checked their CD103 and CD69 expression patterns. Expectedly, T_{RM}-Tregs ($FOXP3^+$) expressed high CD103, while the T_{RM}-Teff cells ($FOXP3^-$) expressed few CD103 (Fig 4G), suggesting CD103 is an indicator of T_{RM}-Treg cells. Furthermore, we discovered the Th1-type T_{RM} ($T\text{-}bet^+$) and Th17-type T_{RM} ($ROR\text{-}\gamma t^+$) expressed less CD103, whereas Th2-type T_{RM}($GATA\text{-}3^+$) expressed relatively high CD103 (Fig 4H), implying the emergence of $GATA\text{-}3^+$ Tregs[30]. Remarkably, the ratio of $CD103^+$ to $CD103^-$ in $CD69^+CD4^+$ T_{RM} cells was lifted with the progression, emphasizing the imbalance within T_{RM} cell subsets related to fibrogenesis (Fig 4I). In synopsis, we proved the cell retention markers CD103 and CD69 can define $CD4^+$ T_{RM} cells into functional distinct lineages, which may provide potential therapeutic targets for alleviating silicosis.

3.5 $CD103^+$ T_{RM}-Tregs exerted an immuno-suppressive but not pro-fibrotic role in the progression of silicosis.

Now that we prove the retention molecules could define $CD4^+$ T_{RM} into relative functional distinct subsets, we aim to explore whether depleting $CD103^+$ T_{RM} subset exerts protective effects on silicosis progression. Taking the notion that $CD103^+$ T_{RM}-Tregs were preferentially recruited from the circulation, we employed FTY720 plus CD103 neutralization treatments to deplete the T_{RM} subsets (Fig S2A). Unexpectedly, we observed semblable phenotype of silicotic lung, suggesting anti-CD103 treatment did not exert protective role (Fig S2B). Significantly, the treatment reduced T_{RM} cell count in the lung, as well as diminished $CD103^+$subsets, resulting in a higher $CD103^-/CD103^+$ ratio (Fig S2C). Furthermore, by checking transcriptional factors, we observed surged ratios of $ROR\text{-}\gamma t^+$ (Th17-type T_{RM}) cells, while $T\text{-}bet^+$ (Th1-type T_{RM}) counterparts were not significantly affected (Fig S2D). To gain more insights, we further explored the proliferation abilities of the $CD103^-$ T_{RM} cells since Tregs exerted an immuno-suppressive role by restraining Teff cells. Significantly, depleting $CD103^+$ T_{RM}-Treg augmented Ki67 levels of the $CD103^-$ T_{RM} subsets (most T_{RM}-Teff cells) (Fig S2E), implying depleting $CD103^+$ T_{RM} cells unleashed the proliferating ability of T_{RM}-Teff cells. Collectively, these results demonstrated that $CD103^+T_{RM}$ subsets could restrain T_{RM}-Teff proliferation while targeting $CD103^+T_{RM}$ cells could not mitigate CS-induced pulmonary fibrosis.

To validate the role of $CD103^+$ T_{RM}Tregs in silicosis, we further depicted the immunological characteristics of the subsets. We observed the $CD103^+$Tregs could secrete higher IL-10 but not TGF-β (detected by LAP, essential for TGF-β cleaved) (Fig 5A), manifesting high anti-inflammation property but not tissue-repair phenotype[31]. Moreover, there were more effector phenotype ($PD\text{-}1^+CD25^+$) and terminally differentiated ($KLRG1^+ICOS^+$) Tregs in the $CD103^+$ subsets (Fig 5B and C)[32,33]. We further expanded our immunophenotyping to the Tregs by introducing CD39 and CD69. Higher CD39 levels further manifested high immune-

suppressive potential, while the higher level of CD69 further confirmed the characteristic of tissue-resident Tregs (Fig 5D), both of which agreed with our previous data demonstrating CD103$^+$ Tregs exerted different phenotypes to CD103$^-$ ones [25].

To directly prove the immune-suppressive role of CD103$^+$ Tregs, we did cell transfer experiments. The CD103$^+$ memory Tregs were sorted from the silicotic lung of FOXP3 reporter mice and transferred into another CS-pre-treated mouse (Fig 5E). We did flow cytometric analysis expectedly demonstrated the appending CD103$^+$T$_{RM}$-Tregs significantly reduced IL-17A producing (Th17) cells and IFN-γ secreting (Th1) cells (Fig 5F). However, this regulatory capacity did not influence IL-13 producing (Th2) cells (Fig 5G), highlighting previously mentioned notion the Th2-like Tregs emerge in the silicotic lung. To complement the above data, we further explored the transcripts of cytokines and found, in accordance with the flow data, that the pro-inflammatory mediators *Il17a, Ifng,* and *Tnfa* were descended, while the suppressive *Il10* augmented in the lung tissue transferred CD103$^+$ T$_{RM}$-Tregs (Fig 5H). In contrast, the CD103$^+$ Treg did not affect *Tgfb1* transcripts, further evidencing that CD103$^+$Tregs did not exert profibrotic roles (Fig 5I). Additionally, histological analysis to the lung section proved a decreased inflammatory infiltration but not collagen deposition, which were further validated by unaffected *Col1a1* and *Fn*transcriptions in the lung (Fig 5J, K and L). Collectively, these results validated that CD103$^+$ Tregs functioned as an immunosuppressive regulator restraining Teff cells rather than profibrotic mediators. Targeting CD103$^+$CD4$^+$ T$_{RM}$ cells could not mitigate CS-induced pulmonary fibrosis.

3.6 Neutralizing IL-7 in lung retarded silicosis progression through disrupting the pathogenic T$_{RM}$-Teff cell maintenance.

Now that the CS-induced pathogenic CD4$^+$T$_{RM}$ cells expanded*in situ* , we next explored interventions targeting their maintenance in the lung, in which IL-7 was reported to be essential[34]. Hence, we first examined the IL-7R (CD127) levels on the CD4$^+$ T$_{RM}$ cells in silicotic lung. Expectedly, CD4$^+$ T$_{RM}$cells expressed higher levels of IL-7R compared to those of the naïve T cells (CD44$^-$). Strikingly, within CD4$^+$ T$_{RM}$ cells, T$_{RM}$- Teff cells but not Tregs expressed a higher level of IL-7R indicating a high demand for IL-7 of those Teff cells (Fig 6A). We then treated the silicotic mice with IL-7-neutralizing antibody through intratracheal (*i.t.*) instillation (Fig 6B). To gain insights, we sorted CD4$^+$ T cells from the lungs and spleens and did qPCR analysis. Significantly, in the treated pulmonary CD4$^+$T cells, we observed alleviated Th1 and Th17-related transcripts (*Tbx21* , *Ifng* , *Il2* ,*Rorc* and *Il17a*), as well as decreased mRNA levels associated with cell activation (*Icos* , *Ctla4* ,*Klrg1* , *and Pdcd1*). Additionally, the cell retention markers (*Cxcr6* and *Itgae*) were decreased, implying a reduction of T$_{RM}$ cells (Fig 6C). Strikingly, the anti-IL-7 treatment augmented *Il10* transcripts, suggesting suppressive inflammatory responses, while markers related to Treg cells,*Foxp3,* and *Areg* were unaltered (Fig 6C). However, these effects were diminished in the splenic CD4+ T cells (Fig 6D), suggesting that pulmonary local IL-7 neutralization did not affect immune response in other organs.

To confirm the transcriptional changes, we further did flow cytometric analysis to the pulmonary CD4$^+$ T$_{RM}$cells but did not observe reduced T$_{RM}$ cells in the lung (Fig 6E), while the ratio of CD103$^-$ to CD103$^+$ in CD69$^+$CD4$^+$ T$_{RM}$ cells was decreased (Fig 6F), suggesting T$_{RM}$-Teff cells were restrained. However, we did not observe a significantly altered ratio of T$_{RM}$-Tregs within CD4$^+$T$_{RM}$ (Fig 6G). To ascertain the effects on T$_{RM}$-Teff cells, we scrutinized the composition of T cell subsets. Consistent with the transcript data, pulmonary IL-7 neutralization blunted T-bet$^+$ Th1-type T$_{RM}$, but not the ROR-γt$^+$ Th17-type T$_{RM}$ cells albeit with an increasing trend (Fig 6H). Additionally, pulmonary IL-7 neutralization decreased Ki67 levels of T$_{RM}$-Teff cells (Fig 6I), confirming that IL-7 plays a positive role in T$_{RM}$ cell proliferation[35].

Last, to gain insight into the profound effect of IL-7 neutralization on silicosis, lung histological analysis was performed. Expectedly, local anti-IL-7 treatment in the lung mitigated disease phenotype with reduced cell accumulation and cellular nodule formation (Fig 6J), as well as attenuated collagen deposition revealed by Masson's trichrome staining (Fig 6K). Consistent with previous sorted cells transcript results, in the lung tissue, the Th1-related cytokine (*Ifng*) and transcriptional factors (*Tbx21*) transcripts were blunted (Fig 6L). The neutralization decreased the transcripts of *Il17a*despite exerting no effects on those of *Rorc* in the

lung (Fig 6L), implying that pulmonary IL-7 neutralization alleviated CS particles-induced inflammatory response. In accordance with alleviated fibrosis, the declined *Col1a1* and *Fibronectin* transcripts in the lung further validated the conclusion (Fig 6M). Collectively, these data demonstrated that the maintenance of T_{RM}-Teff cells depended on IL-7 in the lung. Targeting IL-7 would be a potential intervention against CS-induced pulmonary fibrosis.

4. Discussion

Here, we showed that a substantial accumulation of pulmonary $CD4^+$ T_{RM} cells mediated the pathogenesis of silicosis, who exerted immunological memory and antigen-specific response to the invaded CS particles. The T_{RM} cells, especially T_{RM}-Tregs, rely on continual replenishment from circulating lymphocytes. The defined population by CD103 and CD69 displayed distinct functional phenotypes. Targeting the immunosuppressive $CD103^+T_{RM}$ cells did not exert protective roles to silicosis. Whereas neutralizing IL-7 in the lung disrupted the maintenance of T_{RM}-Teff cells during silicosis progression and exerted protective effects.

Since the invaded CS particles could not be cleared by the pulmonary immune system, it would lead to repeated and cycled antigen stimulation, in which our one-time intratracheal CS instillation gave rise to $CD4^+$ T_{RM} cells. Evidence from the $Rag1^{-/-}$ mice transferred $CD4^+$ T cells that were previously exposed to CS revealed that new CS exposure induced fast T_{RM} cell expansion and potent reaction, demonstrating the T_{RM} cells exerted immunologic memory and antigen-specific characterization. Notably, the CS particles could induce T-cell proliferation by directly activating cells through T-cell antigen receptor (TCR) complexes but not rely on antigen-presenting cells [36]. While research about Be oxide-related toxicological effects demonstrated major histocompatibility complex class II (MHCII) alleles, particularly HLA-DPB1, are strongly associated with susceptibility of the particle-induced lung inflammation[37]. Further study about the structural or molecular mechanism of how CS particles activate T cells, for instance, exploring the CS binding site within the TCR footprint or the presenting MHC molecule and the peptide bound by the MHC, would provide insights into the disease mechanisms and targets for antigen-specific therapies.

By circulatory lymphocyte blockade and parabiosis experiment, we directly proved the circulating T cells contributed to expanded T_{RM} cells, which complies with a general acknowledgment that lymphocytes migrating from lymphoid organs to tissues is likely to be the first step in the formation of T_{RM} cells[12]. Analog to our results, the circulatory T cells were reported to contribute to $CD4^+T_{RM}$ cells exacerbating asthma[38]. Significantly, we noticed circulating lymphocyte recruitment blockage by FTY720 decreased the percentage of Tregs in T_{RM}, implying the T_{RM}-Tregs were in high demand of replenishment from circulating lymphocytes. Additionally, the evidence that fewer Tregs with an impaired immunosuppressive function in the CS-exposed pulmonary $CD4^+$ T cells transferred $Rag1^{-/-}$mice indicated a critical role of circulating T cell to T_{RM}-Tregs, as there was no circulating T cells replenishment in the $Rag1^{-/-}$mice. Ultimately, our parabiosis experiment directly proved T_{RM}-Tregs depended more on circulatory T cell replenishment. In supporting the notion that we previously reported the T_{RM}-Tregs in the silicotic lung expressed high Nrp-1 (markers of thymus-derived Tregs)[25,39], further proving the T_{RM}cells originally came from the thymus and recruited to peripheral tissue[39], but not the conversion of Teff cells to Tregs [40]. All the evidence supported the suggestion that circulating thymus-derived Tregs are required to replenish T_{RM}-Tregs to ensure a tissue-local immunosuppressive environment that promotes tissue remodeling. Remarkably, even though FTY720 could attenuate silicosis by reducing T_{RM} cells in the lung, the FTY720 treatment in silicosis should be cautious. A common adverse effect would be an increased incidence of respiratory infections. Given the association of tuberculosis with silicosis, such treatment would need to be given with caution.

Despite numerous studies exploring the role of Tregs in fibrotic disease, this topic is still debatable [41,42], which may be related to Tregs' heterogeneity in peripheral tissues[43,44]. In pursuing the role of circulatory-derived $CD103^+$ T_{RM}-Tregs in silicosis, we applied FTY720 together with CD103 neutralizing antibody to the silicotic mice expecting to get an alleviated effect. Surprisingly, this treatment did not mitigate disease phenotype, but no aggregated fibrosis was observed, as research reported that depleting $CD103^+$ T_{RM} cells exacerbated pulmonary fibrosis [45]. While the restrained effector T proliferation was unleashed by CD103 neutralization, which was consistent with the notion that $CD103^+T_{RM}$-Tregs restrained pathogenic

$CD4^+$Teff cells in the lung [45]. Although in different models of pulmonary fibrosis, the role of $CD103^+$ Tregs seems to be analog, which exerted an immunosuppressive but not pro-fibrotic phenotype. With the cell transfer method, we ultimately proved the appended $CD103^+$ Tregs role in constraining CS-induced inflammatory response but not promoting CS-induced fibrosis. The subsided tissue local inflammation may be a previous step than tissue repair, during which $CD103^+$Treg attenuated tissue inflammation by limiting Teff cell proliferation, however, it does not promote fibrosis progression. Co-expression of cell retention markers CD103 and CD69 endowed Tregs with stronger tissue resident characteristics since CD103 ($\alpha_E\beta_7$integrin) promotes T cell adhesion through the interaction with E-cadherin expressed by epithelial cells [46,47]. Additionally, the fact that high KLRG1 expression in $CD103^+$Treg also indicated the high adhesive ability to epithelial since the binding site to E-cadherin of KLRG1 is distinct from the CD103 binding site [48]. Further, research about markers distinguishing pro-fibrotic or immunosuppressive Treg would provide insights into treating silicosis.

It should be noted that the CS-stimulated pulmonary $CD4^+$ T_{RM} cells expressed cell retention marker CD69, regardless of Teff cells or Tregs, implying a crucial role of this molecule in mediating silicosis. However, other groups illuminated that genetically deleting CD69 in mice results in a reduction of $CD8^+$ T_{RM} in the skin and lung but does not affect $CD4^+$ T_{RM}cells [49]. These findings established that although CD69 expression can identify T_{RM} in tissues, CD69 per se is not necessarily sufficient for influencing T_{RM} cell maintenance or function. While there is a recent report that CD69 expression on Tregs protects from inflammatory damage after myocardial infarction [50]. Thus, a comprehensive understanding of CD69 on silicosis and other disease models still needs dissection.

IL-2 and IL-7 are crucial cytokines in maintaining lymphocyte viability, T_{RM} cells up-regulate receptors for the homeostatic cytokines [51,52]. The previous study indicated that targeting CD25 (IL-2Rα) aggravated lung inflammation, alleviating fibrogenesis due to Treg depletion [53]. Herein, we illuminated different IL-7R expressions between T_{RM}-Teff cells and T_{RM}-Tregs. Higher receptor levels may indicate high demands for the cytokine. In supporting the notion, a higher cytokine level of IL-7 in the serum was observed in silicosis patients [54], whose role in silicosis was elusive. We demonstrated that IL-7 neutralization in the lung was effective in alleviating CS-induced pulmonary fibrosis by decreasing pathogenic $CD4^+$ T_{RM} cells without affecting T_{RM}-Tregs expressing low IL-7R. Neutralizing IL-7 locally in lung disrupted the maintenance of T_{RM}-Teff cells, attenuated CS-induced pulmonary chronic inflammation, mitigated local inflammatory damage to lung tissue, and thus inhibited fibrosis progression (excessive tissue repair). We did not utilize neutralizing antibodies by intravenous treatment since it may affect immune responses in other organs. Expectedly, lung local IL-7 neutralization did not affect the immune response in the spleen. Besides, bronchoalveolar lavage is often used in silicosis treatment. Clinically, applying IL-7-neutralizing antibodies in this process may provide therapeutic potential. However, in view of the situation that IL-7 was universally required for lymphocyte maintenance, the side effect of neutralization is a problem to be illuminated in the future.

In conclusion, our results provided new insights into CS particle's toxicological effects in the lung, all of which will be essential for the development of new therapeutic strategies for the intractable inflammation-associated fibrotic diseases.

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Author contributions.

Y.C. You: Methodology, Investigation, Visualization, Writing – original draft. X.L. Wu: Investigation, Visualization, Writing – original draft. Y.Y. He: Investigation. H.Y. Yuan: Investigation. Y.H. Chen: Investigation. S.S. Wang: Investigation. H. Min: Investigation. J. Chen: Conceptualization, Supervision. C. Li: Conceptualization, Supervision, Methodology, Visualization, Writing – review & editing, Funding acquisition.

Competing interests.

The authors declare that they have no competing interests.

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Figure Legends

Figure 1. CS particles stimulated $CD4^{+}T_{RM}$ cell emergence and expansion along with silicosis progression.

(A) Schematic showed CD45-APC-Cy7 antibody intravenous (*i.v.*) injection to distinguish tissue-resident or

circulating leukocytes. Circulating leukocytes were labeled. (B) Flow cytometry (FC) analysis of pulmonary $CD4^+$ T_{RM} cells ($CD45_{inject}{}^-CD44^+$) and T_{EM} ($CD45_{inject}{}^+CD44^+$) cells. Percentages and counts of the $CD4^+$ T_{RM}cells were compared at the indicated time points (n = 5). W, week. (C) Representative FC analysis of $CD4^+$ T_{RM} cells for CD69, CD103 and CXCR6 expression respectively. (D) FC analysis of $CD4^+T_{RM}$ cells for CD69 and CD103 expression. The graph showed percentages and counts of $CD69^+CD103^-$ (upper) and $CD69^+CD103^+$ subsets (lower) in $CD4^+$ T_{RM} cells of saline or CS-treated mice at the specified time points (n = 5). (E) FC analysis of lung circulating T_{EM} cells for CD69 and CD103 expression of saline or CS-treated mice at specified time points. The bar graph illustrated ratios of $CD69^+$ in circulating $CD4^+$ T_{EM}cells (n = 4). (F) FC analysis of splenic T_{EM} cells for CD69 and CD103. The graph compared percentages of $CD69^+$ on splenic $CD4^+T_{EM}$ cells of saline or CS-treated mice (n = 6). The bar graphs are the combined results of at least three independent experiments. Individual mice are plotted on the graphs. Values are reported as the mean ± SD. *P* value was determined by one-way ANOVA followed by Tukey's test.

Figure 2. Pulmonary $CD4^+$ T_{RM}cells mediated severe lung inflammatory response to CS particles.

(A) The reconstitution sketch of $Rag1^{-/-}$ mice with different cell origins. $CD4^+$ T cells were sorted from the lung or spleen of CS-treated mice, or from the spleen of saline-treated mice by magnetic-activated cell sorting (MACS) method. The MACS-purified $CD4^+$ T cells and PBS were*i.v.* transferred into $Rag1^{-/-}$ mice 2 weeks before CS instillation. One week after the CS instillation, recipient mice were analyzed. (B) H&E staining to the lung sections of distinct reconstituted $Rag1^{-/-}$ mice. Scale Bar = 500 μm and 200 μm. (C) The inflammation scores were assessed in the lung sections (n = 4 to 5). (D) Relative RNA levels of *Ifng* , *Tnfa* , *Il17a* ,*Tbx21* , and *Il6* in each group (n = 4 to 5). (E) FC analysis of pulmonary resident $CD4^+$ T cells in transferred $Rag1^{-/-}$ mice. The percentages and counts of the resident $CD4^+$ T cells were shown (n = 4 to 5). (F) Flow histogram indicated Ki-67 intensity in lung-resident $CD4^+$ T cells. The graph compared the MFI of Ki-67 (n = 4). (G) FC analysis of CD69 and CD103 expressions within $CD4^+$ T_{RM} cells. The graph compared the proportions of $CD103^-$ to $CD103^+$ in the $CD69^+$ $CD4^+$ T_{RM}cells (n = 4 to 5). (H) FC analysis of T_{RM}-Tregs ($FOXP3^+$). The percentages of T_{RM}-Tregs were displayed (n = 4). (I) FC plots for KLRG1 and CD103 in the T_{RM}-Tregs. The graph compared the ratios of $CD103^+KLRG1^+$ in the T_{RM}-Tregs (n = 4). (J) Flow histogram compared CD25 and ICOS expressions in the T_{RM}-Tregs. The graph showed the MFI (n = 4). The graphs showed the combined results of 4-5 independent experiments. The recipient $Rag1^{-/-}$ mice were littermates. Individual mice are plotted on the graphs. Values are reported as mean ± SD. *P* value was determined by one-way ANOVA followed by Tukey's test.

Figure 3. CS-induced T_{RM} cells, especially T_{RM}-Tregs, depended on replenishment from circulating T cells.

(A) The scheme indicated the time points of FTY720 treatment (20 μg in vehicle).

FTY720 treatment from 0W blocked circulating T cell recruitment. FTY720 treatment from 4W, in which circumstance, lymphocytes could be recruited into the lung at the inflammatory stage but blocked at the fibrogenesis stage. (B) H&E staining to the lung section of different treated mice. Scale Bar = 500 μm and 200 μm. (C) The inflammation scores were assessed in the lung sections (n = 5). (D) FC analysis of $CD4^+T_{RM}$ cells in the lungs of distinct FTY720-challenge. The bar graph showed percentages and counts of $CD4^+$ T_{RM} cells (n = 5). (E) Typical flow histogram indicated Ki-67 expression in $CD4^+T_{RM}$ cells. (F) FC analysis of $CD4^+T_{RM}$ cells for CD69 and CD103 expressions. The bar graph displayed the ratios of $CD103^-$ to $CD103^+$ in the $CD69^+$ T_{RM} cells (n = 5). (G) FC analysis of Tregs ($FOXP3^+$) in the $CD4^+T_{RM}$ cells. The bar graph showed the percentages of T_{RM}-Tregs (n = 5). (H) Schematic overview of parabiosis experiment. CD45.1/1 and CD45.1/2 mice were approximated with sutures. With 14 days' recovery, CD45.1/1 mice were treated by CS particle instillation and analyzed 7 days later. (I) Typical FC plot of circulating blood leukocytes of the parabiont. (J) Flow plot analysis of the $CD4^+$ T_{RM} cells and circulating T_{EM} cells of the CS-treated conjoined mice. $CD45.2^+$ T_{RM} cells indicated the recruited cells from circulating. (K) FC plots demonstrated the CD45.2 expression in T_{RM}-Treg or T_{RM}-Teff cells. (L) FC plots demonstrated CD103

and CD45.2 expressions in T_{RM}-Treg or T_{RM}-Teff cells. N number also indicated independent experimental replicates. Individual mice are plotted on the graphs. Values are reported as the mean ± SD. *P*value was determined by one-way ANOVA followed by Tukey's test.

Figure 4. Differential CD69 and CD103 expressing patterns defined CD4$^+$ T_{RM} cells into relatively functional distinct lineages.

(A) Flow plot indicated CD4$^+$ T_{RM} cells in fibrotic lungs were divided into four subsets by CD69 and CD103 expressions. CD69$^+$CD103$^+$ (Red); CD69$^+$CD103$^-$ (Blue); CD69$^-$CD103$^+$ (Orange); CD69$^-$CD103$^-$ (Green). (B-D) Flow histogram displayed transcriptional factor T-bet (B), GATA-3 (C), and ROR-γt (D) expressions among different subsets. Bar graph below showed the MFI among the indicated CD4$^+$ T_{RM}cell subsets (n = 6). (E) Flow histogram compared ratios of FOXP3$^+$ among different subpopulations. Bar graph below revealed the percentages of FOXP3$^+$ among the indicated subsets (n = 5). (F) Representative flow histogram compared the intensity of CD25, PD-1, ST2, ICOS, and CD39 among four subsets in CD4$^+$ T_{RM} cells. (G) Typical flow plots showed CD69 and CD103 expressing patterns on Teff cells (FOXP3$^-$) or Tregs (FOXP3$^+$) in CD4$^+$ T_{RM} cells. (H) Typical flow plots demonstrated CD69 and CD103 expressing patterns on T-bet$^+$, ROR-γt$^+$, or GATA-3$^+$ subsets in CD4$^+$$T_{RM}$ cells. (I) The bar graph showed the ratios of CD103$^+$ against CD103$^-$ subsets in CD69$^+$CD4$^+$ T_{RM} cells (n = 5). Bar graphs are the combined results of at least three independent experiments. Individual mice are plotted on the graphs. Values are reported as the mean ± SD. *P* value is determined by one-way ANOVA followed by Tukey's test.

Figure 5. CD103$^+$ T_{RM}-Tregs exerted immuno-supressive but not pro-fibrotic roles in the progression of silicosis.

(A) FC analysis of CD103$^+$ and CD103$^-$ Tregs for IL-10 and LAP expressions. The graph compared the percentages of IL-10$^+$ and LAP$^+$ (n = 3 to 4). (B) FC analysis of effector phenotype (CD25$^+$PD-1$^+$) within CD103$^+$ and CD103$^-$ Tregs. The graph compared the proportion (n = 6). (C) FC analysis of terminal differentiated phenotype (ICOS$^+$KLRG1$^+$) within CD103$^+$ and CD103$^-$ Tregs. The graph compared the ratios (n = 6). (D) Flow histogram compared the expression of CD39 and CD69. (E) The schematic diagram describes the process of CD103$^+$$T_{RM}$-Treg (CD4$^+$CD44$^+$FOXP3^{YFP+}CD103$^+$) sorted in silicotic FOXP3^{YFP} mice by the FACS method. CS-pre-challenged mice were *i.v.* transferred with the sorted CD103$^+$ Tregs or PBS. The recipient mice were analyzed 14 days after cell transfer. (F) FC analysis of IFN-γ$^+$ and IL-17A$^+$CD4$^+$ T cells in each group. The chart compared the ratios and counts of IFN-γ$^+$ and IL-17A$^+$ in CD4$^+$ T cells. (G) FC analysis of IL-13$^+$ in CD4$^+$ T cells. The graph compared the ratios and counts of IL-13$^+$ in CD4$^+$ T cells (n = 3 to 4). (H, I) Relative mRNA levels of *Il17a* , *Ifng* , *Tnfa* , and *Il10* (H), and*Tgfb1* (I) in each group (n = 3 to 5). (J) Relative mRNA levels of*Col1a1* and *Fn* in each group (n = 3 to 5). (K) H&E staining to the lung sections of distinct groups. Scale Bar = 600 μm and 300 μm. The graph showed the inflammation score (n = 3 to 5). (L) Masson's trichrome staining to the lung sections of different groups. Scale Bar = 600 μm and 300 μm. The bar graph showed the fibrosis-positive area (n = 3 to 5). Individual mice are plotted on the graphs. Values are reported as the mean ± SD.*P* value was determined using unpaired two-tailed Student's*t* -test.

Figure 6. Neutralizing IL-7 in the lung retarded silicosis progression through disrupting the maintenance of pathogenic T_{RM}-Teff cells.

(A) Flow histogram of IL-7R (CD127) on naïve or T_{RM}-Tregs and Teffs. The graph compared the MFI (n = 5). (B) Bioluminescence of silicotic mice with isotype-control or IL-7 neutralizing (30 μg *i.t.* twice a week from D36). (C) RNA analysis of sorted pulmonary CD4$^+$ T cells in distinct groups. The heatmap contained Th1, Th17, and Treg-related genes, activation, and retention markers (n = 6). (D) RNA analysis of sorted splenic CD4$^+$ T cells in distinct groups. The heatmap contained Th1, Th17, and Treg related genes (n = 3). (E) FC analysis of pulmonary CD4$^+$ T_{RM} cells. Percentages of CD4$^+$ T_{RM} cells were shown (n = 5). (F) FC analysis of CD69 and CD103 expression in CD4$^+$$T_{RM}$ cells. The ratios of CD103$^-$ to CD103$^+$ in the CD69$^+$CD4$^+$ T_{RM} cells were shown (n = 5). (G) FC analysis of Tregs (FOXP3$^+$) in the CD4$^+$ T_{RM} cells. Bar graph compared the percentage of T_{RM}-Tregs (n = 6). (H) FC analysis of CD4$^+$ T_{RM} cells for T-bet

and ROR-γt expression. The graph displayed the percentages of T-bet$^+$ or ROR-γt$^+$T$_{RM}$ cells (n = 6). (I) Flow histogram indicated the Ki-67 levels in the T$_{RM}$-Teffs. The graph compared the MFI (n = 6). (J) H&E staining to the lung sections of different groups. Scale Bar = 500 μm and 200 μm. The inflammation scores were measured (n = 5). (K) Masson's trichrome staining to the lung sections of different groups. Scale Bar = 500 μm and 200 μm. The fibrosis-positive areas were quantified (n = 5). (L) Relative mRNA levels of *Il17a* ,*Rorc* , *Ifng* , and *Tbx21* in mice lung tissue of each group (n = 6). (M) Relative mRNA levels of *Col1a1* and *Fn*in mice lung tissue of each group (n = 6). Individual mice are plotted on the graphs. N number also indicated independent experimental replicates. *P* value was determined using one-way ANOVA followed by Tukey's test or unpaired Student's *t* -test.