Adoptive transfer of CD49a IIssue resident memory cells reverses pulmonary fibrosis in mice.
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Abstract

Pulmonary fibrosis is a devastating disease with no effective treatments to cure, stop or reverse the unremitting, fatal fibrosis. A critical barrier to treating this disease is the lack of understanding of the pathways leading to fibrosis as well as those regulating the resolution of fibrosis. Fibrosis is the patholog pathways: immune effectors, inflammatory mediators and fibroproliferation in the resolution of mosis. Fibrosis is the pathologic side of normal tissue repair that results when the normal
wound healing programs go awry. Successful resolution of tissue injury requires several highly
coordinated pathways, and this rese mound healing programs go awry. Successful resolution of tissue injury requires several highly
coordinated pathways, and this research focuses on the interplay between these overlapping
pathways: immune effectors, inflamma coordinated pathways, and this research focuses on the interplay between these overlapping
pathways: immune effectors, inflammatory mediators and fibroproliferation in the resolution of
fibrosis. Previously we have success pathways: immune effectors, inflammatory mediators and fibroproliferation in the resolution of
fibrosis. Previously we have successfully prevented, mitigated, and even reversed established
fibrosis using vaccinia vaccinati pathways: Previously we have successfully prevented, mitigated, and even reversed established
fibrosis using vaccinia vaccination immunotherapy in two models of murine lung fibrosis. The
mechanism by which vaccinia reverse fibrosis using vaccinia vaccination immunotherapy in two models of murine lung fibrosis. The
mechanism by which vaccinia reverses fibrosis is by vaccine induced lung specific Th1 skewed
tissue resident memory (TRMs) in the mechanism by which vaccinia reverses fibrosis is by vaccine induced lung specific Th1 skewed
tissue resident memory (TRMs) in the lung. In this study, we isolated a population of vaccine
induced TRMs - CD49a⁺ CD4⁺ T ce mechanism by memory (TRMs) in the lung. In this study, we isolated a population of vaccine
induced TRMs - CD49a⁺ CD4⁺ T cells - that are both necessary and sufficient to reverse
established pulmonary fibrosis. Using a induced TRMs - CD49a⁺ CD4⁺ T cells - that are both necessary and sufficient to reverse
established pulmonary fibrosis. Using adoptive cellular therapy, we demonstrate that
intratracheal administration of CD49a⁺ CD4⁺ intratracheal administration of CD49a⁺ CD4⁺ TRMs into established fibrosis, reverses the fibrosis
histologically, by promoting a decrease in collagen, and functionally, by improving lung function,
without the need for without the need for vaccination. Furthermore, co-culture of *in vitro* derived CD49⁺ CD4⁺ human
TRMs with human fibroblasts from individuals with idiopathic pulmonary fibrosis (IPF) results in
the down regulation of I the down regulation of IPF fibroblast collagen production. Lastly, we demonstrate in human IPF
lung histologic samples that CD49a⁺ CD4⁺ TRMs, which can down regulate human IPF fibroblast
function, fail to increase in t lung histologic samples that CD49a⁺ CD4⁺ TRMs, which can down regulate human IPF fibroblast
function, fail to increase in the IPF lungs, thus potentially failing to promote resolution. Thus,
we define a novel unapprec lung histologic samples that CD49a⁻ CD4⁻ TRMs, which can down regulate human IPF fibroblast function, fail to increase in the IPF lungs, thus potentially failing to promote resolution. Thus, we define a novel unappreci Function, failing, failing, failing, failing and prediction, the IPF lunchest of the annovel diagnost established lung fibrosis to promote resolution of fibrosis and re-establish lung homeostasis.
We demonstrate that immun established lung fibrosis to promote resolution of fibrosis and re-establish lung homeostasis.
We demonstrate that immunotherapy, in the form of adoptive transfer of CD49a⁺ CD4⁺ TRMs
into the lungs of mice with establi We demonstrate that immunotherapy, in the form of adoptive transfer of CD49a⁺ CD4⁺ TRMs into the lungs of mice with established fibrosis, not only stops progression of the fibrosis but more importantly reverses the fib more importantly reverses the fibrosis. These studies provide the insight and preclinical rationale for a novel paradigm shifting approach of using cellular immunotherapy to treat lung fibrosis. rationale for a novel paradigm shifting approach of using cellular immunotherapy to treat lung rationale for a novel paradigm shifting approach of units cellular immunotherapy to treat and of
fibrosis.

Introduction

Pulmonary fibrosis is a devastating disease with no effective treatments to cure, stop or reverse the unremitting, fatal fibrosis. A critical barrier to treating this disease is the lack of
understanding of the pathways leading to fibrosis as well as those regulating the resolution of
fibrosis. Pulmonary fibrosis is th understanding of the pathways leading to fibrosis as well as those regulating the resolution of
fibrosis. Pulmonary fibrosis is the common end point for a diverse group of disorders such as
chronic hypersensitivity pneumo monical pullmonary fibrosis is the common end point for a diverse group of disorders such as
chronic hypersensitivity pneumonitis, idiopathic pulmonary fibrosis, silicosis, radiation
pneumonitis, and collagen vascular dise

fibrosic hypersensitivity pneumonitis, idiopathic pulmonary fibrosis, silicosis, radiation
pneumonitis, and collagen vascular diseases^{1,2}
Fibrosis is the pathologic side of normal tissue repair that results when the norm pneumonitis, and collagen vascular diseases^{1,2}
Fibrosis is the pathologic side of normal tissue repair that results when the normal wound
healing programs go awry. Successful resolution of tissue injury requires not onl Fibrosis is the pathologic side of normal tissue repair that results when the normal wound Finaling programs go awry. Successful resolution of tissue injury requires not only the activation
of effector cells and the marked increase in synthesis and deposition of extracellular matrix
(ECM), but also the deactivat of effector cells and the marked increase in synthesis and deposition of extracellular matrix (ECM), but also the deactivation of these effector cells and the clearance of excess ECM to allow return to normal lung structu (ECM), but also the deactivation of these effector cells and the clearance of excess ECM to allow return to normal lung structure and function¹. Thus, fibrosis may result from deviations in one or several of these highl allow return to normal lung structure and function¹. Thus, fibrosis may result from deviations in
one or several of these highly coordinated pathways and this research focuses on the interplay
between these overlapping p allow return to normal lung structure and function⁺. Thus, fibrosis may result from deviations in
one or several of these highly coordinated pathways and this research focuses on the interplay
between these overlapping p

one or several or these overlapping pathways: immune effectors, inflammatory mediators and
fibroproliferation in the resolution of fibrosis.
Supporting the notion of local immune cells interacting with local native lung ce fibroproliferation in the resolution of fibrosis.

Supporting the notion of local immune cells interacting with local native lung cells is our novel

published data demonstrating the critical role of tissue resident memor Supporting the notion of local immune cells in
published data demonstrating the critical ro
resolution of established lung fibrosis^{3,4}. We l
immune response that directs unremitting fil
can be prevented, arrested and ev S Frict published data demonstrating the critical role of tissue resident memory (TRM) cells in the resolution of established lung fibrosis^{3,4}. We have shown that fibrosis is due to a dysregulated immune response that directs u published lung fibrosis^{3,4}. We have shown that fibrosis is due to a dysregulated
immune response that directs unremitting fibroproliferation and that this pathogenic process
can be prevented, arrested and even reversed immune response in the lungs^{3,4}. That is, the induction of robust Th1 TRM cells in the lungs can
T cell response in the lungs^{3,4}. That is, the induction of robust Th1 TRM cells in the lungs can
negatively regulate fibr

T cell response in the lungs^{3,4}. That is, the induction of robust Th1 TRM cells in the lungs can negatively regulate fibrosis and re-establish tissue homeostasis^{3,4}.
While clearly there is a "point of no return" with T cell response in the lungs^{3,4}. That is, the induction of robust Th1 TRM cells in the lungs can negatively regulate fibrosis and re-establish tissue homeostasis^{3,4}.
While clearly there is a "point of no return" with negatively regulate fibrosis and re-establish tissue homeostasis^{3,4}.
While clearly there is a "point of no return" with regard to organ f
clear that lung damage characterized by increased ECM, fik
inflammation may not o ヽヽ ci ドc ド Clear that lung damage characterized by increased ECM, fibroproliferation, and chronic inflammation may not only be arrested but also may be reversed by allowing the normal pathways of wound repair to do their job^{4,5}. U inflammation may not only be arrested but also may be reversed by allowing the normal
pathways of wound repair to do their job^{4,5}. Upon lung infection, TRMs play a critical role in not
only directing the anti-pathogen r pathways of wound repair *to do their job^{4,5}*. Upon lung infection, TRMs play a critical role in not only directing the anti-pathogen response but also in the resolution of inflammation leading to preservation of lung a pathways of wound repair to do their job^{4,5}. Upon lung infection, TRMs play a critical role in not
only directing the anti-pathogen response but also in the resolution of inflammation leading to
preservation of lung arc preservation of lung architecture and function⁶⁻⁸. In addition to playing a critical role in the recall response to pathogens, numerous studies have demonstrated the ability of TRMs to mediate protection against tissue mediate protection against tissue specific challenges such as viral, bacterial, and parasitic
infections ⁶⁻⁸. This protection is achieved by the interaction of TRMs with cells of the adaptive
and innate immune response t infections ⁶⁻⁸. This protection is achieved by the interaction of TRMs with cells of the adaptive
and innate immune response to collectively coordinate and promote immunity and to dictate
the local inflammatory response the local inflammatory response^{9,10}. This local response in turn may affect the recruitment,
proliferation, and activation of not only immune cells but also resident lung cells.
The rationale behind the use of immunother

the local inflammatory response^{9,10}. This local response in turn may affect the recruitment,
proliferation, and activation of not only immune cells but also resident lung cells.
The rationale behind the use of immunother promation, and activitive eventually infinitive cells and also spaced on literature concerning the role of inflammation in coordinating not only the immunt also the response of resident cells in fibrosis¹. Although it is ך
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| ד Interature concerning the role of inflammation in coordinating not only the immune response
but also the response of resident cells in fibrosis¹. Although it is the unregulated
fibroproliferation that leads to fibrosis, but also the response of resident cells in fibrosis¹. Although it is the unregulated fibroproliferation that leads to fibrosis, the mechanisms of fibroblast/myofibroblast recruitment and activation remain poorly understo but also the response of resident cells in fibrosis⁺. Although it is the unregulated
fibroproliferation that leads to fibrosis, the mechanisms of fibroblast/myofibroblast
recruitment and activation remain poorly understo recruitment and activation remain poorly understood. Our group and others have identified a recruitment and activation remain poorly understood. Our group and others have identified a

role for IL-17 and Th17 cells in promoting the inflammation leading to fibrosis^{3,4,11-13}. In contrast,
Th1 immune responses in the lung are associated with resolution of inflammation^{3,4}. Thus, we
sought a mechanism by sought a mechanism by which we could skew the lung environment away from the pro-fibrotic
Th17 toward a pro-resolution Th1 environment. Given the successful use of immunotherapy in
treating cancer, we sought to apply this Th17 toward a pro-resolution Th1 environment. Given the successful use of immunotherapy in
treating cancer, we sought to apply this knowledge to the lungs^{3,14-18}. Indeed, we previously
have successfully prevented, mitig treating cancer, we sought to apply this knowledge to the lungs^{3,14-18}. Indeed, we previously
have successfully prevented, mitigated, and even reversed established fibrosis using
immunotherapy in the form of vaccina vac treating cancer, we sought to apply this knowledge to the lungs^{3,44-2}. Indeed, we previously
have successfully prevented, mitigated, and even reversed established fibrosis using
immunotherapy in the form of vaccina vacc immunotherapy in the form of vaccina vaccination in two models of murine lung fibrosis^{3,4}. In
this study, we further demonstrate that not only does vaccinia induce TRMs that can reverse
fibrosis but so do other vaccines This study, the this study of the vaccines such as influenza vaccine (FluMist®). Furthermore, we have
been able to isolate the population of vaccine induced TRMs – CD49a⁺ CD4⁺ T cells - that are
both necessary and suff fibre a ble to isolate the population of vaccine induced TRMs – CD49a⁺ CD4⁺T cells - that are
both necessary and sufficient to reverse established pulmonary fibrosis. In fact, using adoptive
cellular therapy we demons been able to isolate the population of vaccine induced TRMs – CD49a⁻ CD4' T cells - that are
both necessary and sufficient to reverse established pulmonary fibrosis. In fact, using adoptive
cellular therapy we demonstrat cellular therapy we demonstrate that intratracheal administration of CD49a⁺ CD4⁺ TRMs into established fibrosis, reverses the fibrosis histologically, by promoting a decrease in collagen levels, and functionally, by im CD4 TRMs into
ease in collagen
for vaccination.
an IPF fibroblasts
roblast collagen
nat CD49a⁺ CD4⁺
in IPF lungs, thus levels, and functionally, by improving lung function, without the need for vaccination.
Furthermore, co-culture of *in vitro* derived CD49⁺CD4⁺ human TRMs with human IPF fibroblasts
results in the down regulation of id Furthermore, co-culture of *in vitro* derived CD49⁺CD4⁺ human TRMs with human IPF fibroblasts
results in the down regulation of idiopathic pulmonary fibrosis (IPF) fibroblast collagen
production. Lastly, we demonstrat Furthermore, co-culture of *in vitro* derived CD49 CD4
results in the down regulation of idiopathic pulm
production. Lastly, we demonstrate in human IPF lu
TRMs, which can down regulate human IPF fibroblast f
potentially f onary fibrosis (IPF) fibroblast collagen
ng histologic samples that CD49a⁺ CD4⁺
unction, fail to increase in IPF lungs, thus
performation to the set of production. Lastly, we demonstrate in human IPF lung histologic samples that CD49a⁺ CD4⁺
TRMs, which can down regulate human IPF fibroblast function, fail to increase in IPF lungs, thus
potentially failing to promote

production. Lastly, we demonstrate in human IPF lung histologic samples that CD49a⁻ CD4⁻
TRMs, which can down regulate human IPF fibroblast function, fail to increase in IPF lungs, thus
potentially failing to promote r potentially failing to promote resolution.

In this paper, we define a novel unappreciated role for tissue resident memory T cells in

regulating established lung fibrosis to promote resolution of fibrosis and re-establish pote the control of the this paper, we define a novel unappregulating established lung fibrosis to promote that immum CD4⁺ TRMs into the lungs of mice with explore the subsetion. | r + c f F -In the paper, we demonstrate that immunotherapy, in the form of fibrosis and re-establish lung
homeostasis. We demonstrate that immunotherapy, in the form of adoptive transfer of CD49a⁺
CD4⁺ TRMs into the lungs of mice regulating established in promoting the form of adoptive transfer of CD49a⁺
CD4⁺ TRMs into the lungs of mice with established fibrosis, not only stops progression of the
fibrosis but more importantly reverses the fibro CD4⁺ TRMs into the lungs of mice with established fibrosis, not only stops progression of the fibrosis but more importantly reverses the fibrosis. These studies provide the insight and preclinical rationale for a novel p CD4' TRMs into the lungs of mice with established fibrosis, not only stops progression of the
fibrosis but more importantly reverses the fibrosis. These studies provide the insight and
preclinical rationale for a novel par fibrositional rationale for a novel paradigm shifting approach of using cellular immunotherapy to treat lung fibrosis.
These studies fibrosis. preclinical rationale for a novel paradigm shifting approach of using cellular intervals of using cellular in
munotherapy to using cellular intervals of using cellular intervals of using cellular intervals of using cellula treat lung fibrosis.
The contract lung fibrosis is a set of the contract lung fibrosis.

Materials and Methods

 $\frac{1}{4}$ ($\frac{1}{4}$) C57 BL/6 were purchased from The Jackson Laboratory. 8 to 10-week-old female and male mice were utilized.

Reagents

 $\overline{C57B}$
mice
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BD Biosci ankara virus is a modified vaccinia that contains the full-length ovalbumin protein but lacks lytic
ability. Quadrivalent FluMist® was purchased from AstraZeneca (Wilmington, DE). Bleomycin
was purchased from App Pharmaceu ability. Quadrivalent FluMist® was purchased from AstraZeneca (Wilmington, DE). Bleomycin
was purchased from App Pharmaceuticals (Schumburg, IL). PMA and lonomycin were
purchased from Sigma-Aldrich (St. Louis, MO). Flow cy was purchased from App Pharmaceuticals (Schumburg, IL). PMA and lonomycin were purchased from Sigma-Aldrich (St. Louis, MO). Flow cytometry reagents were purchased from BD Biosciences (Franklin Lakes, NJ). Antibodies utili purchased from Sigma-Aldrich (St. Louis, MO). Flow cytometry reagents were purchased from
BD Biosciences (Franklin Lakes, NJ). Antibodies utilized were from Biolegend (San Diego, CA),
see Supplemental Table 1.
Cell Lines
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purchased from Sigma-Aldrich (St. 2006), Morphology (St. 2007), Congeling More purchased (San Diego, CA),
see Supplemental Table 1.
Cell Lines
Age matched normal and IPF lung fibroblasts were purchased from BioIVT (Westbur See Supplemental Table 1.

See Supplemental Table 1.

Age matched normal and IPF lung fibroblasts were purchased from BiolVT (Westbury, NY).

Fibroblasts were maintained in DMEM supplemented with 10% FBS. For all coculture Cell Lines
Age matched normal and
Fibroblasts were maintaine
fibroblasts were used betw
lymphocytes were isolated <u>(C</u> /
| f |
| s | Age matches

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FBS, antik Fibroblasts were maintained in DMEM supplemented with 10% FBS. For all coculture experiments
fibroblasts were used between passages 3-6. To generate human CD49a⁺ and CD49a⁻ CD4⁺ T cells,
lymphocytes were isolated fro Fibroblasts were used between passages 3-6. To generate human CD49a⁺ and CD49a⁻ CD4⁺ T cells, lymphocytes were isolated from buffy coats of normal donor leukopaks. Bulk lymphocytes were stimulated with anti-CD3/antifibroblasts were used between passages 3-6. To generate human CD49a' and CD49a CD4' T cells,
lymphocytes were isolated from buffy coats of normal donor leukopaks. Bulk lymphocytes were
stimulated with anti-CD3/anti-CD28 (M stimulated with anti-CD3/anti-CD28 (Miltenyi Biotech) in RPMI media supplemented with 10%
FBS, antibiotic, and glutamine. Two days later human IL2 (Peprotech, East Windsor, NJ), was
added at 10 ng/mL. Cultures were maintai FBS, antibiotic, and glutamine. Two days later human IL2 (Peprotech, East Windsor, NJ), was

FBS, and the glutamine of the later hand the complete the control of media as needed and
addition of IL2 every 7 days.
Coculture Experiments
Fibroblasts were harvested and 50,000 were plated per well of a 24 well transwell addition of IL2 every 7 days.

Coculture Experiments

Fibroblasts were harvested and 50,000 were plated per well of a 24 well transwell plate (Corning,

Corning, NY.) Fibroblasts were allowed to adhere for 6 hours before a Coculture Experiments

Fibroblasts were harvested a

Corning, NY.) Fibroblasts w

human lymphocytes in cult

CD4⁺ T cells were then isola くりくりく Fibroblasts were harves
Corning, NY.) Fibrobla
human lymphocytes in
CD4⁺ T cells were then
cells were isolated by m
mouse lgG microbeads Corning, NY.) Fibroblasts were allowed to adhere for 6 hours before addition of T cells. Live
human lymphocytes in culture were isolated by Ficoll-Paque (Fisher Scientific, Waltham, MA).
CD4⁺ T cells were then isolated human lymphocytes in culture were isolated by Ficoll-Paque (Fisher Scientific, Waltham, MA).
CD4⁺ T cells were then isolated by negative magnetic isolation (Biolegend). CD49a⁺ and CD49a⁻
cells were isolated by magnet CD4⁺ T cells were then isolated by negative magnetic isolation (Biolegend). CD49a⁺ and CD49a⁻ cells were isolated by magnetic isolation using Anti-CD49a purified antibody (Biolegend) and anti-mouse IgG microbeads (M Well plates in DMEM media supplemented with 10pg/mL human IL2. Cocultures were incubated
overnight, followed by addition of 10ng/mL TGFB (Peprotech). 24 hours later fibroblast RNA was
harvested.
All Flow cytometry
All Flow overnight, followed by addition of 10ng/mL TGFB (Peprotech). 24 hours later fibroblast RNA was
harvested.
<u>Flow cytometry</u>
All Flow cytometry analysis was performed on a BD FACSCelesta (BD Biosciences) and analyzed
using F

cells were interested by magnetic isolation using Anti-CD49a purified anti-CD49a purification and anti-CD49a purified and anti-CD49a purification and anti-CD49a purification and anti-CD49a purification and anti-CD49a purif Flow cytometry
harvested.
All Flow cytometry
All Flow cytometry analysis was performed on a BD FACSCelesta (BD Biosciences) and analyzed
using FlowJo software (TreeStar Inc, Ashland, OR). Flow cytom
All Flow cy
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All Flow cytome
using FlowJo sof
Pulmonary fibro
Mice were injer All Financy Controlly analyzed one performed on a BD FACSCELEST (Controller pullinomary fibrosis model.

Mice were injected i.p. with 0.8 units bleomycin on days 0, 3, 7, 14, 21, and 28 to induce

pulmonary fibrosis. For s Pulmonary fibrosis model.

Mice were injected i.p. with 0.8 units bleomyci

pulmonary fibrosis. For some experiments mice

of .018 units in 25ul PBS. 上げ ドく Mice were injected i.p. v
pulmonary fibrosis. For so
of .018 units in 25ul PBS. pulmonary fibrosis. For some experiments mice received bleomycin intratracheally at a dose
of .018 units in 25ul PBS.
The State of Network on details and 25 to induce to induce the conduction of the conduction of the condu pulmonary fibrosis. For some experiments microsister at a dose received bleomycin interaction in the central ℓ of ℓ and ℓ at a dose ℓ at a dose ℓ at a dose ℓ and ℓ at a dose ℓ and ℓ and ℓ and

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Quadrivalen
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(H1N1) (A/
(A/Darwin/9
B/Austria/13 Quadrivalent FluMist® was administered intranasally at a dose of 10^{5.5-6.5} fluorescent focus units
of live attenuated influenza virus reassortants of each of the four strains: A/Victoria/1/2020
(H1N1) (A/Victoria/2570/20 (H1N1) (A/Victoria/2570/2019 (H1N1) pdm09-like virus), A/Norway/16606/2021 (H3N2)
(A/Darwin/9/2021 (H3N2)-like virus), B/Phuket/3073/2013 (Yamagata lineage), and
B/Austria/1359417/2021 (Victoria lineage).
<u>ELISA</u>
TNFα and (A/Darwin/9/2021 (H3N2)-like virus), B/Phuket/3073/2013 (Yamagata lineage), and
B/Austria/1359417/2021 (Victoria lineage).
ELISA
TNFα and IFNγ ELISA were purchased from ebioscience (San Diego, CA), and performed
according

ELISA

ELISA

TNFα and IFNy ELISA were purchased from ebioscience (San Diego, CA), and performed

according to manufacturer's instructions. $ELSA$
TNF α and IFNy ELISA were purchased fraccording to manufacturer's instructions.
Histology 【1 ~
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TNFα
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<u>Histole</u>
Lungs
H&E a THE AND INY ELITE WEEP PROMISED WITH ENTERTMIC (SAN DIGG) (SAN PICGO) AND PERFORMED
according to manufacturer's instructions.
Histology
Lungs were inflated to 27cm H₂O with 10% neutral-buffered formalin, sectioned and st Histology
Lungs were inflated to 27cm H₂O with 109
H&E and Masson's trichrome according t
analyzed by microscope at 40x magnificat |
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|-Lungs were
H&E and
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RNA Extra
Total lung

Lungs were analyzed by microscope at 40x magnification.

RNA Extraction and Real Time PCR

Total lung RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and reversed

Total lung RNA was extracted using TRIzo H&E and Masson's trichrome according to previously established procedures⁴⁵. Samples were
analyzed by microscope at 40x magnification.
Total lung RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and reve RNA Extraction and Real Time PCR
Total lung RNA was extracted using TRIzol
transcribed by Reliance (Bio-Rad Laborato
protocol. Real-time PCR was performed on
Applied Biosystems reagents (Carlsbad, CA IT t Fノ Total lung RNA was extracted us
transcribed by Reliance (Bio-Rac
protocol. Real-time PCR was perf
Applied Biosystems reagents (Ca
calculated using the delta Ct me
primers and probe sets used were transcribed by Reliance (Bio-Rad Laboratories, Hercules, CA) as per the manufacturer's protocol. Real-time PCR was performed on an Applied Biosystems 7300 PCR machine using Applied Biosystems reagents (Carlsbad, CA) and no Applied Biosystems reagents (Carlsbad, CA) and normalized to 18s rRNA. Values were
calculated using the delta Ct method in reference to control samples for each primer. All
primers and probe sets used were purchased from A realculated using the delta Ct method in reference to control samples for each primer. All
primers and probe sets used were purchased from Applied Biosystems (Carlsbad, CA).
RNAseq analysis
Wild type mice were vaccinated i

primers and probe sets used were purchased from Applied Biosystems (Carlsbad, CA).

RNAseq analysis

Wild type mice were vaccinated intranasally with FluMist, and 4 weeks later *in vivo* labeled

CD49a⁺ CD4⁺ lung TRM a primers and presents and were purchased from ppins.
Primers analysis
Wild type mice were vaccinated intranasally with FluMist, and 4 weeks later in vive
CD49a⁺ CD4⁺ lung TRM and CD49a⁻ CD4⁺ lung TRM were sorted int |
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| Wild type mice
CD49a⁺ CD4⁺ lun
and subjected tc
gene expression
expressed genes いくこきり CD49a⁺ CD4⁺ lung TRM and CD49a⁻ CD4⁺ lung TRM were sorted into Trizol. RNA was isolated and subjected to RNA seq analysis. Alignments were performed using Hisat2, and differential gene expression was performed usi CD49a' CD4' lung TRM and CD49a CD4'
and subjected to RNA seq analysis. Align
gene expression was performed using D
expressed genes in CD49a⁺ CD4⁺ lung TR
predicted protein-protein interactions. Cl
which contained 25 up ments were performed using Hisat2, and differential
ESeq2 and BiomaRt. The top 500 most differentially
Ms were submitted to STRING analysis of known and
ustering of upregulated proteins resulting in a cluster
with a small and subjected to RNA september 2018 DESeq 2 and BiomaRt. The top 500 most differentially
expressed genes in CD49a⁺ CD4⁺ lung TRMs were submitted to STRING analysis of known and
predicted protein-protein interactions. C expressed genes in CD49a⁺ CD4⁺ lung TRMs were submitted to STRING analysis of known and
predicted protein-protein interactions. Clustering of upregulated proteins resulting in a cluster
which contained 25 upregulated g

predicted present interactions of upregainted present estandon which contained 25 upregulated genes with a small PPI enrichment p-value (< 10e-16), indicating a high likelihood that the proteins are biologically connected. indicating a high likelihood that the proteins are biologically connected.

Analysis of scRNAseq data

Processed scRNAseq data for human lungs with fibrosis were obtained from the Gene

Expression Omnibus database (access Analysis of scRNAseq data
Processed scRNAseq data for human lungs with fibrosis were ob
Expression Omnibus database (accession number GSE16831)²⁰. Are
clustered using the R software package Seurat (v.4.2). CD49a⁺ TRM \ とり にくい The Controller Controller

Repression Commibus data

Contractor Controller Controller

CD69, and ITGA1 (CD49a)

Were identified by CD4, C Expression Omnibus database (accession number GSE16831)²⁰. Annotated T cells were clustered using the R software package Seurat (v.4.2). CD49a⁺ TRM were identified by CD4, CD69, and ITGA1 (CD49a) expression (log-trans clustered using the R software package Seurat (v.4.2). $CD49a⁺ TRM$ were identified by CD4, clustered using the R software package Seurat (v.4.2). CD49a' TRM were identified by CD4,
CD69, and ITGA1 (CD49a) expression (log-transformed expression levels > 0.5). CD103a⁺ TRM
were identified by CD4, CD69, and ITGAE CD69, and ITGA1 (CD49a) expression (log-transformed expression levels > 0.5). CD103a[·] TRM
were identified by CD4, CD69, and ITGAE (CD103) expression. TRMs were plotted as % of
of were identified by CD49, CD49, CD69, and ITGAE (CD103) expression. The plotted as were plotted as \mathcal{L}

In vivo antibody labeling and sorting

annotated T cells in IPF (n=17) and non-fibrotic control (n=10) lungs. Lungs with fewer than 200 CD4⁺ T cells or with no CD49a⁺ or CD103⁺ CD4+ T cells were excluded.

<u>In vivo antibody labeling and sorting</u>

For *in* CD4' T cells or with no CD49a' or CD103' CD4+ T cells were excluded.

In vivo antibody labeling and sorting

For *in vivo* antibody labeling, mice were injected i.v. with 2.5 μ

antibody (clone RM4-5), and after 10 min For *in vivo* antibody labeling, mic
antibody (clone RM4-5), and after 1
in a mixture of 3mg/mL Collagenase
single cell suspensions, and CD4⁺ T c
Biotech (Auburn, CA). Isolated lyn
noncompeting clone of APC-anti-C For *in vivo* antibody labeling, line were injected i.v. with 2.5 μg PE-conjugated anti-CD4
antibody (clone RM4-5), and after 10 minutes, lungs were isolated, rinsed in PBS, and digested
in a mixture of 3mg/mL Collagenase antial antiture of 3mg/mL Collagenase and Dnase I for 45 minutes at 37°C. Cells were filtered into
single cell suspensions, and CD4⁺ T cells were positively selected by magnetic isolation (Miltenyi
Biotech (Auburn, CA). single cell suspensions, and CD4⁺ T cells were positively selected by magnetic isolation (Miltenyi
Biotech (Auburn, CA). Isolated lymphocytes were then stained *in vitro* with a different,
noncompeting clone of APC-anti Biotech (Auburn, CA). Isolated lymphocytes were then stained *in vitro* with a different, noncompeting clone of APC-anti-CD4 (clone RM4-4), along with antibodies to other surface markers with fluorochrome-conjugated antib Iymphocytes were then stained *in vitro* with a different,
ti-CD4 (clone RM4-4), along with antibodies to other surface
ijugated antibodies. Stained cells were sorted using a BD
in Lakes, NJ). CD4⁺ T cells which were si Biotech (Auburn, CA). Isolated lymphocytes were then stained in vitro with a different,
noncompeting clone of APC-anti-CD4 (clone RM4-4), along with antibodies to other surface
markers with fluorochrome-conjugated antibodi markers with fluorochrome-conjugated antibodies. Stained cells were sorted using a BD
FacsAria (BD Biosciences, Franklin Lakes, NJ). CD4⁺ T cells which were singly stained with APC
anti-CD4 *in vitro* but not stained wi FacsAria (BD Biosciences, Franklin Lakes, NJ). CD4⁺ T cells which were singly stained with APC
anti-CD4 *in vitro* but not stained with PE anti-CD4 *in vivo* were considered to be tissue resident.
For TRM transfer experi FacsAria (BD Biosciences, Franklin Lakes, NJ). CD4' T cells which were singly stained with APC
anti-CD4 *in vitro* but not stained with PE anti-CD4 *in vivo* were considered to be tissue resident.
For TRM transfer experime

For TRM transfer experiments, sorted T cells were resuspended in PBS at $1x10^6$ per ml and mice
received 50ul intratracheally.
Diffusion factor for CO measurement.
To assess the efficiency of gas exchange in the lungs fo Diffusion factor for CO measurement.
To assess the efficiency of gas exchange in the lungs following bleomycin-induced injury,
measurement of the diffusion factor for CO (DFCO) was performed as described previously²¹.
B Diffusion factor for CO measur
To assess the efficiency of
measurement of the diffusion
Briefly, mice were anesthetize
i.p. injection. Once sedated, <u>Diffusion factor for CO measurement</u>.
To assess the efficiency of gas exc
measurement of the diffusion factor
Briefly, mice were anesthetized with a
i.p. injection. Once sedated, mice w
lungs were quickly inflated with a measurement of the diffusion factor for CO (DFCO) was performed as described previously²¹.
Briefly, mice were anesthetized with a mixture of ketamine (100 mg/kg)/xylazine (15 mg/kg) via
i.p. injection. Once sedated, mice i.p. injection. Once sedated, mice were intubated with a 20-gauge IV angiocatheter. Mouse
lungs were quickly inflated with a 0.8 ml gas mixture (0.5% neon, 1% CO and 98.5% air). After a
9-second breath hold, 0.8 m of gas ilungs were quickly inflated with a 0.8 ml gas mixture (0.5% neon, 1% CO and 98.5% air). After a
9-second breath hold, 0.8 m of gas was quickly withdrawn from the lung and diluted to 2 ml
with room air. The neon and CO con 9-second breath hold, 0.8 m of gas was quickly withdrawn from the lung and diluted to 2 ml
with room air. The neon and CO concentrations in the diluted air were measured by gas
chromatography (INFICON, Model 3000A) to asse with room air. The neon and CO concentrations in the diluted air were measured by gas
chromatography (INFICON, Model 3000A) to assess DFCO. The dilution to 2 ml was needed,
since the gas chromatograph required a minimal sa

with restrict and VINFICON, Model 3000A) to assess DFCO. The dilution to 2 ml was needed,
since the gas chromatograph required a minimal sample size of 1 ml.
Pulmonary Mechanics Measurements
After DFCO assessment, mice wer chromatography (interest), the distribution of the significant in the distribution since the gas chromatograph required a minimal sample size of 1 ml.

Pulmonary Mechanics Measurements

After DFCO assessment, mice were con Pulmonary Mechanics Measurements
After DFCO assessment, mice were connected to a flexi-Vent ventilat
with a tidal volume of 0.2 ml of 100% oxygen at a rate of 150 Hz. with
pressure (PEEP) of 3 cmH₂O. Mice were subjected トノ ドミミ After DFCO assessment, mice were co
with a tidal volume of 0.2 ml of 100%
pressure (PEEP) of 3 cmH₂O. Mice v
seconds and returned to normal ventil
system resistance (Rrs), compliance
oscillation technique via the SnapSho with a tidal volume of 0.2 ml of 100% oxygen at a rate of 150 Hz. with a positive end–expiratory
pressure (PEEP) of 3 cmH₂O. Mice were subjected to deep inspiration at 30 cmH₂O for 5
seconds and returned to normal ven pressure (PEEP) of 3 cmH₂O. Mice were subjected to deep inspiration at 30 cmH₂O for 5 seconds and returned to normal ventilation for 1 minute. Baseline measurements of respiratory system resistance (Rrs), compliance (presents and returned to normal ventilation for 1 minute. Baseline measurements of respiratory
system resistance (Rrs), compliance (Crs) and elastance (Ers) were measured using forced
oscillation technique via the SnapShot system resistance (Rrs), compliance (Crs) and elastance (Ers) were measured using forced
oscillation technique via the SnapShot perturbation, a single 2.5-Hz sinusoidal waveform which
is fit to the single compartment model sponsillation technique via the SnapShot perturbation, a single 2.5-Hz sinusoidal waveform which
is fit to the single compartment model via linear regression. Following measurements
angiocatheters were removed and mice wer

or the single compartment model via linear regression. Following measurements
angiocatheters were removed and mice were observed until they woke up from anesthesia.
Immunofluorescence Staining
Formalin-fixed, paraffin-embe angiocatheters were removed and mice were observed until they woke up from anesthesia.

Immunofluorescence Staining

Formalin-fixed, paraffin-embedded (FFPE) human IPF and normal tissue slides were incubated

with primary anglem
Immunofluorescence Staining
Formalin-fixed, paraffin-embedded (FFPE) human IPF and normal tissue slides were incubate
with primary antibody to CD4, CD49a, and CD103 in 5% goat serum, 0.5% BSA, in PBS at 4°C
overnigh ||
|}
|} Immunofluorescence Staining
Formalin-fixed, paraffin-embedded (FFPE) human IPF and normal tissue slides were incubated
with primary antibody to CD4, CD49a, and CD103 in 5% goat serum, 0.5% BSA, in PBS at 4°C
overnight. Ima with primary antibody to CD4, CD49a, and CD103 in 5% goat serum, 0.5% BSA, in PBS at 4°C
overnight. Images were collected at x20 magnification using a Nikon Eclipse 80i microscope an
Nikon DS-fi1 camera.
<u>Statistical Analy</u> $\frac{1}{2}$ overing in angle at 20 checked at x20 magnification using a nikon Engles 80i microscope and
Nikon DS-fi1 camera.
Statistical Analysis

<u>Statistical Analysis</u>
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All experiments were performed in biological triplicate and results represent the mean \pm standard deviation. All experiments were replicated at least three times. Statistical analysis was performed using either 1-way A performed using either 1-way ANOVA followed by Tukey's test or a paired Student's tperformed using either 1-way ANOVA followed by Tukey's test or a paired Student's*t-*
test; p<0.05 was considered statistically significant.
Results
CD49a and CD103 expression differentiates populations of lung CD4⁺ TR

Results

CD49a and CD103 expression differentiates populations of lung CD4+

test; p<0.05 was considered statistically significant.
Results
CD49a and CD103 expression differentiates popula
We previously demonstrated that immuno
virus induced a nool of lung, tissue resident, me We previously demonstrated that immunotherapy in the form of intranasal vaccinia virus induced a pool of lung, tissue resident, memory Th1 CD4⁺ T cells in mice^{3,4}. We also
demonstrated that generation of lung TRM was required to mitigate bleomycin induced
pulmonary fibrosis^{3,4}. To further unders virus induced a pool of lung, tissue resident, memory Th1 CD4⁺ T cells in mice^{3,4}. We also
demonstrated that generation of lung TRM was required to mitigate bleomycin induced
pulmonary fibrosis^{3,4}. To further underst pulmonary fibrosis^{3,4}. To further understand the effect of immunotherapy induced lung TRM in
mice we intranasally administered PBS, quadrivalent FluMist[®], or vaccinia virus. To analyze TRM
generation during the develo pulmonary fibrosis^{3,42}. To further understand the effect of immunotherapy induced lung TRM in
mice we intranasally administered PBS, quadrivalent FluMist®, or vaccinia virus. To analyze TRM
generation during the developm generation during the development of fibrosis we also treated a group of mice with
intratracheal bleomycin. Twenty-eight days later we intravenously labeled circulating T cells
with CD4 antibody to identify and isolate lu intratracheal bleomycin. Twenty-eight days later we intravenously labeled circulating T cells
with CD4 antibody to identify and isolate lung tissue resident CD4⁺ T cells. Mice were sacrificed
and lungs were removed, pro with CD4 antibody to identify and isolate lung tissue resident CD4⁺ T cells. Mice were sacrificed
and lungs were removed, processed to single cell suspensions, and stained for CD4 and the
surface markers CD49a and CD103 surface markers CD49a and CD103. Lung CD4⁺ T cells which were not stained *in vivo* and thus
only singly stained with CD4 *in vitro* were considered tissue resident. CD49a⁺ and CD103⁺ CD4⁺
lung TRM were sorted and

surface markers CD49a and CD103. Lung CD4' T cells which were not stained *in vivo* and thus
only singly stained with CD4 *in vitro* were considered tissue resident. CD49a⁺ and CD103⁺ CD4⁺
lung TRM were sorted and st only singly stained with CD4 *in vitro* were considered tissue resident. CD49a⁻ and CD103⁻ CD4⁻

lung TRM were sorted and stimulated for 4 hours to perform intracellular cytokine staining (ICS)

TRM from bleomycin tr $\ddot{}$ TRM from bleomycin treated mice were predominantly CD103⁺, whereas TRM
from vaccinated mice were predominantly CD49a⁺ (Figure 1a, b). PBS treated mice also had
predominantly CD49a⁺ TRM, however the percentages and t from vaccinated mice were predominantly CD49a' (Figure 1a, b). PBS treated mice also had
predominantly CD49a⁺ TRM, however the percentages and total numbers of these cells were
significantly lower than vaccinated mice. I significantly lower than vaccinated mice. ICS analysis of sorted $CD4^+$ TRM demonstrated
increased expression of IL17a and minimal expression of IFNy by $CD103^+$ TRM regardless of how
mice were treated (Figure 1c). ICS an IFNy production was required for the therapeutic effect observed by vaccination induced significantly lower than vaccinated mice. ICS analysis of sorted CD4⁻ TRM demonstrated
increased expression of IL17a and minimal expression of IFNy by CD103⁺ TRM regardless of how
mice were treated (Figure 1c). ICS an IFNY production was required for the therapeutic effect observed by vaccination induced

TRM^{3,4}. Based on this finding and these ICS data, we hypothesize that CD49a⁺ CD4⁺ TRM are

responsible for the therapeutic eff

CD49a⁺ CD4⁺ TRM are therapeutic in pulmonary fibrosis CD4+ TRM are therapeutic in pulmonary fibrosis

TRM^{3,4}. Based on this finding and these ICS data, we hypothesize that CD49a⁺ CD4⁺ TRM are
responsible for the therapeutic effect of vaccination on fibrosis resolution in the lung.
CD49a⁺ CD4⁺ TRM are therapeuti TRM^{3,4}. Based on this finding and these ICS data, we hypothesize that CD49a⁺
responsible for the therapeutic effect of vaccination on fibrosis resolution in the
CD49a⁺ CD4⁺ TRM are therapeutic in pulmonary fibros CD4' TRM are
|ung.
|injected mice
|nong term
|in long term **CD49a^t CD4^t TRM are therapeutic in pulmonary fibrosis**
To further analyze the effect of CD49a^t CD4^t TRM on fibrosis we inject
intraperitoneally with bleomycin on days 0, 3, 7, 14, 21, and 28 to induce pulmonar
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s To further analyze the effect of CD49a⁺ CD4⁺ TRM on fibrosis we injected mice

ritoneally with bleomycin on days 0, 3, 7, 14, 21, and 28 to induce pulmonary fibrosis.

the intratracheal model of pulmonary fibrosis, th Unlike the intratracheal model of pulmonary fibrosis, this model results in long term
progressive lung fibrosis which better mimics human disease ⁵. To generate TRM we
intranasally vaccinated another cohort of mice with progressive lung fibrosis which better mimics human disease 5 . To generate TRM we
intranasally vaccinated another cohort of mice with FluMist® and 28 days later sorted lung CD4⁺
TRM into two populations, CD49a⁺ CD4 TRM into two populations, CD49a CD4 T cells, and CD49a CD4 T cells (figure 2a). We
intratracheally transferred 50,000 sorted CD49a⁺ or CD49⁻ CD4⁺ T cells into the IP bleomycin
treated mice 42 days after the initiatio intratracheally transferred 50,000 sorted CD49a' or CD49 CD4'
treated mice 42 days after the initiation of bleomycin treatmer
mice underwent pulmonary function testing. Mice that
demonstrated increased tissue resistance, d nt. Thirty days later, (day 72),
received CD49a⁻ CD4⁺ TRM
nce, and decreased diffusion mice underwent pulmonary function testing. Mice that received CD49a CD4⁺ TRM
demonstrated increased tissue resistance, decreased compliance, and decreased diffusion
demonstrated increased tissue resistance, decreased com demonstrated increased tissue resistance, decreased compliance, and decreased diffusion

capacity of carbon monoxide (CO) relative to PBS treated control mice (figure 2b). In contrast, mice which received CD49a⁺ CD4⁺ TRM demonstrated no notable change in tissue resistance, lung compliance or diffusion cap lung compliance or diffusion capacity relative to PBS control mice, indicating a therapeutic role resolution of the fibrosis. When we analyzed lung RNA for collagen expression, we observed for these TRM in preventing the progression of fibrosis but more importantly in promoting the
resolution of the fibrosis. When we analyzed lung RNA for collagen expression, we observed
significantly increased levels of co resolution of the fibrosis. When we analyzed lung RNA for collagen expression, we observed
significantly increased levels of collagen 1 and collagen 3 in the lungs of mice which received
CD49a⁻ CD4⁺ TRM. However, lung significantly increased levels of collagen 1 and collagen 3 in the lungs of mice which received
CD49a⁻ CD4⁺ TRM. However, lungs from mice which received CD49a⁺ CD4⁺ TRM failed to
demonstrate a significant increase Significant increase in collagen 1 or collagen 3 relative to PBS treated to demonstrate a significant increase in collagen 1 or collagen 3 relative to PBS treated mice.
These data again support a therapeutic role for CD49a

These data again support a therapeutic role for CD49a⁺ CD4⁺ TRM in preventing the progression
of pulmonary fibrosis (Figure 2c).
To further confirm the therapeutic role of CD49a⁺ CD4⁺ T cells in our model of bleomy These data again support a therapeutic role for CD49a

of pulmonary fibrosis (Figure 2c).

To further confirm the therapeutic role of CD49

and to rule out unforeseen effects on mice lung b

bleomycin injections, we perfor CD4⁺ TRM in preventing the progression
a⁺ CD4⁺ T cells in our model of bleomycin
nealth or experimental variability from
periment. We again induced pulmonary
performed baseline pulmonary function
of TRMs. Only mice w To further confirm the the
and to rule out unforeseen eff
bleomycin injections, we perform
fibrosis by repeat I.P. injections of
testing on mice at day 42 before a
decreased pulmonary function a To further confirm the therapeutic role of CD49a['] CD4['] T cells in our model of bleomycin

rule out unforeseen effects on mice lung health or experimental variability from

cin injections, we performed a time course ex bleomycin injections, we performed a time course experiment. We again induced pulmonary
fibrosis by repeat I.P. injections of bleomycin and then performed baseline pulmonary function
testing on mice at day 42 before any ad fibrosis by repeat I.P. injections of bleomycin and then performed baseline pulmonary function
testing on mice at day 42 before any adoptive transfer of TRMs. Only mice which demonstrated
decreased pulmonary function as d testing on mice at day 42 before any adoptive transfer of TRMs. Only mice which demonstrated
decreased pulmonary function as determined by increased tissue resistance, decreased lung
compliance, and decreased diffusion ca decreased pulmonary function as determined by increased tissue resistance, decreased lung
compliance, and decreased diffusion capacity were selected for CD4⁺ T cell transfer. To induce
CD4⁺ T cells for transfer, a dif compliance, and decreased diffusion capacity were selected for CD4⁺ T cell transfer. To induce CD4⁺ T cells for transfer, a different cohort of mice received intranasal FluMist® and 4 weeks later lung CD49a⁺ CD4⁺ later lung CD49a' CD4' TRM and CD49a⁻ CD4+ TRM were isolated by sorting. On day 49, mice
with decreased lung function received either no CD4⁺ T cells, 50,000 CD49a⁻ CD4⁺ TRM, or
50,000 CD49a⁺ CD4⁺ TRM intratrac with decreased lung function received either no CD4' T cells, 50,000 CD49a CD4' TRM, or
50,000 CD49a⁺ CD4⁺ TRM intratracheally. Mice were followed for 31 more days and on Day 80,
pulmonary function testing was again pe 50,000 CD49a² CD4² TRM intratracheally. Mice were followed for 31 more days and on Day 80, pulmonary function testing was again performed. Mice that received bleomycin without T cell transfer or bleomycin followed by C pulled in the member of bleomycin followed by CD49a⁻ CD4⁺ TRM demonstrated increased resistance, decreased lung compliance, and decreased diffusion capacity relative to their function observed at day 42 and to PBS cont transfer or bleomycin followed by CD49a⁻ CD4⁺ TRM demonstrated increased resistance,
decreased lung compliance, and decreased diffusion capacity relative to their function observed
at day 42 and to PBS controls, consis decreased lung compliance, and the continued development of lung fibrosis
(figure 2d). However, mice which received bleomycin followed by CD49a⁺ CD4⁺ TRM
demonstrated decreased tissue resistance, increased lung complia (figure 2d). However, mice which received bleomycin followed by $CD49a^+$ CD4⁺ TRM demonstrated decreased tissue resistance, increased lung compliance and increased diffusion capacity at day 80 relative to their function (figure 2d). However, mice which received bleomycin followed by CD49a⁻ CD4⁻ TRM
demonstrated decreased tissue resistance, increased lung compliance and increased diffusion
capacity at day 80 relative to their function expacity at day 80 relative to their function observed at day 42. More specifically, the mice that
received CD49a⁺ TRM had improved lung function between days 42 and 80 indicating reversal
of the progressive fibrosis see received CD49a⁺ TRM had improved lung function between days 42 and 80 indicating reversal
of the progressive fibrosis seen in the other experimental conditions (Figure 2d). Histology of
lungs isolated at day 100 confirme lungs isolated at day 100 confirmed our pulmonary function data, with lungs from mice which
received no T cells or CD49a CD4⁺ TRM demonstrating increased collagen deposition and
cellular infiltration, whereas lungs from received no T cells or CD49a⁻ CD4⁺ TRM demonstrating increased collagen deposition and cellular infiltration, whereas lungs from mice which received CD49a⁺ CD4⁺ TRM resembled lungs from PBS treated mice (figure 2e) received no T cells or CD49a-CD4' TRM demonstrating increased collagen deposition and
cellular infiltration, whereas lungs from mice which received CD49a⁺ CD4⁺ TRM resembled lungs
from PBS treated mice (figure 2e). The fibrosis as determined by increased resistance, decreased compliance, and decreased diffusion
capacity observed at day 42, transfer of CD49a⁺ CD4⁺ TRM is capable of halting and reversing
the development of pulmonary f capacity observed at day 42, transfer of CD49a⁺ CD4⁺ TRM is capable of halting and reversing
the development of pulmonary fibrosis as demonstrated by the improvements of lung function
observed at day 80.
Human CD49a⁺ CD4 TRM is capable of halting and reversing

nstrated by the improvements of lung function

gene expression

were therapeutic in our bleomycin model of

this was also occurring in human idiopathic

Human CD49a+ CD4+

the development of pulmonary fibrosis as demonstrated by the improvements of lung function
observed at day 80.
Human CD49a⁺ CD4⁺ T cells suppress profibrotic gene expression
Having shown that CD49a⁺ CD4⁺ T cells we the development of pulmonary fibrosis as dependent of pulmonary fibrosis as demonstrated by the improvements of
Human CD49a⁺ CD4⁺ T cells suppress profibrotic gene expression
pulmonary fibrosis, we wanted to determine Human CD49a⁺ CD4
Having show
pulmonary fibrosis,
pulmonary fibrosis.
human CD4⁺ T cells 计时间 T cells suppress profibrotic gene expression Having shown that CD49a' CD4

pulmonary fibrosis, we wanted to dete

pulmonary fibrosis. To accomplish this,

human CD4⁺ T cells and lung fibroblasts o

duman CD4⁺ T cells and lung fibroblasts o There is the therapeutic in the term, there is the term
mine if this was also occurring in human idiopathic
we elected to perform coculture experiments with
cultured from biopsies of IPF patients. We felt that this pulmonary fibrosis. To accomplish this, we elected to perform coculture experiments with
human CD4⁺ T cells and lung fibroblasts cultured from biopsies of IPF patients. We felt that this
human CD4⁺ T cells and lung fib pulmonary find the accomplishment of the perform of perform continues in the human CD4⁺ T cells and lung fibroblasts cultured from biopsies of IPF patients. We felt that this with the state of the state of the state of t human CD4+ The cells and lung fibroblasts cultured from biopsies of IPF patients. We felt that the this this this this th
This is the contract that the contract

experimental design was appropriate since we had observed decreased levels of collagen 1 and
3 expression in mice which received CD49a⁺ CD4⁺ T cells, and lung fibroblasts are known to be
significant producers of collag 3 expression in mice which received CD49a[.] CD4[.] T cells, and lung fibroblasts are known to be significant producers of collagen in the fibrotic lung.

To generate CD49a⁺ CD4⁺ T cells, human PBMCs were stimulated w To generate CD49a⁺ CD4⁺ T cells, human PBN
CD28 in the presence of interlukin-2 for four weeks.
then the cells were magnetically separated based o
resulted in the upregulation of CD49a on approxi
CD49a⁺ T cells were performed ICS analysis. Functionally CD49a⁺ CD4⁺ T cells produced more of the classical Th1 cytokines TNF α , granzyme b, and IFNy than CD49a⁻ CD4⁺ T cells(Figure 3b). CD28 in the presence of interlukin-2 for four weeks. CD4
then the cells were magnetically separated based on CD
resulted in the upregulation of CD49a on approximate
CD49a⁺ T cells were effectively isolated by magnetic s The magnetically action of PBMCs

Iy 25% of isolated CD4⁺ T cells, and

eparation (Figure 3a). To characterize

^F T cells with PMA and lonomycin and

s produced more of the classical Th1

T cells(Figure 3b). The cells were effectively isolated by magnetic separation (Figure 3a). To characterize

cD49a⁺ T cells were effectively isolated by magnetic separation (Figure 3a). To characterize

human CD49a⁺ CD4⁺ T cells, we st resulted in the upregulation of CD49a on approximately 25% of isolated CD4
CD49a⁺ T cells were effectively isolated by magnetic separation (Figure 3a). To
human CD49a⁺ CD4⁺ T cells, we stimulated isolated CD4⁺ T c characterize
nomycin and
classical Th1
vell coculture
ng fibroblasts

CD49a T cells were effectively isolated by magnetic separation (Figure 3a). To characterize
human CD49a⁺ CD4⁺ T cells, we stimulated isolated CD4⁺ T cells with PMA and lonomycin and
performed ICS analysis. Functiona from IPF patients. IL2 was added to stimulate the CD4[.] T cells and cells were incubated
overnight before the addition of TGFβ to induce profibrotic gene expression. Fibroblast RNA
was collected after 24 hours of TGFβ st vas collected after 24 hours of TGFβ stimulation. The addition of TGFβ induced a significant
increase in collagen 1, smooth muscle actin, and elastin gene expression (Figure 3c). Coculture
with CD49a CD4⁺ T cells had n increase in collagen 1, smooth muscle actin, and elastin gene expression (Figure 3c). Coculture
with CD49a⁻ CD4⁺ T cells had no effect on profibrotic gene expression relative to TGFβ addition
alone, however coculture with CD49a CD4⁺ T cells had no effect on profibrotic gene expression relative to TGFβ addition
alone, however coculture with CD49a⁺ CD4⁺ T cells resulted in a significant reduction in
profibrotic gene expression su alone, however coculture with CD49a⁺ CD4⁺ T cells resulted in a significant reduction in profibrotic gene expression supporting a role for these T cells in the setting of decreasing fibrosis. When we assayed coculture Fibrosis. When we assayed coculture supernatants for cytokines we observed increased levels
of TNF α and IFNy in wells to which CD49a⁺ CD4⁺ T cells were added. While overall TNF α levels
were barely detectable, th of TNFα and IFNγ in wells to which CD49a⁺ CD4⁺ T cells were added. While overall TNFα levels
were barely detectable, the levels of IFNγ were significantly increased in wells which contained
CD49a⁺ CD4⁺ T cells, co CD49a⁺ CD4⁺ T cells, confirming our ICS analysis data (Figure 3d). Since we observed increased granzyme b levels following stimulation of CD49a⁺ CD4⁺ T cells we elected to determine if the observed decrease in pro granzyme b levels following stimulation of CD49a⁻ CD4⁻ T cells we elected to determine if the observed decrease in profibrotic gene expression was due to killing of IPF fibroblasts. To accomplish this IPF fibroblasts w accomplish this IPF fibroblasts were isolated post coculture and stained with a live/dead dye
and flow cytometry was performed. Coculture with either CD49a⁻ or CD49a⁺ CD4⁺ T cells did not
result in increased death of and flow cytometry was performed. Coculture with either CD49a⁻ or CD49a⁺ CD4⁺ T cells did not
result in increased death of IPF fibroblasts as determined by equivalent exclusion of dye (Figure
3e). These data support 3e). These data support a therapeutic role for CD49a⁺ CD4⁺ T cells against fibrotic progression
and specifically demonstrate the ability of human CD49a⁺ CD4⁺ T cells to suppress profibrotic
gene expression by IPF f 3e). These data support a therapeutic role for CD49a' CD4' T cells against fibrotic progression
and specifically demonstrate the ability of human CD49a⁺ CD4⁺ T cells to suppress profibrotic
gene expression by IPF fibro

and specifically demonstrate the ability of human CD49a
gene expression by IPF fibroblasts.
CD49a+ TRM alter the fibrotic lung environment
Although we have demonstrated that in mice intra
TRM can improve lung function, r CD4 T cells to suppress profibrotic
atracheally transferred CD49a⁺ CD4⁺
sis *in vivo,* and can suppress human
to determine how these TRMs were CD49a+ TRM alter the fibrotic lung
Although we have demons
TRM can improve lung function, refibroblast profibrotic gene express
modifying the fibrotic lung environ - /
| ||
| || CD49a+ TRM alter the fibrotic lung environment Although we have demonstrated that in mice intratracheally transferred CD49a⁻ CD4⁻
TRM can improve lung function, reverse established fibrosis *in vivo*, and can suppress human
fibroblast profibrotic gene expression *i* This can improve lung function, reverse established fibrosis in vivo, and can suppress human
fibroblast profibrotic gene expression in vitro, we wanted to determine how these TRMs were
modifying the fibrotic lung environm modifying the fibrotic lung environment. To accomplish this, we vaccinated wild type mice with
intranasal FluMist® and 4 weeks later sorted CD49a⁺ CD4⁺ TRM and CD49a⁻ CD4⁺ TRM and then
performed RNA sequence analys intranasal FluMist® and 4 weeks later sorted CD49a⁺ CD4⁺ TRM and CD49a⁻ CD4⁺ TRM and then
performed RNA sequence analysis. Differential gene analysis comparing lung CD49a⁺ CD4⁺ TRM
to lung CD49a⁻ CD4⁺ TRM d decreased expression of the cytokines IL17a and IL17f consistent with the phenotype we observed previously (Figure 4a). To identify upregulated molecular pathways, we submitted the observed previously (Figure 4a). To identify upregulated molecular pathways, we submitted the

top 500 most significantly upregulated differentially expressed genes by CD49a⁻ CD4
STRING analysis of known and predicted protein-protein interactions. Clustering of up
proteins resulting in a cluster which contained 25 top 500 most significantly upregulated differentially expressed genes by CD49a⁺ CD4⁺ T cells to STRING analysis of known and predicted protein-protein interactions. Clustering of upregulated proteins resulting in a cl proteins resulting in a cluster which contained 25 upregulated genes with a small PPI
enrichment p-value (< 10e-16), indicating a high likelihood that the proteins are biologically
connected (Figure 4b). This cluster cont enrichment p-value (< 10e-16), indicating a high likelihood that the proteins are biologically connected (Figure 4b). This cluster contained many proteins involved in extracellular matrix and collagen binding. In addition connected (Figure 4b). This cluster contained many proteins involved in extracellular matrix and
collagen binding. In addition, the cluster contained several proteases and metallopeptidases.
These data suggest that CD49a collagen binding. In addition, the cluster contained several proteases and metallopeptidases.
These data suggest that CD49a⁺ CD4⁺ TRM are acting directly on the fibrotic extracellular matrix,
potentially binding to dep These data suggest that CD49a⁺ CD4⁺ TRM are acting directly on the fibrotic extracellular matrix,
potentially binding to deposited collagen. Through this binding, CD49a⁺ CD4⁺ T cells may be
inducing degradation of potentially binding to deposited collagen. Through this binding, CD49a
inducing degradation of collagen and other extracellular matrix proteins
quantities during fibrosis through the expression of metallopeptidases.
Prese CD4' T cells may be
present in increased
pable of suppressing
e CD49a⁺ CD4⁺ T cells

Presence of CD49a+ CD4+ T cells in healthy and fibrotic human lungs

inducing degradation of collagen and other extracellular matrix proteins present in increased
quantities during fibrosis through the expression of metallopeptidases.
**Presence of CD49a⁺ CD4⁺ T cells in healthy and fib Presence of CD49a⁺ CD4⁺ T cells in healthy and fibrotic human lungs
We have observed that human CD49a⁺ CD4⁺ T cells are
profibrotic gene expression** *in vitro* **(Figure 3), and that transfer of mu
can both prevent f トレード くせく** We have observed that human CD49a⁺ CD4⁺ T cells are capable of suppressing
profibrotic gene expression *in vitro* (Figure 3), and that transfer of murine CD49a⁺ CD4⁺ T cells
can both prevent further lung fibrosis a treated mice (Figure 2). We have also observed that bleomycin treated mice have an increase in CD103⁺ CD4⁺ TRM relative to CD49a⁺ CD4⁺ TRM, whereas control and vaccinated mice have ratios favoring CD49a⁺ CD4⁺ T CD103⁺ CD4⁺ TRM relative to CD49a⁺ CD4⁺ TRM, whereas control and vaccinated mice have
ratios favoring CD49a⁺ CD4⁺ TRM to CD103⁺ CD4⁺ TRM (Figure 1b). These findings indicate that
during fibrosis there is a CD103⁺ CD4⁺ TRM relative to CD49a⁺ CD4⁺
ratios favoring CD49a⁺ CD4⁺ TRM to CD103⁺
during fibrosis there is a skewing of lung
CD49a⁺ expression, which we believe repres
reverse this skewing by adoptive trans ratios favoring CD49a⁺ CD4⁺ TRM to CD103⁺ CD4⁺ TRM (Figure 1b). These findings indicate that
during fibrosis there is a skewing of lung TRM towards CD103⁺ expression and away from
CD49a⁺ expression, which we b ratios favoring CD49a' CD4' TRM to CD103' CD4' TRM (Figure 1b). These findings indicate that
during fibrosis there is a skewing of lung TRM towards CD103⁺ expression and away from
CD49a⁺ expression, which we believe re reverse this skewing by adoptive transfer of CD49a' CD4' TRM may be a therapeutic phenotype.
As such, we were interested in determining if this skewing of lung TRM cells was also present in
human lungs. For this, we analyz As such as such a such a such a such as such as such as the means along the series of the sense is expression Omnibus database (accession number GSE16831)²⁰. Analysis of the data from annotated T cells indicated that the Expression Omnibus database (accession number GSE16831)²⁰. Analysis of the data from
annotated T cells indicated that there were significantly more CD103⁺ TRMs in IPF lungs
compared to control lungs, whereas the perce annotated T cells indicated that there were significantly more CD103⁺ TRMs in IPF lungs
compared to control lungs, whereas the percentage of CD49a⁺ TRMs was relatively unchanged
(Figure 5a). Thus, (at least based on t compared to control lungs, whereas the percentage of CD49a⁺ TRMs was relatively unchanged (Figure 5a). Thus, (at least based on transcriptomic data), it appears that there is a similar skewing of human lung $CD4^+$ TRMs Frame in the large

relatively unchanged

at there is a similar

4[†] TRMs relative to

cells in healthy and

s from age matched

cD103[†] CD4[†] T cells skewing of human lung CD4⁺ TRMs favoring an increase in CD103⁺ CD4⁺ TRMs relative to CD49a⁺ CD4⁺ TRMs in human IPF. To further assess the presence of these cells in healthy and diseased lungs, we performed immuno appears that there is a similar
CD103⁺ CD4⁺ TRMs relative to
nce of these cells in healthy and
opsy samples from age matched
nstrated few CD103⁺ CD4⁺ T cells
d large numbers of CD4⁺ T cells, Exercise of human lung CD4⁺ TRMs favoring an increase in CD103⁺ CD4⁺ TRMs relative to CD49a⁺ CD4⁺ TRMs in human IPF. To further assess the presence of these cells in healthy and diseased lungs, we performed immun CD49a⁺ CD4⁺ TRMs in human IPF. To turther assess the presence ot these cells in healthy and
diseased lungs, we performed immunofluorescence on lung biopsy samples from age matched
normal controls and IPF patients. IHC normal controls and IPF patients. IHC of CD4 and CD103 demonstrated few CD103⁺ CD4⁺ T cells
in normal lungs (Figure 5b). However, IHC of IPF lungs showed large numbers of CD4⁺ T cells,
many of which co-stained with C populations of CD49a⁺ CD4⁺ T cells in normal lungs, but few CD4⁺ T cells co-staining with CD49a
in the lungs of IPF patients. These data strongly support a similar increase in CD103⁺ CD4⁺ TRMs
relative to CD49a⁺ alteration in the phenotype of lung TRMs during fibrosis which permits the progression of this disease state and that transfer of $CD49a⁺CD4⁺TRM$ into the lung represents a therapeutic intervention to swing the bal disease state and that transfer of CD49a⁺CD4⁺ TRM into the lung represents a therapeutic
intervention to swing the balance back toward homeostasis. disease state and that transfer of CD49a'CD4'
intervention to swing the balance back toward ho
that that the balance back toward ho $\begin{array}{ccc} \texttt{D} & \texttt{D} & \texttt{D} \end{array}$ is a therm into the lung representation of $\begin{array}{ccc} \texttt{D} & \texttt{D} & \texttt{D} \end{array}$ intervention to swing the balance back toward homeostasis.

Discussion

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r In this paper, we demonstrate that immunotherapy, in the form of adoptive transfer of CD49a⁺ CD4⁺ TRMs into the lungs of mice with established fibrosis, not only stops progression of fibrosis but more importantly reve novel paradigm shifting approach of utilizing immunotherapy for lung fibrosis in general and re-establish lung homeostasis. These studies provide the insight and preclinical rationale for a
novel paradigm shifting approach of utilizing immunotherapy for lung fibrosis in general and
specifically IPF, a deadly dise

Current medications for IPF at best slow down the fibrosis without impacting mortality or novel paradigm shifting approach of utilizing immunotherapy for lung fibrosis in general and
specifically IPF, a deadly disease with no treatment to stop or reverse fibrosis.
Current medications for IPF at best slow down t specifically IPF, a deadly disease with no treatment to stop or reverse fibrosis.
Current medications for IPF at best slow down the fibrosis without impacting mortality or
quality of life²²⁻²⁴. Although the exact cause Current medications for IPF at best slow down the fibrosis without impactuality of life²²⁻²⁴. Although the exact cause of the disease is unknown, it appet do dysregulated wound healing. The immune microenvironment in th (くれ) quality of life²²⁻²⁴. Although the exact cause of the disease is unknown, it appears to be related to dysregulated wound healing. The immune microenvironment in the lung is constantly exposed to the external environment to dysregulated wound healing. The immune microenvironment in the lung is constantly exposed to the external environment and thus has developed a robust immune response to potential threats. As such, alterations in this re

immunotherapy, in the form of vaccinia vaccination, not only prevents and halts lung fibrosis, potential threats. As such, alterations in this response may result in excessive responses and
lack of appropriate downregulation and healing²⁵.
We have previously demonstrated in a chronic mouse model of lung fibrosis From the interation and healing²⁵.
lack of appropriate downregulation and healing²⁵.
We have previously demonstrated in a chronic mouse model of lung fibrosis that
immunotherapy, in the form of vaccinia vaccination, n ヽikrsf. immunotherapy, in the form of vaccinia vaccination, not only prevents and halts lung fibrosis,
but it also actually reverses established lung fibrosis^{3,4}. The mechanism by which vaccinia
reverses fibrosis is via vaccine but it also actually reverses established lung fibrosis^{3,4}. The mechanism by which vaccinia reverses fibrosis is via vaccine induced lung specific Th1 skewed TRMs in the lung^{3,4}. In this study, we further demonstrate reverses fibrosis is via vaccine induced lung specific Th1 skewed TRMs in the lung^{3,4}. In this study, we further demonstrate that not only does vaccinia induce TRMs that can reverse fibrosis but so do other vaccines such study, we fibrosis but so do other vaccines such as an influenza vaccine (FluMist®). Furthermore, we now have been able to isolate the exact vaccine induced TRMs that are both sufficient and necessary, CD49a⁺ CD4⁺, to have been able to isolate the exact vaccine induced TRMs that are both sufficient and necessary,
CD49a⁺ CD4⁺, to reverse the established fibrosis. In fact, using adoptive cellular therapy we
demonstrate that intratrach CD49a⁺ CD4⁺, to reverse the established fibrosis. In fact, using adoptive cellular therapy we demonstrate that intratracheal administration of CD49a⁺ CD4⁺ T cells into established fibrosis, reverses the fibrosis h CD49a' CD4', to reverse the established fibrosis. In fact, using adoptive cellular therapy we
demonstrate that intratracheal administration of CD49a⁺ CD4⁺ T cells into established fibrosis,
reverses the fibrosis histo demonstrate that intratracheal administration of CD49a[.] CD4[.] T cells into established fibrosis, reverses the fibrosis histologically, by promoting a decrease in collagen levels, and functionally without the need for va without the need for vaccination. In addition, we demonstrate the paucity of these cells in
histologic samples from patients with IPF as well as that co-culture of *in vitro* derived CD49a⁺
CD4⁺ TRMs with human IPF fib CD4⁺ TRMs with human IPF fibroblasts results in a down regulation of IPF fibroblast collagen

histologic samples from patients with IPF as well as that co-culture of *in vitro* derived CD49a⁻
CD4⁺ TRMs with human IPF fibroblasts results in a down regulation of IPF fibroblast collagen
production.
No mouse model CD4' TRMs with human IPF fibroblasts results in a down regulation of IPF fibroblast collagen
production.
No mouse model of fibrosis is perfect, however in our model, chronic fibrosis is induced with IP
injections of bleomy No mouse m
injections of
infiltration,
model differ
phase but injections of bleomycin over four weeks leading to progressive low-grade immune cell
infiltration, collagen deposition and fibrotic changes in the mouse lungs at 72⁺ days^{3,4}. This
model differs from an acute IT bleomy infiltration, collagen deposition and fibrotic changes in the mouse lungs at 72^+ days^{3,4}. This
model differs from an acute IT bleomycin model because it never causes an acute inflammatory
phase but rather promotes su infiltration, collagen deposition and fibrotic changes in the mouse lungs at 72⁺ days^{3,4}. This
model differs from an acute IT bleomycin model because it never causes an acute inflammatory
phase but rather promotes sub phase but rather promotes sub-acute inflammation that progresses to fibrosis over
months^{3,4,26,27}. As an acute inflammatory stage is often unrecognized in humans and the lung
fibrosis progresses slowly and continuously months^{3,4,26,27}. As an acute inflammatory stage is often unrecognized in humans and the lung
fibrosis progresses slowly and continuously over time, the IP bleomycin model appears to more
closely approximate human diseas months^{3,4,20,27}. As an acute inflammatory stage is often unrecognized in humans and the lung
fibrosis progresses slowly and continuously over time, the IP bleomycin model appears to more
closely approximate human diseas closely approximate human disease. Although numerous animal studies using the <u>acute</u>
intratracheal bleomycin model of lung fibrosis have demonstrated benefit from several
therapeutic agents, these often have not translat intratracheal bleomycin model of lung fibrosis have demonstrated benefit from several
therapeutic agents, these often have not translated into human disease^{28,29}. This may be in part
be due to the fact that the intratra therapeutic agents, these often have not translated into human disease^{28,29}. This may be in part
be due to the fact that the intratracheal bleomycin model causes <u>acute l</u>ung injury that heals
with variable fibrosis²⁸⁻ with variable fibrosis²⁸⁻³⁰. This model is often neither progressive beyond the first few weeks with variable fibrosis²⁸⁻³⁰. This model is often neither progressive beyond the first few weeks with variable fibrosis²⁹⁻³⁹. This model is often neither progressive beyond the first few weeks
in the first few weeks of the first few weeks of the first few weeks $\frac{1}{2}$

and has been demonstrated to be reversible without any intervention over time²⁰¹². Indeed,
most drug interventions in this model are tested during this acute/subacute injury and may
indeed only reflect accelerated resolu indeed only reflect accelerated resolution of lung injury thus preventing fibrosis. In humans, IPF
likely develops over many years prior to presentation. Simply put, human disease is not
temporally associated with acute lu likely develops over many years prior to presentation. Simply put, human disease is not
temporally associated with acute lung injury and is also not punctuated with recurrent acute
episodes in the majority of patients³¹. temporally associated with acute lung injury and is also not punctuated with recurrent acute
episodes in the majority of patients³¹. Our model of chronic lung fibrosis better approximates
the relentless, progressive, hum episodes in the majority of patients³¹. Our model of chronic lung fibrosis better approximates
the relentless, progressive, human disease. In our model, fibrosis arises after subacute insults of
intraperitoneal bleomyci intraperitoneal bleomycin over a month, followed by progressive fibrosis (in the absence of
continued bleomycin insult) over the subsequent months ³. When we therapeutically intervene,
it is temporally far removed from continued bleomycin insult) over the subsequent months ³. When we therapeutically intervene, it is temporally far removed from any acute phase. In fact, we quantitate the levels of lung fibrosis via survival pulmonary f fibrosis via survival pulmonary function testing on all mice on $\frac{day}{2}$ after bleomycin and then randomize the mice to therapy with adoptive transferred CD49a⁺ TRM or CD49a⁻ TRM. Follow up is on $\frac{day}{80}$ with repe randomize the mice to therapy with adoptive transferred CD49a⁻ TRM or CD49a-TRM. Follow
up is on <u>day 80</u> with repeat lung function testing, histology, FACS and cytokine expression. Thus,
our data-show that immunotherapy

Although some memory T cells circulate in the blood and amongst secondary lymphoid organs our data show that immunotherapy via adoptive transfer of CD49a⁺ TRM reverses established
lung fibrosis in mice.
Although some memory T cells circulate in the blood and amongst secondary lymphoid organs
as effector memo Although some mem
as effector memory
tissue compartments
site specific infection
tissues³²⁻³⁵. Numero ノミ しょし as effector memory cells (Tem), memory T cells also take up permanent residence in specific
tissue compartments³²⁻³⁵. These tissue resident memory cells (TRM), generated in response to
site specific infections in lungs, tissue compartments³²⁻³⁵. These tissue resident memory cells (TRM), generated in response to site specific infections in lungs, skin, etc., are non-migratory and specifically maintained in these tissues³²⁻³⁵. Numerous tissues³²⁻³⁵. Numerous studies have demonstrated an essential role for TRM in the recall
response to mediate protection both against tissue specific and non-specific challenges such as
viral, bacterial, and parasitic in First, bacterial, and parasitic infections⁶⁻⁸. For example, in the lungs both CD4⁺ and CD8⁺ TRM
are important for protection against influenza^{8,9}. Interestingly, although both injectable
inactivated influenza vacc viral, bacterial, and parasitic infections⁶⁵. For example, in the lungs both CD4' and CD8' TRM
are important for protection against influenza^{8,9}. Interestingly, although both injectable
inactivated influenza vaccine (I are important for protection against influenza^{9,9}. Interestingly, although both injectable
inactivated influenza vaccine (IIV) and intranasal live attenuated influenza vaccine (LAIV)
generate neutralizing strain-specific materate neutralizing strain-specific antibodies, only the intranasally administered LAIV generates lung localized, virus specific T cell responses similar to what is generated with influenza infection⁸. More importantl generates lung localized, virus specific T cell responses similar to what is generated with
influenza infection⁸. More importantly, only the intranasal LAIV generates TRM that mediate
cross-strain protection, independent influenza infection⁸. More importantly, only the intranasal LAIV generates TRM that mediate cross-strain protection, independent of circulating T cells and neutralizing antibodies⁸. Thus, intranasal LAIV generation of

cross-strain protection, independent of circulating T cells and neutralizing antibodies". Thus,
intranasal LAIV generation of lung TRM not only protects from future infection to the specific
vaccinated viral strain but als vaccinated viral strain but also provides heterosubtypic protection to non-vaccine viral strains⁸.
In this regard, we too have published that only intranasal vaccination of live vaccinia generates TRMs that are essential es
dan
ic $\frac{1}{7}$ in the set of $\frac{1}{7}$ TRMs that are essential both to the protection from and the reversal of bleomycin-induced
fibrosis^{3,4}. Reversal of pulmonary fibrosis by vaccinia immunotherapy requires creation of a
lung-specific memory CD4⁺ Th1 cell fibrosis^{3,4}. Reversal of pulmonary fibrosis by vaccinia immunotherapy requires creation of a
lung-specific memory CD4⁺ Th1 cell³. Furthermore, our data show that vaccinia vaccination
induced IFN_Y producing TRM in fibrosis^{3,4}. Reversal of pulmonary fibrosis by vaccinia immunotherapy requires creation of a
lung-specific memory CD4⁺ Th1 cell³. Furthermore, our data show that vaccina vaccination
induced IFN_Y producing TRM in th lung-specific memory CD4' Th1 cell". Furthermore, our data show that vaccinia vaccination
induced IFNy producing TRM in the bleomycin-injured lungs whereas the unvaccinated fibrotic
control lungs have a predominance of IL1 control lungs have a predominance of $|L17^+ \text{TRM}^3|$. In this study, we demonstrate that IT adoptive transfer of CD49a⁺ TRMs are necessary and sufficient to reverse established murine fibrosis in the absence of vaccin

control lungs have a predominance of IL17' TRM". In this study, we demonstrate that IT adoptive transfer of CD49a⁺ TRMs are necessary and sufficient to reverse established murine fibrosis in the absence of vaccination.
T The development of fibrosis is a coundeed, numerous studies, both modepithelial and immune cells but also a $\begin{bmatrix} 1 \\ 1 \end{bmatrix}$ Indeed, numerous studies, both mouse and human, have implicated not only fibroblasts,
epithelial and immune cells but also a host of cytokines, chemokines and transcription factors
prichalism and immune cells but also a ho Indeed, numerous studies, when the detailed in the implication of our optical and human, in the studies, epithelial and immune cells but also a host of cytokines, chemokines and transcription factors epithelial and immune cells but also a host of cytokines, chemokines and transcription factors as playing essential roles in the excess accumulation of ECM characteristic of lung fibrosis. We
believe that these tissue-specific adoptively transferred TRMs interact with host immune cells
to promote normal resolution o to promote normal resolution of lung injury. Our bulk RNA seq data would also suggest that the
CD49a⁺ TRM may be having broad effects on these processes that promote and maintain
fibrosis. STRING analysis suggests that C CD49a⁺ TRM may be having broad effects on these processes that promote and maintain fibrosis. STRING analysis suggests that CD49a⁺ TRM upregulate distinct molecular pathways involved in collagen binding and signaling, CD49a⁻ TRM may be having broad effects on these processes that promote and maintain fibrosis. STRING analysis suggests that CD49a⁺ TRM upregulate distinct molecular pathways involved in collagen binding and signaling, fibrosis. STRING analysis suggests that CD49a
involved in collagen binding and signaling, extra-
and growth factor and glycosaminoglycan bir
fibrosis by promoting remodeling of the ECM,
wound healing. Interestingly, the da TRM EXPLUSING METHEM METHEM PRINCE,
cellular matrix organization, metalloproteinases
iding. Thus, CD49a⁺ TRM reverse established
perhaps resetting the imbalance of injury to
rating the paucity of CD49a⁺ cells in IPF lu involved in collagen binding. Thus, CD49a⁺ TRM reverse established
fibrosis by promoting remodeling of the ECM, perhaps resetting the imbalance of injury to
wound healing. Interestingly, the data demonstrating the paucit and growth factor and glycosaminoglycan binding. Thus, CD49a'
fibrosis by promoting remodeling of the ECM, perhaps resetting t
wound healing. Interestingly, the data demonstrating the paucity of
consistent with our data th The imbalance of injury to
CD49a⁺ cells in IPF lungs is
lungs supporting a strategy
of promoting resolution of motion of promoting removes the ECM, permany promoting the Dava' cells in IPF lungs is
consistent with our data that these cells are relatively absent in IPF lungs supporting a strategy
of augmenting CD49a⁺ TRMs, via ado consistent with our data that these cells are relatively absent in IPF lungs supporting a strategy
of augmenting CD49a⁺ TRMs, via adoptive transfer as a means of promoting resolution of
fibrosis and normal healing.
Over pporting a strategy
oting resolution of
the deadly human
hysiology, or a cure

consistent with the that these cells are relatively absent in the range trippering a trial of
of augmenting CD49a⁺ TRMs, via adoptive transfer as a means of promoting resolution of
fibrosis and normal healing.
Over the l of augmenting CD49a[.] TRMs, via adoptive transfer as a means of promoting resolution of
fibrosis and normal healing.
Over the last 30 years, much has been learned about lung fibrosis and the deadly human
disease IPF. Howe Fibrosis and normal healing.
Over the last 30 years, mudisease IPF. However, we stift this invariably fatal disease a potential role for imm C C f s a c . disease IPF. However, we still have not determined the cause, exact pathophysiology, or a cure
for this invariably fatal disease. Like the recent advances using immunotherapy for cancer, we
see a potential role for immunot for this invariably fatal disease. Like the recent advances using immunotherapy for cancer, we see a potential role for immunotherapy in IPF as well. Adoptive Cellular Therapy is emerging as a powerful means of treating c see a potential role for immunotherapy in IPF as well. Adoptive Cellular Therapy is emerging as
a powerful means of treating certain cancers³⁶. Our data provide the scientific rationale for the
development of a novel par a powerful means of treating certain cancers³⁶. Our data provide the scientific rationale for the
development of a novel paradigm shifting therapeutic approach to lung fibrosis in the form of
immunotherapy of CD49a⁺ TR a powerful means of treating certain cancers³⁰. Our data provide the scientific rationale for the
development of a novel paradigm shifting therapeutic approach to lung fibrosis in the form of
immunotherapy of CD49a⁺ TR immunotherapy of CD49a⁺ TRMs derived from a patient's own T cells to be adoptively
transferred into the lung to not only halt but reverse the progressive deadly fibrosis.
Transferred into the lung to not only halt but r transferred into the lung to not only halt but reverse the progressive deadly fibrosis. TRMs dependence only halt but reverse the progressive deadly fibrosis.

The control of the progressive deadly fibrosis.

The control of the progressive deadly fibrosis. transferred into the lung to not only halt but reverse the progressive deadly fibrosis.

Figure Legends

Figure 1. CD49a and CD103 expression differentiates populations of lung CD4⁺ TRM

Figure 1. CD49a and CD103 expression differentiates populations of lung CD4
Mice received intranasal PBS, intratracheal bleomycin, intranasal FluMis
vaccinia virus. 28 days later mice were injected i.v. with CD4 antibody i Mice received intranasal PBS, intratracheal bleomycin, intranasal FluMist®, or intranasal Mother and Survey in the Universal A. Thought order to identify tissue t CD4⁺ T cells, sacrificed, and lungs were isolated. A. Flow plots of tissue resident CD4⁺ T ained with CD49a and CD103 antibodies **B**. Numbers of resident CD4⁺ T cells, sacrificed, and lungs were isolated. A. Flow plots of tissue resident CD4⁺ T cells stained with CD49a and CD103 antibodies **B**. Numbers of lung CD49a⁺ and CD103⁺ CD4⁺ TRM. C. Intracellular 4⁺
Л.
5. cells stained with CD49a and CD103 antibodies **B**. Numbers of lung CD49a and CD103 CD4

TRM. C. Intracellular staining of IL17a and IFNy from sorted CD49a⁺ and CD103⁺ lung CD4⁺ TRM.

Error bars represent standard de TRM. C. Intracellular staining of IL17a and IFNγ from sorted CD49a⁻ and CD103⁻ lung CD4⁻

Error bars represent standard deviation. * indicates statistical significance where p

Experiments were performed three times

l CD4
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 $< .05.$
tions.
ceived Experiments were performed three times with 3 mice per group.

Figure 2. CD49a⁺ CD4⁺ TRM are therapeutic in pulmonary fibrosis

Pulmonary fibrosis was established in mice using intraperitoneal bleomycin injections.

CD Figure 2. CD49a⁺ CD4⁺ TRM are therapeutic in pulmonary fibrosis
Pulmonary fibrosis was established in mice using intrape
CD49a⁺ CD4⁺ T cells, and CD49a⁻ CD4⁺ T cells were sorted from lur
intranasal FluMist® 28 Figure 2. CD49a
Pulmonai
CD49a⁺ CD4⁺ T ce
intranasal FluMis
established pulm
were subjected
transfer. B. Pulm Pulmonary fibrosis was established in mice using intraperitoneal bleomycin injections. CD49a⁺ CD4⁺ T cells, and CD49a⁻ CD4⁺ T cells were sorted from lungs of mice which had received
intranasal FluMist® 28 days prior. Sorted TRM were transferred intratracheally into mice with
established pulmonary fib established pulmonary fibrosis on day 42 of the I.P. bleomycin model. Thirty days later mice
were subjected to pulmonary function testing. A. Flow plots of sorted CD4⁺ TRM prior to
transfer. **B**. Pulmonary function testi were subjected to pulmonary function testing. A. Flow plots of sorted CD4⁺ TRM prior to transfer. B. Pulmonary function testing of I.P. bleomycin treated mice. C. Lung Collagen RNA expression 45 days following T cell tra transfer. **D.** Pulmonary function testing of I.T. bleomycin treated mice. **C.** Lung Conagen RNA expression 45 days following T cell transfer. **D.** Changes in pulmonary function from day 42 (pre-TRM transfer) and day 80 (p Expression 45 days following T cell transfer. D. Changes in pullificially function from day 42
(pre-TRM transfer) and day 80 (post-TRM transfer). E. Represent histology of I.P. bleomycin
treated mice sacrificed on day 100 (pre-TRM transfer) and day 80 (post-TRM transfer). E. Represent instology of I.T. bleomycin
treated mice sacrificed on day 100. Error bars represent standard deviation, * indicates
statistical significance where $p < .05$.

CD4
+- CD+

statistical significance where $p < .05$. Experiments were performed three times with 8 mice per
group.
Figure 3. Human CD49a⁺ CD4⁺ T cells suppress lung fibroblast profibrotic gene expression
In order to generate CD49a Figure 3. Human CD49a⁺ CD4⁺ T cells suppress lung fibroblast profibrotic gene expression
In order to generate $CD49a^+$ CD4⁺ T cells, human PBMCs were stimulated with anti-CD3
and anti-CD28 in the presence of interlu Figure
Figure
and an
cells w
isolated $rac{1}{2}$ Figure 3. Human CD49a

In order to genera

and anti-CD28 in the pres

cells were separated base

isolated human CD4⁺ T ce

T cells. C. RNA expression

or CD49a⁺ CD4⁺T cells in In order to generate $CD49a^+$ CD4⁺ T cells, human PBMCs were stimulated with anti-CD3 In order to generate CD49a⁻ CD4⁻ T cells, human PBMCs were stimulated with anti-CD3

and anti-CD28 in the presence of interlukin-2 for four weeks. CD4⁺ T cells were isolated, and

cells were separated based on CD49a isolated human CD4⁺ T cells. **B.** Flow analysis of cytokine production by CD49a or CD49a⁺ CD4⁺
T cells. **C.** RNA expression of pro-fibrotic genes by IPF lung fibroblasts cocultured with CD49a⁺ CD4⁺
or CD49a⁺ C isolated human CD4 T cells. **B.** Flow analysis of cytokine production by CD49a- or CD49a⁻ CD4

T cells. **C.** RNA expression of pro-fibrotic genes by IPF lung fibroblasts cocultured with CD49a⁻

or CD49a⁺ CD4⁺T ce $\frac{1}{2}$ or CD49a⁺ CD4⁺T cells in transwell plates. **D.** TNF α and IFNy concentration of coculture supernatants measured by ELISA. **E.** Live/Dead staining of IPF fibroblasts following no coculture, or coculture with CD49a⁻ $\frac{1}{2}$ or CD49a⁺ CD4⁺T cells in transwell plates. **D.** TNFα and IFNγ concentration of coculture
supernatants measured by ELISA. **E.** Live/Dead staining of IPF fibroblasts following no coculture,
or coculture with CD49a⁺ a supernatants measured by ELISA. E. Ewe/Dead staining of IPF hibroblasts following no coculture,
or coculture with CD49a⁻ and CD49a⁺ CD4⁺ T cells. Error bars represent standard deviation, *
indicates statistical sign

l, Figure 4. CD49a+ CD4+ TRM gene signature indicates the ability to bind and alter the

or coculture with CD49a-and CD49a⁻ CD4

indicates statistical significance where $p < .0$
 **Figure 4. CD49a⁺ CD4⁺ TRM gene signat

extracellular matrix.**

CD49a-and CD49a⁺ CD4⁺ T cells we

intranasal FluMist® 28 d 5. Experiments were performed three times.

ture indicates the ability to bind and alter the

ture sorted from lungs of mice which had received

as isolated and subjected to RNA seq analysis. A. Figure 4. $CD49a^+$ $CD4^+$ TRM gene signature indicates the ability to bind and alt
extracellular matrix.
CD49a and CD49a⁺ CD4⁺ T cells were sorted from lungs of mice which had re
intranasal FluMist® 28 days prior. RNA CD49a and CD49a⁺ CD4⁺ T cells were sorted from lungs of mice which had received CD49a⁻ and CD49a⁻ CD4⁻ T cells were sorted from lungs of mice which had received
intranasal FluMist® 28 days prior. RNA was isolated and subjected to RNA seq analysis. **A.**
STRING clustering figure of the 500 most di INTRING Clustering figure of the 500 most differentially expressed genes by CD49a⁺ CD4⁺ TRM. **B.**
Differential gene expression analysis of CD49a⁺ CD4⁺ TRM versus CD49a⁻ CD4⁺ TRM. **B.**
Figure 5. Imbalanced CD49a Differential gene expression analysis of CD49a⁺ CD4⁺ TRM versus CD49a⁻ CD4⁺ TRM. CD4
1. TRM. B. $CD4$ ⁺

Differential gene expression analysis of CD49a' CD4'
Figure 5. Imbalanced CD49a⁺/CD103⁺ CD4⁺ T cells in
-TRM versus CD49a
human IPF lungs
- Figure 5. Imbalanced CD49a /CD103 CD4 I cells in human IPF lungs

A. Percentage of CD49a⁺ and CD103⁺ CD4⁺ T cells in control and IPF lungs as determined
by analysis of single cell RNA-seq dataset from Adams et al (2020). Error bars represent
standard deviation, * = p value of 0.00 standard deviation, $* = p$ value of 0.0001. **B.** Immunohistochemistry was performed on biopsies
of normal and IPF human lungs in order to determine densities of CD49a⁺ and CD103⁺ CD4⁺ T
cells. Images were taken at 40 standard deviation, * = p value of 0.0001. B. Immunohistochemistry was performed on biopsies
of normal and IPF human lungs in order to determine densities of CD49a⁺ and CD103⁺ CD4⁺ T
cells. Images were taken at 40X. of normal and IPF human lungs in order to determine densities of CD49a+ and $CD103^{\circ}$ $CD4$ ⁺ cells. Images were taken at 40X.
Images were taken at 40X.

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Figure 4.

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