

# Telomerase RNA component knockout exacerbates *S. aureus* pneumonia by extensive inflammation and dysfunction of T cells

Reviewed Preprint

v2 • November 6, 2024

Revised by authors

Reviewed Preprint

v1 • August 22, 2024

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## eLife Assessment

In this manuscript, the authors sought to elucidate mechanistic intricacies of inflammatory responses, with emphasis on T cell dysfunction, to *S. aureus*-induced pneumonia in the context of aging process using *Terc* deficient mice. Conceptually, the study is very interesting with a set of **useful** findings. Although some experimental approaches are appropriate, the work as shown in the revised manuscript remains significantly underpowered and the absence of rigorous controls make this study **incomplete** in support of its claims.

<https://doi.org/10.7554/eLife.100433.2.sa3>

## Abstract

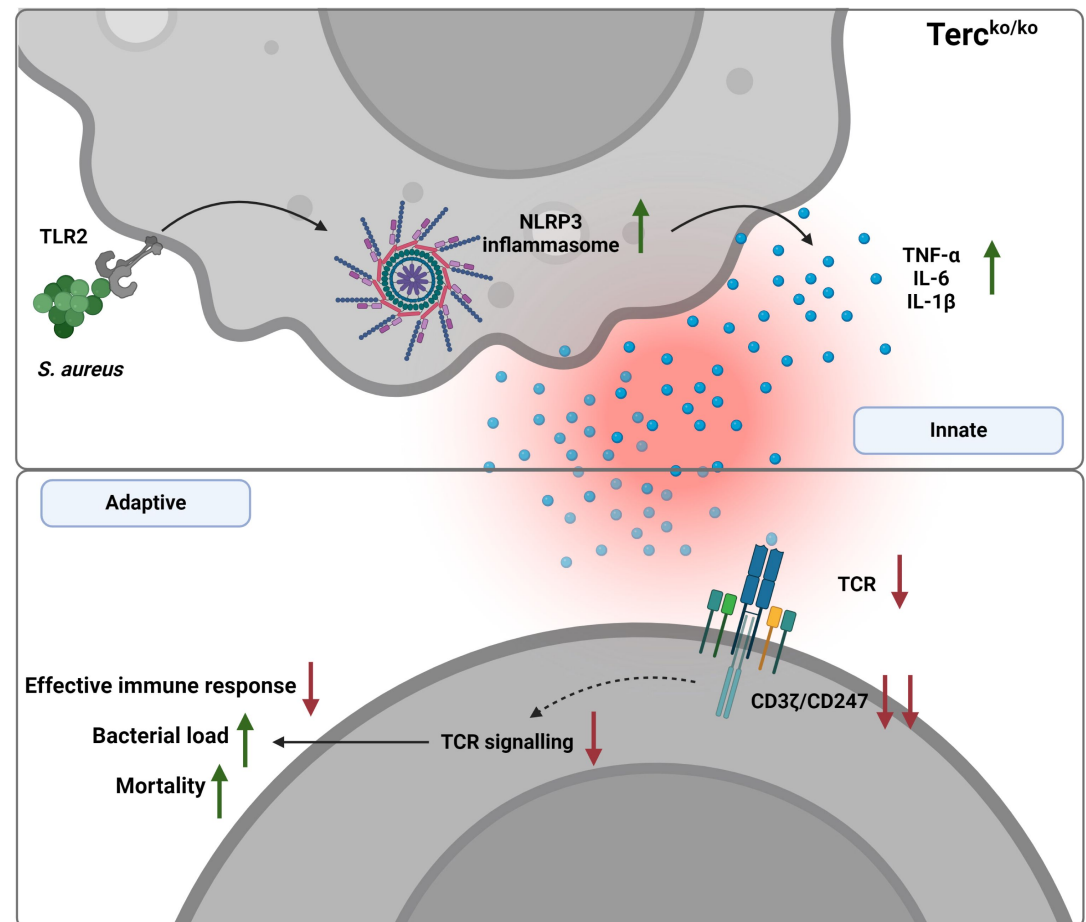
The telomerase RNA component (*Terc*) constitutes a non-coding RNA critical for telomerase function, commonly associated with aging and pivotal in immunomodulation during inflammation.

Our study unveils heightened susceptibility to pneumonia caused by *Staphylococcus aureus* (*S. aureus*) in *Terc* knockout (*Terc*<sup>ko/ko</sup>) mice compared to both young and old infected counterparts. The exacerbated infection in *Terc*<sup>ko/ko</sup> mice correlates with heightened inflammation, manifested by elevated interleukin-1 $\beta$  (IL-1 $\beta$ ) levels and activation of the NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome within the lung.

Employing mRNA sequencing methods alongside *in vitro* analysis of alveolar macrophages (AMs) and T cells, our study elucidates a compelling correlation between *Terc*<sup>ko/ko</sup>, inflammation, and impaired T cell functionality. *Terc* deletion results in compromised T cell function, characterized by dysregulation of the T cell receptor and absence of CD247, potentially compromising the host's capacity to mount an effective immune response against *S. aureus*.

This investigation provides insights into the intricate mechanisms governing increased vulnerability to severe pneumonia in the context of *Terc* deficiency, which might also contribute to aging-related pathologies, while also revealing for the first time the influence of *Terc* on T cell function.

## Graphical abstract



## Introduction

As the global population ages, the impact of infectious diseases on the aging population becomes increasingly significant. Among the most relevant risk factors leading to higher susceptibility as well as severity in both community-acquired (CA) and healthcare-associated (HA)-pneumonia is advanced age ( $\geq 60$  years) (Torres et al., 2021). Particularly severe is pneumonia caused by *Staphylococcus aureus* (*S. aureus*) (Lee et al., 2022). *S. aureus* is a gram-positive bacterium that frequently colonizes humans but can manifest various pathologies, ranging from mild to more severe infections, including pneumonia (Howden et al., 2023; Jong, Kessel, & Strijp, 2019). It accounts for a notable proportion of CA and HA cases (Lee et al., 2022; Torres et al., 2021). The bacterium exhibits a broad spectrum of virulence factors, including toxins, adhesins, and immune evasion strategies, contributing to its ability to establish infections in the lower respiratory tract (Howden et al., 2023). Aging is a central factor predisposing individuals to a higher risk for *S. aureus* pneumonia (Torres et al., 2021), as aging is impacting several important functions of the body such as the immune response and thus the capability to mount an effective defensive

response (Montecino-Rodriguez, Berent-Maoz, & Dorshkind, 2013). Age-related dysregulation of the immune response is also characterized by inflammaging, defined as the presence of elevated levels of pro-inflammatory cytokines in the absence of an obvious inflammatory trigger (Franceschi et al., 2000; Mogilenko, Shchukina, & Artyomov, 2022). Additionally, immune cells, such as macrophages, exhibit an activated state that alters their response to infection (Canan et al., 2014). In contrast, the immune response of macrophages to infectious challenges has been shown to be initially impaired in aged mice (Boe, Boule, & Kovacs, 2017). Thus aging is a relevant factor impacting the pulmonary immune response. This highlights the vital connection between aging and *S. aureus* pneumonia, as well as the need for further research investigating this connection.

The process of aging is a multifaceted phenomenon that involves various underlying mechanisms, known as hallmarks (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2023). Among these hallmarks, telomere shortening is a significant factor in cellular aging. Telomeres serve as protective caps at the end of chromosomes, which shorten with every cell division until they reach a critical length where replication becomes impossible, eventually resulting in either apoptosis or senescence (López-Otín et al., 2023). However, this process can be counteracted by the telomerase, which can add new repeats to the ends of chromosomes and thus prevent the shortening of telomeres (Greider & Blackburn, 1985). The telomerase mainly consists of two parts: the telomerase reverse transcriptase (Tert), which harbors the enzymatic activity, and Terc, which serves as a template for the addition of new repeats (Sahin & DePinho, 2010; Shay & Wright, 2019). Telomerase activity is restricted to specific tissues and cell types, largely dependent on the expression of *Tert*. While *Tert* is highly expressed in stem cells, progenitor cells, and germline cells, its expression is minimal in most differentiated cells (Chakravarti, LaBella, & DePinho, 2021). Consequently, the impact of telomerase dysfunction on tissues varies according to their self-renewal rate (Chakravarti et al., 2021).

One important aspect of telomere dysfunction is the impact of telomere shortening on the immune system as well as the hematopoietic system. Tissues or organ systems that are highly replicative, such as the skin or the hematopoietic system, are affected first by telomere shortening (Chakravarti et al., 2021). Due to the loss of the protective telomere caps, mutations can accumulate, and the cells ultimately become senescent (Chakravarti et al., 2021). Interestingly, while most differentiated cells do not exhibit telomerase activity, T cells display a high activity of this enzyme (Hodes, Hathcock, & Weng, 2002). Aging T cells become senescent, which is characterized by short telomeres and the absence of CD28 expression (Hohensinner, Goronzy, & Weyand, 2011). Furthermore, telomere length and T cell functionality are closely connected. T cells with shorter telomeres are implicated in diseases related to aging like arthritis (Hohensinner et al., 2011). Additionally, CD4<sup>+</sup> T cells from *Terc* knockout (*Terc*<sup>ko/ko</sup>) mice displayed several characteristics of aged T cells such as a reduction in CD28 expression and changed secretion of cytokines. Furthermore the naïve T cell population was found to be reduced (Matthe, Thoma, Sperka, Neurath, & Waldner, 2022). This highlights the importance of intact telomeres and telomerase on immune cell function.

While most differentiated cells lack the expression of *Tert*, almost all human cells and several murine tissues express *Terc* (Zhang et al., 2018). However, the function of ubiquitous *Terc* expression remains elusive (Chakravarti et al., 2021; Shay & Wright, 2019). Interestingly, recent studies identified a variety of functions of *Terc*, which are independent of its function as part of the telomerase. Notably, a study could show that *Terc* triggers inflammation via the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Liu, Yang, Ge, Liu, & Zhao, 2019). Furthermore, *Terc* was identified to be involved in cell proliferation by enhancing the expression of several genes belonging to the Phosphoinositide 3-kinase (PI3K) – Protein Kinase B (AKT) pathway (Wu et al., 2022). The same study could show that this mechanism also contributed to CD4<sup>+</sup> T cell expansion (Wu et al., 2022). Additionally, *Terc* deletion in mice induced NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome activation in

macrophages upon pulmonary infection with *S. aureus*, thereby revealing another connection between immune modulation and the presence of *Terc* (Kang et al., 2018 [DOI](#)). Thus, it appears that *Terc* could serve as a relevant immunomodulatory factor, potentially implicated in *S. aureus*-induced pathologies. Interestingly, downregulation of *Terc* and *Tert* expression in tissues of aged mice and rats has been found (Tarry-Adkins, Aiken, Dearden, Fernandez-Twinn, & Ozanne, 2021 [DOI](#); Zhang et al., 2018 [DOI](#)). Therefore, as a potential immunomodulatory factor reduced *Terc* expression could be connected to age-related pathologies.

Knockout of *Terc* in mice is a well-established model for premature aging, as telomeres of *Terc*<sup>ko/ko</sup> mice shorten with every generation (Wong et al., 2008 [DOI](#)). Additionally, the telomeres shorten during each generation with increasing age of the mice (Wong et al., 2008 [DOI](#)). Furthermore, third-generation (G3) *Terc*<sup>ko/ko</sup> mice with a C56Bl/6 background have a shorter lifespan, a smaller litter size and a generally reduced body size and weight (Wong et al., 2008 [DOI](#)). Although G3 *Terc*<sup>ko/ko</sup> mice with shortened telomeres were used in this study, they were infected at a young age (8 weeks). This approach allowed for the investigation of *Terc* deletion effects rather than telomere dysfunction. As control cohort age-matched young WT mice were utilized. To investigate whether *Terc* deletion, beyond critical telomere shortening, impacts the pulmonary immune response, we used young *Terc*<sup>ko/ko</sup> mice. Additionally, naturally aged mice (2 years old) were infected to explore the potential link to a fully developed aging phenotype.

In this study, we aimed to investigate the impact of *Terc* deletion, as an essential immunomodulatory factor, on the immune response to *S. aureus*-induced pneumonia in mice. For this purpose, G3 *Terc*<sup>ko/ko</sup> mice were infected with *S. aureus*, and the progression of disease and immune response were evaluated. Strikingly, our data highlights that the deletion of *Terc* resulted in a more severe disease outcome and disrupted the innate and adaptive immune response. These findings suggest a possible connection between *Terc* and immune cell homeostasis.

## Materials and methods

### Bacteria

All *in vivo* and *in vitro* experiments were carried out with the *S. aureus* strain USA300. *S. aureus* was cultivated in brain heart infusion (BHI) medium (Thermo Fisher Scientific, Waltham, MA, US) at 37°C while shaking at 150 rpm. The bacteria were harvested during the mid-logarithmic phase and adjusted to the appropriate optical density (OD) at 600nm for the respective experiments in phosphate-buffered saline (PBS). The bacteria were then either used freshly for *in vivo* infection of mice or stored at -80 °C until further usage in *in vitro* experiments.

For heat-killing, the adjusted bacteria stock was incubated at 95°C for 5 minutes while shaking at 500 rpm. A fraction of the heat-killed bacteria was plated on Müller-Hinton (MH) plates. Plates were incubated for 5 days at 37°C to confirm heat-killing of the bacteria.

### Determination of bacterial load

Organs were homogenized in the appropriate amount of PBS (adjusted to their weight in mg) using a SpeedMill Plus (Analytik Jena, Jena, Germany). Homogenized organs and bronchoalveolar lavage (BAL) fluid were diluted in a decadic dilution series and plated onto MH plates. Plates were incubated overnight at 37°C. Colonies were counted, and colony-forming units (CFU) per ml was calculated.

### Mouse models and infection

Mouse *in vivo* experiments were approved by the Office for Consumer Protection of Thuringia (TV-Number: UKJ-19-028 and UKJ-22-023).

G3 female *Terc*<sup>ko/ko</sup> mice with a C57Bl/6 background were bred from homozygous parents in the animal facility of the Jena University Hospital. To confirm knockout of *Terc*, DNA was extracted from the tail of newborn mice using the DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. The DNA was then used for genotyping via PCR using the following primer sequences: mTR-R: 5'-TTC TGA CCA CCA CCA ACT TCA AT-3'; mTR-WT-F: 5'-CTA AGC CGG CAC TCC TTA CAA G-3'; 5PPgK(-F): 5'-GGG GCT GCT AAA GCG CAT-3'. The results were analyzed with an Agilent 2200 TapeStation system using a Agilent D1000 ScreenTape (both Agilent Technologies, Santa Clara, CA, USA). The WT *Terc* product had a size of 200 bp while the knockout product had a size of 180 bp. Only mice that showed a single band at 180 bp were used for the further breeding process.

*Terc*<sup>ko/ko</sup> mice aged 8 weeks, were used for infection studies (n = 8; non-infected = 3; infected = 5). Female young WT (age 8 weeks) and old WT (age 24 months) C57Bl/6 mice (both n = 10; non-infected = 5; infected = 5) were purchased from Janvier Labs (Le Genest-Saint-Isle, France). All infected mouse cohorts were compared to their respective non-infected controls, as well as to the infected groups from other cohorts. Additionally, comparisons were made between the non-infected cohorts across all groups.

All mice were anesthetized with 2% isoflurane before intranasal infection with *S. aureus* USA300 ( $1 \times 10^8$  CFU/20  $\mu$ l) per mouse. After 24 hours, the mice were weighed and scored as previously described (Hornung et al., 2023 [\[4\]](#)). Infected *Terc*<sup>ko/ko</sup> mice were grouped into different degrees of severity based on their clinical score, fatal outcome of the disease (fatal) and the presence of bacteria in organs other than the lung (systemic infection) for the indicated analysis. Mice with fatal infections were excluded from subsequent analyses, with only their final scores being reported. The mice were sacrificed via injection of an overdose of xylazine/ketamine and bleeding of axillary artery after 24 hpi. BAL was collected by instillation and subsequent retrieval of PBS into the lungs. Serum and organs were collected. Bacterial load in the BAL, kidney and liver was determined by plating of serially diluted sample as described above. For this organs were previously homogenized in the appropriate volume of PBS. Gene expression was analyzed in the right superior lung lobe. Lobes were therefore homogenized in the appropriate amount of TriZol LS reagent (Thermo Fisher Scientific, Waltham, MA, US) prior to RNA extraction. The left lung lobe was embedded into Tissue Tek O.C.T. (science services, Munich, Germany) and stored at -80°C until further processing for histological analysis. Cytokine measurements were performed using the right inferior lung lobe. Lobes were previously homogenized in the appropriate volume of PBS. Remaining organs were stored at -80°C until further usage.

Mouse studies were conducted without the use of randomization or blinding.

## Telomere length measurement

The length of telomeres in the lung of young WT, old WT and *Terc*<sup>ko/ko</sup> mice was measured using the Absolute Mouse Telomere Length Quantification qPCR Assay Kit (ScienCell research Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. Genomic DNA from the lungs was extracted by DNeasy Blood & Tissue Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. For the PCR 2ng of each sample was used. Average telomere length per chromosome end was calculated as described by the manufacturer.

## Histology and Immunofluorescence

Preparation and staining of tissue cryosections was performed as described previously (Hornung et al., 2023 [\[4\]](#)).

For staining of *S. aureus*, rabbit anti-*S. aureus* polyclonal antibody (Cat. # PA1-7246, Thermo Fisher Scientific, Waltham, MA, US) was diluted 1:400 in antibody diluent with background reducing components (Agilent, Santa Clara, CA, US). Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG

(Cat. #711-545-152, Jackson Immuno Research, West Grove, PA, US) and BODIPY 558/568 phalloidin, which stains actin fibers, (Cat. # B3475, Thermo Fisher Scientific, Waltham, MA, US) were diluted in antibody diluent 1:500 and 1:400 respectively and applied to the slides as secondary antibodies.

## Immunocytochemistry

Murine primary macrophages were fixed with 4% paraformaldehyde (PFA) for 15 minutes at 37°C, subsequently permeabilized with 0.1% Triton-X, and blocked with 3% bovine serum albumin (BSA) in PBS for each 30 minutes at room temperature (RT). Cells were incubated overnight with either rabbit anti-*S. aureus* polyclonal antibody or rabbit anti-CD68 polyclonal antibody (Cat. # BOSSBS-0649R, VWR, Radnor, PA, US) diluted 1:400 or 1:200 in 3% BSA in PBS respectively. The following day, the slides were washed three times with PBS. Cy3-conjugated AffiniPure Donkey Anti-Rabbit IgG (Cat. #711-165-152, Jackson Immuno Research, West Grove, PA, US) and Alexa Fluor™ Plus 647 Phalloidin (Cat. # A30107, Thermo Fisher Scientific, Waltham, MA, US) were applied to the cells diluted 1:500 and 1:400 in 3% BSA in PBS respectively and incubated for 1 hour at RT. The cells were then mounted with DAPI Fluoromount-G.

The immunofluorescence pictures were taken with an AxioObserver Z.1 microscope (Carl Zeiss AG, Oberkochen, Germany) and analyzed with the Zen software (Zen Pro v3.3).

## RNA extraction and mRNA sequencing

RNA was extracted from murine lung tissue using the Trizol/Chloroform method. First, about 15 mg of lung tissue was homogenized in TriZol LS reagent (Thermo Fisher Scientific, Waltham, MA, US) using the SpeedMill Plus. The tissue-free supernatant of the homogenate was transferred into a new tube after centrifugation. 50 µl of chloroform was added to 250 µl TriZol Reagent, incubated for 3 minutes at RT, and centrifuged at 12000xg for 15 minutes at 4°C. The aqueous phase was mixed with isopropanol in a 1:2 ratio and incubated at RT for 10 minutes before centrifugation at 12000xg for 10 minutes at 4°C. Afterward, the RNA pellet was washed twice with 75% ethanol. Finally, ethanol was removed, and the pellet was air-dried for 5-10 minutes. The dry pellet was then dissolved in sterile distilled water, and RNA concentration was determined by an ND-1000 NanoDrop spectrophotometer (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Before sequencing, RNA integrity was measured using a 5400 Fragment Analyzer (Agilent Technologies, Santa Clara, CA, US). RNA concentration and RNA integrity of the samples can be found in Supplemental Table 1.

Library construction and mRNA sequencing were performed by Novogene Co., LTD. (Beijing, China) using the Illumina platform Novaseq 6000 S4 flowcell V1.0, based on the sequencing by synthesis (SBS) mechanism and PE150 strategy. For bioinformatics analysis, raw reads in FASTQ format were first processed using fastp. Reads containing adapters and poly N sequences were removed as well as low quality reads to generate clean data. Clean reads were then mapped to the reference genome (*Mus Musculus* (GRCm39/mm39)) with the HISAT2 software. Gene expression levels were quantified using the FPKM method. DESeq2 software as well as negative binomial distribution was used for analysis of differential gene expression. For FDR (False discovery rate) calculation the Benjamini-Hochberg procedure was used. A summary of the quality of the sequencing data of each sample can be found in Supplemental Table 2.

Heatmaps of the differentially regulated genes (DEG) were constructed using R (v4.2.2; R Foundation for Statistical Computing, <https://www.r-project.org/>). Only significant DEGs (p-value < 0.05) were displayed in the heatmaps. Non-significant genes were set to zero and are shown in white. All DEGs used for construction of the respective heatmaps are summarized in Supplemental Table 3a-i.



## Immunophenotyping of the spleen

Mice were sacrificed as described above, and spleens were freshly stored in cold 2% FCS in PBS. For isolation of immune cells, spleens were mashed through a 70  $\mu$ m cell strainer (Miltenyi Biotec, Bergisch Gladbach, Germany), and red blood cell lysis was performed by adding 5 ml of Ammonium–chloride–potassium (ACK) lysis buffer (Thermo Fisher Scientific, Waltham, MA, US) for 5 minutes at 4°C to the pelleted cells. Cells were then counted, adjusted to  $2 \times 10^6$  per ml PBS, and stained with BD Fixable Viability Stain 780 (BD Biosciences, Heidelberg, Germany) diluted 1:1000 in PBS for 15 minutes at RT. Non-specific binding of antibodies was blocked by incubating the samples with 3% rat serum in BD staining buffer (BD Biosciences, Heidelberg, Germany) for 5 minutes at RT. Anti-CD11c (Cat. #557401), anti-CD19 (Cat. #566412), anti-CD335 (Cat. #564069), anti-CD34 (Cat. #742971), anti-CD3 $\epsilon$  (Cat. #553066), anti-Ly6G (Cat. #560601), and anti-F4/80 (Cat. #565411) were added to the samples in a dilution of 1:100 and incubated for 20 minutes at 4°C. All antibodies were purchased from BD Biosciences. Stained samples were measured with a BD Symphony A1 (BD Biosciences, Heidelberg, Germany), and analysis was performed with FlowJo v10.8.1. Detailed information on the gating strategy is presented in Supplemental Figure 3A.

## Isolation and infection of murine T cells and alveolar macrophages

*Terc*<sup>ko/ko</sup> and young WT mice were sacrificed. Killing for scientific purposes was conducted in accordance with the German animal welfare regulations. Alveolar macrophages (AMs) were isolated as published elsewhere (Rios, Touyz, & Montezano, 2017 [DOI](#)). In short, the murine lungs were washed out several times with PBS containing 0.5 mM Ethylenediaminetetraacetic acid (EDTA). The lavage was centrifuged at 350xg for 10 minutes at 4°C. The cell pellet was dissolved in Roswell Park Memorial Institute (RPMI) medium containing 20 mM HEPES and 5% fetal calf serum (FCS) and counted. Subsequently, cells were seeded and incubated at 37°C for 2 hours.

Lymphocytes were isolated from the spleen as described above. To subsequently isolate CD4<sup>+</sup> T cells, cells were separated via magnetic cell separation. These cells were mixed with CD4<sup>+</sup> beads and separated via MACS LS Columns (both Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The purity of the resulting CD4<sup>+</sup> T cell population was measured via flow cytometry using a BD Symphony A1 (BD Biosciences, Heidelberg, Germany). T cells were added to AMs for co-culture in a 1:1 ratio or cultured alone.

For infection with heat-killed (HK) *S. aureus*, a multiplicity of infection (MOI) of 5 was calculated, and the appropriate bacteria volume was added to each well. Cells were then incubated for 24 hours and harvested for downstream analysis.

## Cytokine measurement

For cytokine measurements, the right inferior lung lobe was homogenized using a SpeedMill Plus in the appropriate volume of PBS to adjust the organs to their weight in mg. Then, the cytokines were measured with the LEGENDPlex<sup>TM</sup> mouse inflammation panel (BioLegend, San Diego, CA, US) according to the manufacturer's instructions. Samples were run in duplicates on the same plate. For the young WT and *Terc*<sup>ko/ko</sup> cohort three biological replicates and for old WT cohorts four biological replicates were measured. Supernatants of co-cultured and single-cultured T cells were measured with the LEGENDPlex<sup>TM</sup> mouse T helper cytokine panel (BioLegend, San Diego, CA, US). For each condition three biological replicates were measured. All samples were run on the same plate. Flow cytometry analysis was performed with a BD Symphony A1, and data analysis was carried out with the Qognit software v2023-02-15 (BD Biosciences, Heidelberg, Germany).

## Flow cytometry

Co-cultured and single-cultured T cells were transferred to FACS tubes and centrifuged at 500xg for 5 minutes at 4°C. The supernatant was frozen for cytokine measurements, and the cell pellet was stained with PBS containing 1:1000 diluted BD Fixable Viability Stain 780 (Cat. # 565388, BD Biosciences, Heidelberg, Germany) for 15 minutes at RT. Staining of surface markers was performed as described above. Samples were stained with anti-CD3ε (1:100, Cat. #553066), anti-CD25 (1:800, Cat. #553866), anti-CD69 (1:400, Cat. # 551113) and anti-CD44 (1:100, Cat. # 560569) for 30 minutes at 4°C. All antibodies were purchased from BD Pharmingen. Subsequently, samples were permeabilized and fixed. For this, the samples were centrifuged for 5 minutes at 500xg and 4°C, resuspended in BD Cytofix/Cytoperm buffer (BD Biosciences, Heidelberg, Germany), and incubated for 20 minutes at 4°C. Samples were washed once with BD Perm/Wash Buffer (BD Biosciences, Heidelberg, Germany). For intracellular staining, samples were stained with anti-CD247 (1:100, Cat. # ab91493, Abcam, Cambridge, UK) antibody in BD Perm/Wash Buffer for 30 minutes at 4°C. Lastly, the samples were washed and resuspended in BD Perm/Wash Buffer. Stained samples were measured with a BD Symphony A1 and analyzed with FlowJo v10.8.1. Detailed information on the gating strategy is presented in Supplemental Figure 4B.

## Scanning electron microscopy (SEM)

T cells and AMs were isolated as described above. Cells were grown on poly-L-lysine (Merck-Millipore, Burlington, MA, US) coated glass coverslips in a 1:1 ratio. T cells were allowed to attach to the coverslips for 30 minutes before adding *S. aureus*. After adhesion of T cells, *S. aureus* USA300 was added at a MOI1 and incubated at 37°C. After 3.5 hours, the samples were fixed with freshly prepared modified Karnovsky fixative (4% w/v paraformaldehyde, 2.5 % v/v glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for 1 hour at RT. After washing each 3 times for 15 minutes with 0.1 M sodium cacodylate buffer (pH 7.4), the cells were post-fixed with 2% (w/v) osmium tetroxide for 1 hour at RT. Subsequently, the samples were dehydrated in ascending ethanol concentrations (30, 50, 70, 90, and 100%) for 15 minutes each. Next, the samples were critical-point dried using liquid CO<sub>2</sub> and sputter coated with gold (thickness approx. 2 nm) using a CCU-010 sputter coater (safematic GmbH, Zizers, Switzerland). Finally, the specimens were investigated with a field emission SEM LEO-1530 Gemini (Carl Zeiss NTS GmbH, Oberkochen, Germany).

## Statistical analysis and scheme design

Data are presented as mean ± SD, or as median with interquartile range for violin and box plots, with up to four levels of statistical significance indicated. P-values were calculated using Kruskal-Wallis test. Individual replicates are represented as single data points. Statistical analysis was carried out using GraphPad Prism v 9.0. Illustrations were created with BioRender.com.

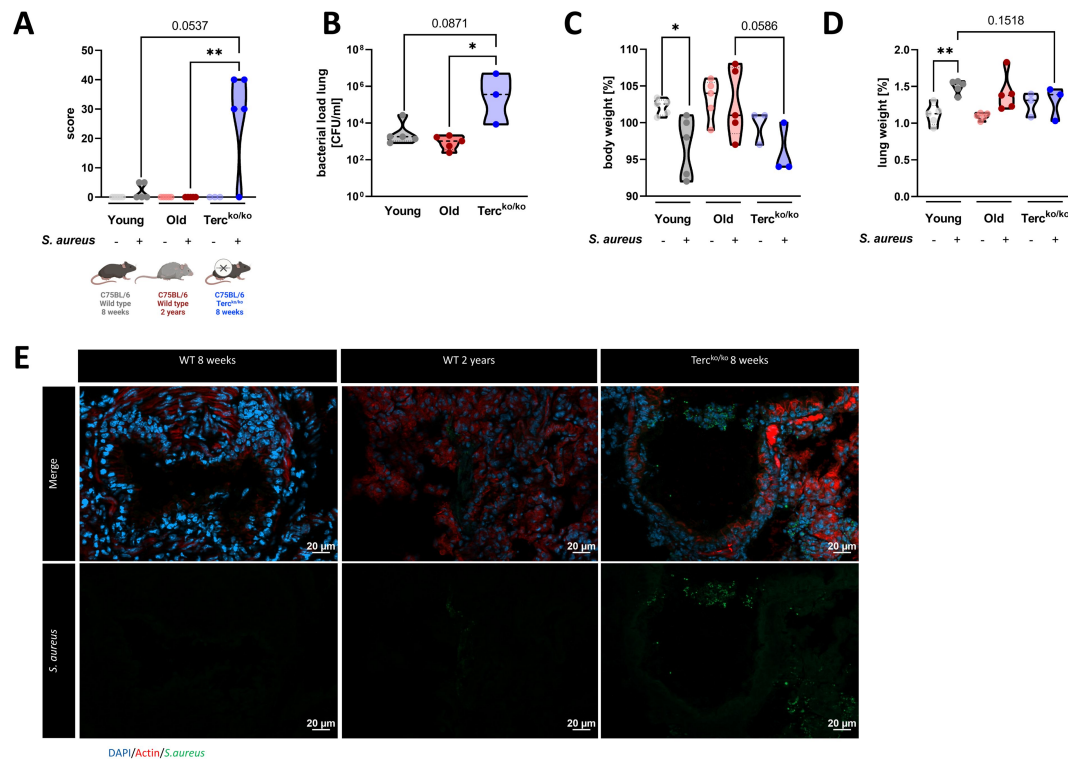
# Results

## *Terc* knockout is associated with severe pneumonia in mice

To investigate the impact of *Terc* on the course of *S. aureus* pneumonia, we compared young *Terc*<sup>ko/ko</sup> mice to young and old wild type (WT) mice. For this purpose, we performed an intranasal infection of *Terc*<sup>ko/ko</sup> mice at the age of 8 weeks and naturally aged mice at the age of 2 years (old WT) with the *S. aureus* strain USA300. WT mice at the age of 8 weeks (young WT) were additionally infected and utilized as the control group (**Figure 1A** [↗](#)).

The clinical score of infected *Terc*<sup>ko/ko</sup> mice was increased compared to the other two infected mice cohorts at 24 hours post infection (hpi) (**Figure 1A** [↗](#)). The bacterial load was significantly higher in the BAL of *Terc*<sup>ko/ko</sup> mice compared to old WT mice (**Figure 1B** [↗](#)). Interestingly, three





**Figure 1**

### ***Terc*<sup>ko/ko</sup> mice exhibit more severe pneumonia with increased mortality and a higher bacterial load.**

A) Clinical score of non-infected and infected young WT (n=10, 5 non-infected and 5 infected), old WT (n=10, 5 non-infected and 5 infected), and *Terc*<sup>ko/ko</sup> (n=8, 3 non-infected and 5 infected) mice at 24 hpi. The age and genetic background of the different groups is depicted below.

B) Bacterial load of the lungs of infected mice at 24 hpi. Data is displayed as logarithmic.

C) Relative body weight of young WT, old WT and *Terc*<sup>ko/ko</sup> mice. Relative body weight displays body weight at 24 hpi as a percentage of the body weight at the time of infection.

D) Relative lung weight of young WT, old WT, and *Terc*<sup>ko/ko</sup> mice. Relative lung weight displays lung weight at 24 hpi as a percentage of the current body weight.

E) Immunofluorescence staining of lung tissue of infected young (WT 8 weeks), old (WT 2 years), and *Terc*<sup>ko/ko</sup> mice. Lungs were stained for *S. aureus* (green), actin (red), and DAPI (blue). Representative pictures are shown for each group.

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. P calculated by Kruskal-Wallis-test (A-D). Data is displayed as violin plot showing the median as well as lower and upper percentile of each dataset. Each replicate is displayed as a data point.

distinct degrees of severity were observed in the infected  $Terc^{ko/ko}$  mice ( $n = 5$ ): mild infection, systemic infections, or fatal infections (Supplemental Figure 1A). Mice exhibiting systemic infection were characterized by the presence of bacteria in extra pulmonary organs, specifically observed in the liver and kidney tissues of some  $Terc^{ko/ko}$  mice (Supplemental Figure 1B).

Infection of young WT mice resulted in a significant decrease in bodyweight at 24 hpi. The body weight of infected  $Terc^{ko/ko}$  mice was reduced compared to the infected old WT mice and the non-infected  $Terc^{ko/ko}$  mice (Figure 1C). In contrast, at 24 hpi the relative weight of the lung was increased in infected compared to the non-infected mice for all cohorts, indicating inflammation of the lung (Figure 1D). However, this effect was the most pronounced in young WT mice.

Based on our observation of the different degrees of severity displayed by the infected  $Terc^{ko/ko}$  mice, we categorized them according to their clinical score and the presence of bacteria in extra pulmonary organs, distinguishing between mice with and without systemic infection. This grouping resulted in more pronounced differences between the mice cohorts regarding bacterial load and reduction of relative body weight (Supplemental Figure 1C-D). The relative lung weight increased for all groups compared to the respective non-infected mice, except for the  $Terc^{ko/ko}$  mouse without systemic infection (Supplemental Figure 1E). Additionally, average telomere length of the lungs of young and old WT as well as  $Terc^{ko/ko}$  mice was measured. As shortened telomeres are a hallmark of aging, impacting other cellular mechanisms such as cellular senescence, they could have an influence on the disease progression (López-Otín et al., 2023). Notably, the telomeres of  $Terc^{ko/ko}$  mice were the shortest among the three different mouse models, even shorter than those of naturally aged mice (Supplemental Figure 1F).

In order to investigate gene expression patterns, mRNA sequencing of the lungs was performed. This data supported the grouping of infected  $Terc^{ko/ko}$  mice into different groups, as the principal component analysis (PCA) plot showed a separate cluster containing  $Terc^{ko/ko}$  mice with systemic infection (Supplemental Figure 1G).

Immunofluorescence (IF) staining of *S. aureus* within the lung tissue of all three infected mice cohorts was carried out. The increased signal for *S. aureus* in sections of  $Terc^{ko/ko}$  mice compared to sections of young WT and old WT mice closely reflects the quantified CFU detected in the BAL (Figure 1E).

$Terc^{ko/ko}$  mice were characterized by an increased bacterial load in the lungs, a higher clinical score as well as mortality (Figure 1A-B; Supplemental Figure 1A). Additionally, we could observe different degrees of severity in the infected  $Terc^{ko/ko}$  cohort and could identify a subgroup of mice with systemic infection (Supplemental Figure 1A-B and G).

## **$Terc^{ko/ko}$ mice showed excessive inflammation with inflammasome activation**

In order to investigate the underlying pathomechanisms leading to a more severe course of disease in the  $Terc^{ko/ko}$  mice, inflammation parameters in the lungs were measured. In the course of this analysis, we applied the previously outlined groups of mice, differentiating between those with and without systemic infection in the  $Terc^{ko/ko}$  cohort. Several pro-inflammatory cytokines were significantly upregulated in  $Terc^{ko/ko}$  mice with systemic infection, such as Interleukin-6 (IL-6), Granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-1 $\beta$  (IL-1 $\beta$ ). Other important pro-inflammatory factors like Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and Interleukin-1 $\alpha$  (IL-1 $\alpha$ ) were also elevated (Figure 2A). A significant upregulation of pro-inflammatory mediators due to infection was, however, solely detected in infected  $Terc^{ko/ko}$  mice compared to the corresponding non-infected control cohort (Figure 2A).



We performed mRNA sequencing of the murine lung tissue of infected and non-infected mice at 24 hpi to elucidate potential differentially expressed genes that contribute to the more severe illness of *Terc*<sup>ko/ko</sup> mice. Comparison of overall gene expression pattern of the different groups revealed that non-infected young WT and *Terc*<sup>ko/ko</sup> mice cluster together and show a similar expression pattern. In contrast, the infected mice of those cohorts display different expression patterns (**Figure 2B**). This highlights that most differences between the knockout and WT mice are only present after infection with *S. aureus*. Furthermore, both non-infected and infected old WT mice show a completely distinct expression profile compared to young WT as well as *Terc*<sup>ko/ko</sup> mice (**Figure 2B**).

Intriguingly, several genes related to the NLRP3 inflammasome pathway, such as *NLRP3* or Thioredoxin Interacting Protein (*TXNIP*), were upregulated in infected *Terc*<sup>ko/ko</sup>, indicating activation of the inflammasome (**Figure 2C**; Supplemental Figure 2A). Notably, expression of genes encoding for TNF $\alpha$ , IL-1 $\beta$ , and IL-6 were heightened. Thus, the increased inflammation in the lungs of *Terc*<sup>ko/ko</sup> mice is likely induced by activation of the NLRP3 inflammasome (**Figure 2C**; Supplemental Figure 2A). Supporting this, the Nucleotide oligomerization domain (NOD)-like receptor signaling pathway was among the most significantly enriched pathways when comparing infected *Terc*<sup>ko/ko</sup> and young WT mice (**Figure 2D**).

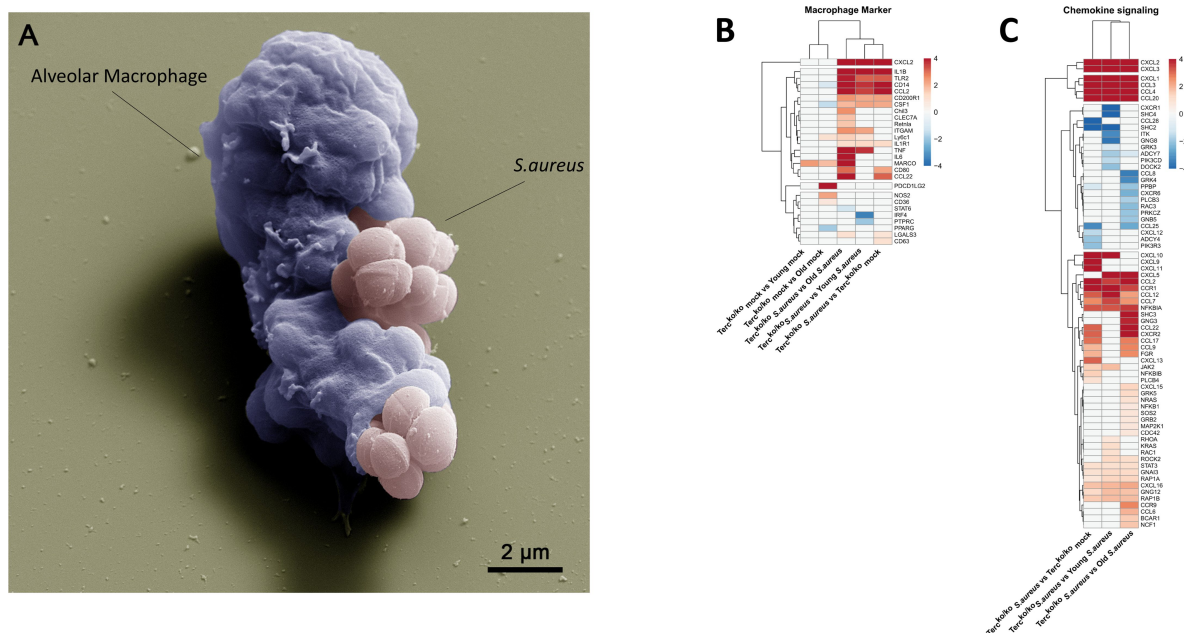
Macrophages are part of the first line of defense against bacterial infections as they can phagocytose bacteria and support pathogen clearance. AMs are present in the lung and can directly react to the pathogen at the site of infection via the production of inflammatory mediators or attraction of other immune cells (Malainou, Abdin, Lachmann, Matt, & Herold, 2023). To specifically investigate the role of AMs during *S. aureus* infection, we isolated them from lungs of young WT and *Terc*<sup>ko/ko</sup> mice. By using scanning electron microscopy (SEM) we could strikingly visualize the ability of AMs to phagocytose *S. aureus* (**Figure 3A**). Additionally, we identified the AMs via IF staining of the macrophage marker CD68 (Supplemental Figure 2B).

Notably, our sequencing data revealed an upregulation of pro-inflammatory M1 macrophage markers, such as C-X-C Motif Chemokine Ligand 2 (*CXCL2*), in *Terc*<sup>ko/ko</sup> mice following infection (**Figure 3B**; Supplemental Figure 2C). Anti-inflammatory M2 markers, e.g., Interferon Regulatory Factor 4 (*IRF4*), were mainly downregulated. Additionally, cluster of differentiation 14 (*CD14*), a general macrophage marker, was increased in the infected *Terc*<sup>ko/ko</sup> mice (**Figure 3B**; Supplemental Figure 2C). Furthermore, the expression of multiple chemokines such as C-X-C Motif Ligand 1-3 (*CXCL1-3*) and C-C Motif Chemokine Ligand 2 (*CCL2*) was elevated, suggesting increased migration of leukocytes such as macrophages and neutrophils to the lung (**Figure 3C**; Supplemental Figure 2D). Additionally, the TNF, NF- $\kappa$ B and IL-17 signaling pathways were among the most significantly enriched pathways in infected *Terc*<sup>ko/ko</sup> mice compared to young WT mice (**Figure 2D**).

Combining the findings above, *S. aureus* infection in *Terc*<sup>ko/ko</sup> mice leads to excessive inflammation induced by NLRP3 inflammasome activation and infiltration of pro-inflammatory immune cells, such as M1 macrophages.

## The T cell receptor is partially downregulated in infected *Terc*<sup>ko/ko</sup> mice

In addition to macrophages, T cells and their activation play a crucial role in the immune response and are vital for resolving infections. Surprisingly, multiple general T cell markers such as *CD2*, *CD5*, and *CD4* were downregulated in the infected *Terc*<sup>ko/ko</sup> mice compared to young WT mice (**Figure 4A**; Supplemental Figure 2E). However, these distinctions are not evident in comparisons between non-infected *Terc*<sup>ko/ko</sup> and young WT mice. This further emphasizes that the observed variations in immune response activation arise specifically in response to infection



**Figure 3**

### Elevated expression of pro-inflammatory M1 macrophages markers and chemokines are present in the lungs of infected *Terc*<sup>ko/ko</sup> mice.

A) Colorized SEM picture of an AM isolated from young WT mice phagocytosing *S. aureus*. Heatmap of differentially expressed macrophage markers (B) and DEGs belonging to the chemokine signaling pathway (C). Red indicates upregulation, and blue indicates downregulation of the gene. Differential gene expression analysis was conducted using the DESeq2 software as well as negative binomial distribution. For FDR calculation the Benjamini-Hochberg procedure was used. Heatmaps were constructed using R Studio. Only significant DEGs (p-value < 0.05) were displayed in the heatmap. Non-significant genes were set to zero and are shown as white. DEGs are displayed as log2fold-change. For each group three biological replicates were sequenced and used for analysis. The respective groups used for the comparison are indicated below each column. All heatmaps display mRNA sequencing data of non-infected and infected lungs of the three different mice cohorts at 24 hpi.

(Figure 4A). Some T cell markers, such as *CD5*, that were downregulated in infected *Terc*<sup>ko/ko</sup> mice were also downregulated in infected old WT mice compared to young WT mice (Supplemental Figure 2E).

To rule out that this effect is caused by a reduction of T cells in *Terc*<sup>ko/ko</sup> mice, the immune cells of non-infected *Terc*<sup>ko/ko</sup>, young WT and old WT mice were analyzed via flow cytometry. However, no significant difference was observed between the groups (Figure 4B).

Additionally, several genes belonging to the T cell receptor (TCR) signaling pathway were downregulated in infected *Terc*<sup>ko/ko</sup> mice, such as *CD3G* (encoding CD3γ) and *CD247* encoding CD3ζ (Figure 4C, Supplemental Figure 2F). Expression of downstream factors of the TCR like Lymphocyte Cell-Specific Protein-Tyrosine Kinase (*LCK*), linker of activation (*LAT*) and IL-2 inducible T cell kinase (*ITK*) was also reduced (Figure 4C, Supplemental Figure 2F). We also noted several of these distinctions, including the downregulation of *LCK* and *ITK* when comparing infected old WT and young WT mice (Figure 4C). Since there was no reduction of T cells in *Terc*<sup>ko/ko</sup> mice, the change in TCR signaling and marker expression is likely induced by the infection.

Specifically, *CD247* was the only gene downregulated compared to infected young WT, old WT, and non-infected *Terc*<sup>ko/ko</sup> mice (Figure 4C). Additionally, *CD247* was downregulated in infected old WT mice compared to infected young WT mice (Figure 4C). *CD247* is an essential factor for the assembly of the TCR and the downstream signaling after stimulation with an antigen (Jin, Yuan, Mehta, Ezenwa, & Morel, 2024; Pitcher et al., 2003). In young WT mice gene expression levels of *CD247* were increased with infection, likely due to proliferation and infiltration of T cells. (Figure 4D). Notably, in infected *Terc*<sup>ko/ko</sup> mice, the expression of *CD247* is absent. Moreover, there is no difference in *CD247* expression between non-infected and infected old WT mice (Figure 4D). Similar trends can be seen for other T cell markers, such as Programmed Cell Death 1 (*PDCD1*) and *CD4* (Figure 4E and F). Additionally, the TCR signaling pathway and Th1 and Th2 differentiation pathway are among the most enriched pathways in infected young WT mice compared to non-infected young WT mice. In contrast, those pathways are not upregulated when comparing infected *Terc*<sup>ko/ko</sup> with non-infected *Terc*<sup>ko/ko</sup> mice (Supplemental Figure 4A).

These findings indicate a malfunction in the activation of T cells as components of the TCR signaling pathway were partially downregulated. The complete absence of *CD247*, an essential factor for downstream activation of the TCR, indicates aberrant T cell function of *Terc*<sup>ko/ko</sup> mice during infection.

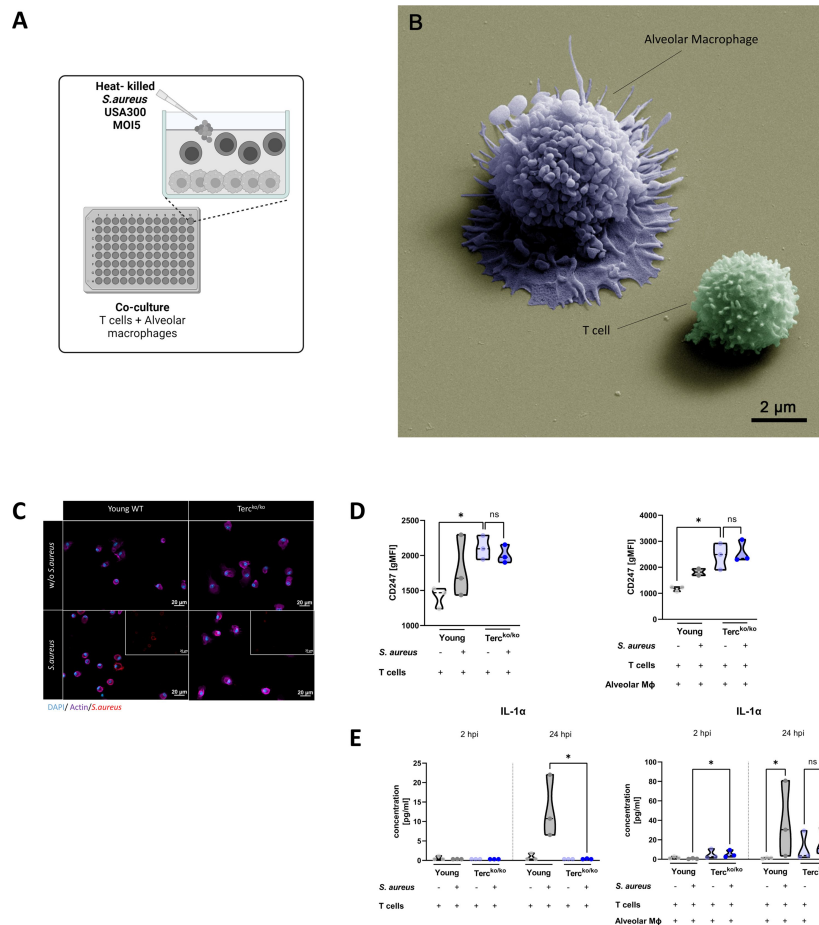
## T cells of *Terc*<sup>ko/ko</sup> mice do not adequately react to infectious challenge

To investigate the functionality of T cells and their *CD247* expression in *Terc*<sup>ko/ko</sup> mice during infection, murine T cells and AMs were isolated. An overactive inflammatory response could be a potential explanation for the dysregulated TCR signaling. Therefore, to study the impact of AMs on T cells we infected the co-culture of both isolated cells with a multiplicity of infection 5 (MOI5) of heat-killed (HK) *S. aureus* for 24 hours (Figure 5A). All components of the experimental setup were visualized using SEM. In particular, the close association between AMs and T cells was notable (Figure 5B). IF staining of AMs of *Terc*<sup>ko/ko</sup> and young WT mice revealed the successful internalization of *S. aureus* as well as a change of phenotypic appearance to a more activated shape (Figure 5C).

Utilizing flow cytometry, we observed a substantial elevation in the baseline expression of *CD247* in T cells from *Terc*<sup>ko/ko</sup> mice in comparison to T cells from young WT mice at 24 hpi (Figure 5D). However, there was no increase in *CD247* expression upon stimulation with *S. aureus* in the *Terc*<sup>ko/ko</sup> T cells (Figure 5D). In contrast, observation of T cells of young WT mice revealed a







**Figure 5**

### T cells of Terc<sup>ko/ko</sup> mice do not adequately react to bacterial challenge.

(A) Schematic representation of experiment. Murine T cells and AMs were isolated, co-cultured, and stimulated with heat-killed *S. aureus* with an MOI5 for 24 hours.

(B) Colorized SEM picture of the experimental setup. An AM close to a T cell, is shown.

(C) Immunofluorescence staining of infected and non-infected AMs from young WT and Terc<sup>ko/ko</sup> mice. AMs were stained with antibodies for *S. aureus* (red), actin (purple), and DAPI (blue).

(D) CD247 expression measured with flow cytometry of non-infected and infected T cells (left) and co-cultured T cells and AMs (right) of young WT (each n=3) and Terc<sup>ko/ko</sup> mice (each n=3) after 24 hpi. CD247 expression is displayed as geometric mean fluorescence intensity (gMFI).

(E) IL-1α from supernatants of T cells (left) and co-cultured T cells and AMs (right) of young WT (each n=3) and Terc<sup>ko/ko</sup> mice (each n=3) at 2 and 24 hpi. IL-1α levels were measured using a flow cytometry based LEGENDPlex<sup>TM</sup> mouse T helper cytokine panel.

ns ≥ 0.05, \*p < 0.05. P calculated by Kruskal-Wallis-test (D-E). Data is displayed as violin plot showing the median as well as lower and upper percentile of each dataset. Each replicate is displayed as a data point.

tendency towards an increase in CD247 expression in response to *S. aureus* stimulation (**Figure 5D** [↗](#)). This indicated improper activation of TCR signaling after *S. aureus* stimulation in T cells of  $Terc^{ko/ko}$  mice.

We could also detect differences in cytokine production when comparing the AMs and T cells from young WT and  $Terc^{ko/ko}$  mice. At 24 hpi, IL-1 $\alpha$  production was significantly higher in T cells from young WT mice stimulated with *S. aureus* when compared to the T cells from  $Terc^{ko/ko}$  mice (**Figure 5D** [↗](#), left). Notably, in the co-culture, IL-1 $\alpha$  production was significantly higher in  $Terc^{ko/ko}$  cells 2 hpi (**Figure 5D** [↗](#), right). On the contrary, there was a significant induction of IL-1 $\alpha$  in the young WT co-culture but no increase in the  $Terc^{ko/ko}$  co-culture with *S. aureus* stimulation after 24 hours (**Figure 5D** [↗](#), right).

Our data indicate that T cells from  $Terc^{ko/ko}$  mice do not effectively react to the infectious stimulus and thus seem dysfunctional due to TCR downregulation.

## Discussion

Shortening of telomeres is a key factor in the aging process and has a significant impact on other hallmarks of aging, such as mitochondrial dysfunction and cellular senescence ([Birch, Barnes, & Passos, 2018](#) [↗](#); [López-Otín et al., 2023](#) [↗](#)). In this context, the discussion surrounding the importance of *Terc* is primarily confined to its role within telomerase. Consequently, other potential functions of *Terc* in various cellular processes have been largely overlooked. Interestingly, various studies have linked *Terc* to inflammation, as it has been shown to activate the NF- $\kappa$ B and PI3K-AKT pathways ([Liu et al., 2019](#) [↗](#); [Wu et al., 2022](#) [↗](#)). Another study could show that the deletion of *Terc* in mice resulted in NLRP3 inflammasome activation after infection with *S. aureus*, thus contributing to more severe pneumonia ([Kang et al., 2018](#) [↗](#)). These studies suggest a close association between *Terc*, inflammation, and the response to bacterial lung infections.

In our study, we utilized a  $Terc^{ko/ko}$  *S. aureus* pneumonia model to investigate this connection. In accordance with German animal welfare regulations and the 3R principle, we only had a reduced number of  $Terc^{ko/ko}$  mice available for the experiment, as some infections were fatal. This reduced number of mice is a limitation of our study. However, we could demonstrate that  $Terc^{ko/ko}$  mice presented with a more severe pneumonia, higher bacterial load, and increased mortality compared to old and young WT mice (**Figure 1** [↗](#)). Additionally, we could show that infected  $Terc^{ko/ko}$  mice are rather heterogeneous and display several degrees of severity, which we investigated separately. Of 5 infected mice, 2 died, 2 displayed signs of a systemic infection and only 1 had a mild infection. The mice with systemic infection were grouped based on the presence of bacteria in organs other than the lung, as well as the sequencing data, where they clustered separately. The detection of bacteria in extra pulmonary organs is of particular interest, as bacteremia is a symptom of severe pneumonia and is associated with high mortality ([De la Calle et al., 2016](#) [↗](#)). The in this study conducted subclassification into different degrees of severity of  $Terc^{ko/ko}$  mice is lacking in similar studies, highlighting the here gained detailed insights.  $Terc^{ko/ko}$  mice were previously shown to have higher susceptibility to *S. aureus* pneumonia, although they exhibited a reduced bacterial load in their lungs ([Kang et al., 2018](#) [↗](#)). These differences may be attributed to the missing separation of infected  $Terc^{ko/ko}$  mice into different degrees of severity. Additionally, this heterogeneity in disease manifestation points towards the limitations of mouse models to study *S. aureus* pathologies. There are a variety of differences between *S. aureus* infections in humans and mice. For instance several relevant virulence factors (e.g. Pantone-Valentine leukocidine) function less efficient in mice than in humans ([Zheng et al., 2023](#) [↗](#)). Therefore, higher infection doses are needed to successfully establish an infection that is comparable to humans ([Zheng et al., 2023](#) [↗](#)).

Inflammation and the activation of the NLRP3 inflammasome are necessary components to fight bacterial infections of the lung. However, excessive inflammation and over-activation of the NLRP3 inflammasome lead to lung injury and can impede pathogen clearance, thus contributing to *S. aureus*-induced pathologies (McVey, Steinberg, & Goldenberg, 2021 [\[1\]](#); Wang et al., 2022 [\[2\]](#)). Upon examining the lungs of infected *Terc*<sup>ko/ko</sup> mice with systemic infection, we identified a marked increase in inflammation parameters (**Figure 2** [\[3\]](#)). Furthermore, we discovered that genes associated with the NLRP3 inflammasome pathway were elevated in the infected *Terc*<sup>ko/ko</sup> mice but not in the corresponding non-infected control cohort. As published previously, this implies that *Terc* knockout on its own does not result in heightened inflammation and inflammasome activation but rather requires an external trigger (Kang et al., 2018 [\[4\]](#)). Inflammasome activation in *Terc*<sup>ko/ko</sup> mice was connected to mitochondria dysfunction and an elevated reactive oxygen species (ROS) production. Additionally, regulation of the inflammasome seems to be dysfunctional as TNFAIP3, an important negative regulator of the inflammasome was downregulated in the *Terc*<sup>ko/ko</sup> mice in this study (Kang et al., 2018 [\[4\]](#)). However, we did not observe downregulation of TNFAIP3, but rather an upregulation in infected *Terc*<sup>ko/ko</sup> mice (**Figure 2C** [\[3\]](#)). These differences may however be attributed to the different study design. Kang et al. focused on telomere dysfunction studying macrophages of old *Terc*<sup>ko/ko</sup> mice, while our study investigated the entire lung of young *Terc*<sup>ko/ko</sup> mice. Of particular note were the heightened levels of IL-1 $\beta$ , a factor known for its ability to amplify the inflammatory response and facilitate the infiltration of pro-inflammatory immune cells, thereby fostering pulmonary injury (Chen et al., 2018 [\[5\]](#); Hu et al., 2020 [\[6\]](#)). Additionally, pro-inflammatory macrophages infiltrating the lung are critical to inflammation-mediated lung injury (Chen et al., 2018 [\[5\]](#); Guan, Zhou, Zhou, & Lin, 2023 [\[7\]](#)). In our study, we could identify the upregulation of several pro-inflammatory macrophage markers in infected *Terc*<sup>ko/ko</sup> mice, as well as several chemokine markers further exacerbating the inflammation in the lung via attraction of immune cells (**Figure 3** [\[3\]](#)). Notably, we did not observe upregulation of inflammation parameters or activation of the inflammasome in the infected old WT mice. Although *CD14* showed a slight increase in non-infected old WT mice compared to non-infected young WT mice, there was no upregulation of macrophage markers in the infected cohorts of these groups. Innate immune cells of aged individuals especially macrophages are associated with chronic low grade inflammation contributing to injury during infection (Duong et al., 2021 [\[8\]](#)). However studies could also show that macrophages of aged mice had a reduced response to stimulation of pathogen recognition receptors such as Toll-like receptor 2 (TLR2). This results in an initially diminished and delayed response of macrophages to infectious challenge, which could be a possible explanation for the lack of inflammation at 24 hpi (Boe et al., 2017 [\[9\]](#); Hinojosa, Boyd, & Orihuela, 2009 [\[10\]](#)).

An overactive inflammatory response not only causes tissue damage but also leads to T cell dysfunction (Guan et al., 2023 [\[7\]](#)). A possible mechanism is the downregulation of CD247, which is the CD3 $\zeta$  chain of the TCR. CD247/CD3 $\zeta$  is necessary for the assembly of the receptor at the cell surface. Furthermore due to its multiple immunoreceptor tyrosine-based activation (ITAM) motifs, it is a central factor in the signal transduction (Bronstein-Sitton et al., 2003 [\[11\]](#); Jin et al., 2024 [\[12\]](#)). The downregulation or absence of CD247/CD3 $\zeta$  is linked to reduced responsiveness in T cells and is commonly present in autoimmune diseases like rheumatoid arthritis and lupus (Dexiu, Xianying, Yingchun, & Jiafu, 2022 [\[13\]](#)). This mechanism also occurs in various chronic infectious diseases, including HIV and Hepatitis C (Dexiu et al., 2022 [\[13\]](#)). However, it can also be a general regulatory process in T cells during infection, helping to maintain immune homeostasis and prevent excessive inflammation (Baniyash, 2004 [\[14\]](#); van der Donk et al., 2021 [\[15\]](#)). Thus, the connection between CD247/CD3 $\zeta$  downregulation during chronic inflammation and chronic infectious diseases has been well documented (Dexiu et al., 2022 [\[13\]](#)). Interestingly, we could show that expression of *CD247* is entirely absent in infected *Terc*<sup>ko/ko</sup> mice, while in the non-infected *Terc*<sup>ko/ko</sup> mice expression of *CD247* could be detected after 24 hours of infection. Hence, our data indicates that infection combined with deletion of *Terc* is associated with reduction of *CD247* expression. Our study provides the first evidence of the total absence of *CD247* expression during acute bacterial infection and inflammation (**Figure 4** [\[3\]](#)).

Previous work has already reported that constant stimulus with bacterial antigens reduces the expression of CD247/CD3 $\zeta$  and impairs T cell function (Bronstein-Sitton et al., 2003 [DOI](#)). However, the complete absence of CD247/CD3 $\zeta$  after a single antigenic stimulus, without chronic inflammation as well as the connection to knockout of *Terc*, are novel findings. These findings require further studies and investigations to gain a deeper understanding of the underlying mechanisms.

Downregulation of CD247/CD3 $\zeta$  is facilitated by myeloid suppressor cells (MSC), which accumulate at the site of inflammation (Baniyash, 2004 [DOI](#); Dexiu et al., 2022 [DOI](#); Ezernitchi et al., 2006 [DOI](#)). MSC are cells which exert an immunosuppressive function on T cells in response to inflammation or infection (Medina & Hartl, 2018 [DOI](#)). Thus MSC contribute towards the regulation of the immune response to pathogens to prevent an overshooting reaction of the immune system (Medina & Hartl, 2018 [DOI](#)). However their immunosuppressive function can also exacerbate infections. For instance, elevated levels of MSC induced T cell dysfunctionality and caused an exacerbated and sustained chronic infection in an *S. aureus* mouse model (Tebartz et al., 2015 [DOI](#)). Immunosuppression of T cells is conveyed by multiple mechanisms, one of which is the downregulation of CD247/CD3 $\zeta$  (Ezernitchi et al., 2006 [DOI](#)). This process is facilitated among others by the ability of MSCs to metabolize the amino acid arginine, as arginine seems to be essential for expression of CD247 in T cells (Ezernitchi et al., 2006 [DOI](#); Rodriguez et al., 2004 [DOI](#)). Interestingly, we could identify several MSC markers, such as lymphocyte antigen 6 family member G (*LY6G*), to be upregulated in the infected *Terc*<sup>ko/ko</sup> mice. This suggests a potential role of MSCs in the downregulation of CD247/CD3 $\zeta$  (Supplemental Figure 4D). Notably, we could identify reduced expression of *CD247* in infected old WT mice compared to young WT mice. Moreover, our sequencing data did not show an increase in *CD247* expression in infected old WT mice compared to the non-infected control group, which further suggests that the T cells of old WT mice may also exhibit some level of dysfunction (Figure 4 [DOI](#)). One potential underlying mechanism is the reduced ability of aged macrophages to activate T cells due to diminished expression of the receptor required for presentation of the antigen (Herrero, Marqués, Lloberas, & Celada, 2001 [DOI](#)). Furthermore, T cells of aged individuals become increasingly deficient due to immunosenescence and exhaustion (Mittelbrunn & Kroemer, 2021 [DOI](#)). Supporting this, we could observe downregulation of *CD27* compared to infected Young WT mice, while *CTLA4* was upregulated in infected old WT compared to uninfected mice (Supplemental Figure 2E and F). Downregulation of CD28 and CD27 is part of T cell aging and an essential marker of T cell senescence, while upregulation of CTLA4 is part of a signature pointing to T cell exhaustion (Zhao, Shao, & Peng, 2020 [DOI](#)). Interestingly, senescent T cells have shorter telomeres and exhibit reduced telomerase activity, which indicates a possible connection between *Terc* and T cell function (Mittelbrunn & Kroemer, 2021 [DOI](#)). Interestingly, *CD28* expression was downregulated in infected *Terc*<sup>ko/ko</sup> mice compared to old WT infected mice (Figure 4A and C [DOI](#)). However, we did not observe any other relevant changes of T cell senescence or exhaustion markers in infected *Terc*<sup>ko/ko</sup> mice (Figure 4A and C [DOI](#)). Thus T cell senescence could be a contributing factor to the T cell dysfunctionality, but likely does not explain the complete phenotype. The differences in the expression of T cell senescence and exhaustion markers, in addition to the lack of increased inflammation in old WT mice, indicates that mechanisms causing downregulation of parts of the TCR and T helper cell markers in old WT and *Terc*<sup>ko/ko</sup> mice differ from each other. As T cell senescence is however a known consequence of *Terc* deletion, the lack of in depth investigation of this process in the present study is a limitation (Matthe et al., 2022 [DOI](#)). It is important to note that telomere shortening has a significant impact on the immune system (Chakravarti et al., 2021 [DOI](#)). Although young *Terc*<sup>ko/ko</sup> mice were used in this study, telomere shortening is still likely to be a contributing factor. Therefore, further experiments investigating the role of T cell senescence in this model should be conducted.

Nevertheless, old WT mice exhibit certain immune cell dysfunction similarities. However, given the intricate nature of aging, other mechanisms contribute to the distinct response observed in *Terc*<sup>ko/ko</sup> and old WT mice following infection. This highlights the necessity of employing a

knockout model to specifically examine the influence of *Terc* on infection, isolating it from the multifaceted processes involved in aging.

We observed an increase in pro-inflammatory macrophages in the lungs of *Terc*<sup>ko/ko</sup> mice during infection. To explore the role of macrophages in regulating T cell function during infection, we investigated whether AMs were sufficient to induce CD247/CD3ζ downregulation in T cells. While CD247/CD3ζ expression increased with bacterial stimulation in T cells from young WT mice, we failed to induce such an increase in CD247/CD3ζ expression in T cells from *Terc*<sup>ko/ko</sup> mice (**Figure 5**). The expression pattern of CD247/CD3ζ was similar to that observed in the sequencing data, as initial levels of CD247/CD3ζ expression in non-infected *Terc*<sup>ko/ko</sup> mice were higher than those of young WT mice. However, there was no significant reduction of CD247/CD3ζ after stimulation with bacteria in the T cells of *Terc*<sup>ko/ko</sup> mice. One potential explanation for this finding is the absence of MSCs and other infiltrating cells, which can contribute to the inflammatory environment and lead to T cell dysfunction. Furthermore, after 24 but not 2 hours of infection, we observed a reduced release of the pro-inflammatory cytokine IL-1α from *Terc*<sup>ko/ko</sup> T cells, suggesting a time-dependent reduction of T cell function. The results of the experiment conducted on T cells of *Terc*<sup>ko/ko</sup> mice revealed an insufficient response to the bacterial stimulation, as there was no significant increase in CD247/CD3ζ expression or IL-1α release after 24 hpi. As total CD4<sup>+</sup> T cells were analyzed in this study, it would be useful to investigate specific T cell populations such as memory and effector T cells to elucidate the potential mechanism leading to T cell dysfunctionality in further detail. Additionally, analysis of differences in immune cell recruitment to the lungs between young WT and *Terc*<sup>ko/ko</sup> mice would be relevant. Thus, additional experiments are needed to validate and further investigate the underlying mechanisms leading to T cell dysfunctionality in *Terc*<sup>ko/ko</sup> mice.

Our data points towards the pivotal role of *Terc* in the regulation of inflammation as well as maintaining immune homeostasis during infection. We could demonstrate that *S. aureus* pneumonia in *Terc*<sup>ko/ko</sup> mice resulted in an overshooting inflammation and activation of the NLRP3 inflammasome. The elevated inflammation eventually leads to T cell dysfunction, which impairs the host's ability to mount an effective immune response against the pathogen, resulting in increased bacterial load and mortality. As T cell dysfunctionality is caused by the excessive inflammation, other pathogens inducing high inflammatory responses in the host could also lead to a similar phenotype in *Terc*<sup>ko/ko</sup> mice.

This study linked T cell dysfunction to *Terc* deletion for the first time and thus provided insights into possible mechanisms contributing to a more severe *S. aureus* pneumonia in the aging population.

## Acknowledgements

Breeding pairs of *Terc*<sup>ko/ko</sup> mice were kindly provided by Lenhard Rudolph (Leibniz Institute on Aging, Jena, Germany). We thank Sylvia Hänßgen, Yvonne Ozegowski, and Lea Herrmann for their excellent technical assistance.

## Additional information

### Conflicts of interest

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



## Funding

This work is supported by the BMBF, funding program Photonics Research Germany (“LPI-BT1-FSU”, FKZ 13N15466; “LPI-BT2-IPHT”, FKZ 13N15704) and is integrated into the Leibniz Center for Photonics in Infection Research (LPI). The LPI initiated by Leibniz-IPHT, Leibniz-HKI, UKJ, and FSU Jena is part of the BMBF national roadmap for research infrastructures. We also want to thank the BMBF for the funding for the “ADA” (13GW0456A). In addition, this work was supported by funding from the Foundation “Else Kröner-Fresenius-Stiftung” within the Else Kröner Graduate School for Medical Students “Jena School for Ageing Medicine (JSAM). This research was also supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany’s Excellence Strategy-EXC 2051 (Project ID No. 390713860).

## Author Contributions

Conceptualization, S.D.-E.; methodology, Y.R.; F.H., A.H., T.L., S.N., writing—original draft preparation, Y.R.; S.D.-E. writing—review and editing, all authors; visualization: Y.R., S.D.-E.; resources and supervision: B.L., S.D.-E.; funding acquisition, B.L.; S.D.-E. All authors have read and agreed to the published version of the manuscript.

## Data availability statement

The data supporting this study’s findings are available from the corresponding author upon reasonable request.

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**Reviewer #1 (Public review):**

Summary:

This work sets out to elucidate mechanistic intricacies in inflammatory responses in pneumonia in the context of aging process (Terc deficiency - telomerase functionality).

Strengths:

Very interesting, conceptually speaking, approach that is by all means worth pursuing. An overall proper approach to the posited aim.

Weaknesses:

The work is heavily underpowered and may have statistical deficits. This precludes at its current state drawing unequivocal conclusions.

I remain at my initial position regarding the weaknesses.

<https://doi.org/10.7554/eLife.100433.2.sa2>



**Reviewer #2 (Public review):**

## Summary

The authors demonstrate heightened susceptibility of Terc-KO mice to *S. aureus*-induced pneumonia, perform gene expression analysis from the infected lungs, find an elevated inflammatory (NLRP3) signature in some Terc-KO but not control mice, and some reduction in T cell signatures. Based on that, they conclude that dysregulated inflammation and T cell dysfunction play a major role in these phenomena.

The strengths of the work did not change, and include a problem not previously addressed (the role of Terc component of the telomerase complex) in certain aspects of resistance to bacterial infection and innate (and maybe adaptive) immune function.

The weaknesses of this revised version still outweigh the strengths, because the authors did not substantially or experimentally answer the main criticism points, and have rather tried to argue away that which cannot be argued away. In summary, the most germane conclusions of this study remain plagued by flaws in experimental design, by lack of rigorous controls and by incomplete and inadequate approaches to testing of immune function.

I will devote the rest of the comments to the revised manuscript and its success or lack thereof in responding to prior criticisms. Prior criticisms are again listed below in italics, to provide context for the attempts of the investigators to respond.

(1) Reviewer 1 has justifiably criticized the exceptionally low power of the study, with 5 control and 3 experimental animals. The responding author has replied that the animal welfare laws preclude them from doing more experiments. That is unfortunate, and I sympathize with the authors. Nonetheless, in the absence of robust corroboration the rigor of the study remains severely compromised and the work is reduced to what I have pointed above - a preliminary and inconclusive study that is in need of deeper and more serious mechanistic investigation.

(2) Terc-KO mice are a genomic knockout model, and therefore the authors need to carefully consider the impact of this KO on a wide range of tissues. This, however, is not the case. There are no attempts to perform cell transfers, use irradiation chimera or crosses that would be informative.

In response to this criticism, the authors have quoted a whole bunch of papers characterizing different aspects of biology of these same mice. The most important paper in that regard would be the one by Matthe et al. on CD4 cells from these same mice. That study was limited and simply diagnosed *in situ* the changes in T cell pool, but did not decipher whether and to what extent such defects are cell-intrinsic or a byproduct of similarly altered microenvironments. Most importantly, none of that answers the original critique question of which cell types are truly the culprits in the Terc deletion phenotype presented here. As I indicated, one has to perform cell transfers, bone marrow irradiation chimera, additional genetic crosses and combinations thereof to substantiate whether the defects are ascribable to the lung tissue itself, the infiltrating myeloid cells, including macrophages, the T cells or a combination thereof. The authors provided none of this.

(3) Throughout the manuscript the authors invoke the role of telomere shortening in aging, and according to them their Terc-KO mice should be one potential model for aging. Yet the authors consistently describe major differences between young Terc-KO and naturally aging old mice, with no discussion of the implications. This further confuses the biological significance of this work as presented.

(4) Related to #2, group design for comparisons lacks a clear rationale. The authors stipulate that Terc-KO will mimic natural aging, but in fact, the only significant differences seen

between groups in susceptibility to *S. aureus* are, contrary to the authors' expectation, between young Terc-KO and naturally old mice (Fig. 1A and B, no difference between young Terc-KO and young wt); or there are no significant differences at all between groups (Fig. 1, C, D.). I have also raised the issue of non-physiological nature of a germline Terc-KO, that does not mimic any known physiological or pathological state.

The authors provided a non-response to this criticism. They argue in their response under (2) of their rebuttal that they included old mice as controls not for aging, because their experimental Terc-deletion mice were G3 and do not exhibit as much of a progeroid phenotype as G5 or G6 mice. But they still say in the revised formulation that these mice were infected "to explore the potential link to a fully developed aging phenotype". They just never conclude that no such link is substantiated by the vast majority of their data. Moreover, they come back to state in their response (4) that because the literature reported ".... reduction of Terc and Tert in tissues of old mice and rats. Therefore, as a potential immunomodulatory factor reduced Terc expression could be connected to age-related pathologies." So either they have used old mice here to compare aging phenotypes, and found that Terc-KO mice diverge massively from aging phenotypes, in which case they have to state so, or they are not using them as age comparators (in which case I am not sure what their purpose is).

(5) (originally part of criticism #4) I have criticized inadequate group design is when the authors begin dividing their Terc-KO groups by clinical score into animals with or without "systemic infection" (the condition where a bacterium spreads uncontrollably across the many organs and via blood, which should be properly called sepsis), and then compare this sepsis group to other groups (Suppl Fig. 1G; Fig. 2; lines 374-376 and 389-391). .... Most importantly, methodologically it is highly inappropriate to compare one mouse with sepsis to another one without. If Terc-KO mice with sepsis are a comparator group, then their controls have to be wild type mice with sepsis, who are dealing with the same high bacterial load across the body and are presumably forced to deploy the same set of immune defenses. The authors responded by making me aware of the 2016 JAMA definition of sepsis that invokes "a life-threatening organ dysfunction caused by a dysregulated host response to infection". I appreciate the correction, and note that in a human setting and globally, such a definition may make sense. The authors stated that bacteremia and not sepsis should be used as a criterion. I agree, and per my original criticism, believe it will be appropriate to compare bacteremic wt and KO mice.

(6) I am shortening my prior critique to make it more to the point that was not addressed: The authors conclude that dysregulated inflammation and T cell dysfunction play a major role in *S. aureus* susceptibility. This may or may not be an important observation, because many KO mice are abnormal for a variety of reasons, and until such reasons are mechanistically dissected, the physiological importance of the observation will remain unclear. ...., the authors truly did not examine the key basic features of their model, including the features of basic and induced inflammatory and immune response. This analysis could be done either using model antigens in adjuvants, defined innate immune stimuli (e.g. TLR, RLR or NLR agonists), or microbial challenge. The only data provided along these lines are the baseline frequencies of total T cells in the spleen of the three groups of mice examined (not statistically significant, Fig. 4B). We do not know if the composition of naïve to memory T cell subsets may have been different, and more importantly, we have no data to evaluate whether recruitment of the immune response (including T cells) to the lung upon microbial challenge is similar or different. So, what are the numbers and percentages of T cells and alveolar macrophages in the lung following *S. aureus* challenge and are they even comparable or are there issues in mobilizing the T cell response to the site of infection ? If, for example, Terc-KO mice do not mobilize enough T cells to the lung during infection, that would explain paucity in many T cell -associated genes in their transcriptomic set that they authors report. That in turn may not mean dysfunction of T cells but potentially a whole different set of defects in coordinating the response in Terc-KO mice.

The authors did not respond to this criticism other than to provide more frequencies of

different subsets. The key here are the NUMBERS of cells present at the peak of challenge, or better yet the kinetics of cell accumulation (again numbers), as well as transfer experiments to establish where the defect actually lies (mobilization, activation, proliferation, etc.).

(7) Related to that, immunological analysis is also inadequate. First, the authors pull signatures from the total lung tissue, which is both imprecise and potentially skewed by differences not in gene expression but in types of cells present and/or their abundance, a feature known to be affected by aging and perhaps by *Terc* deficiency during infection. Second, to draw any conclusions about immune responses, the authors would have to track antigen-specific T cells, which is possible for a wide range of microbial pathogens using peptide-MHC multimers. This would allow highly precise analysis of phenomena the authors are trying to conclude about. Moreover, it would allow them to confirm their gene expression data in populations of physiological interest.

The authors agreed that this would be of interest but did nothing to provide it. They provided a sentence in the discussion stating that this (as well as many other experiments needed to interpret the results) would be of interest.

(8) Overall, the authors begun to address the role of *Terc* in bacterial susceptibility, but to what extent that specifically involves inflammation and macrophages, T cell immunity or aging remains unclear at the present.

My conclusion from the prior review remains unchanged in the face of the revision that did not answer most of the previous criticism. The study as it stands is inconclusive and highly preliminary, with lack of clearly defined mechanistic underpinnings.

<https://doi.org/10.7554/eLife.100433.2.sa1>

#### Author response:

The following is the authors' response to the original reviews.

##### **Public Reviews:**

##### **Reviewer #1 (Public Review):**

###### *Summary:*

*This work sets out to elucidate mechanistic intricacies in inflammatory responses in pneumonia in the context of the aging process (Terc deficiency - telomerase functionality).*

###### *Strengths:*

*Very interesting, conceptually speaking, approach that is by all means worth pursuing. An overall proper approach to the posited aim.*

We want to thank the reviewer for taking the time to review our manuscript and for providing positive feedback regarding our research question.

###### *Weaknesses:*

*The work is heavily underpowered and may have statistical deficits. This precludes it in its current state from drawing unequivocal conclusions.*

Thank you for this essential and valuable comment. We fully accept that the small sample size of the *Tercko/ko* mice is a major limitation of our study and transparently discuss this in our manuscript. However, due to Animal Welfare regulations, only a reduced number of mice

were approved because of the strong burden of disease. Consequently, only three non-infected and five infected mice were available to us. This reduced number of mice presents a clear limitation to our study. However, due to ethical considerations related to animal welfare and sustainability, as well as compliance with German animal welfare regulations, it is not possible to obtain additional Tercko/ko mice to increase the dataset.

The animal studies are an important aspect of our study; however, our hypothesis was also investigated at multiple levels, including in an *in vitro* co-culture model (Figure 5), to ensure comprehensive analysis. Thus, we clearly demonstrated that *S. aureus* pneumonia in Tercko/ko mice leads to a more severe phenotype, orchestrated by the dysregulation of both innate and adaptive immune response.

#### **Reviewer #2 (Public Review):**

##### *Summary:*

*The authors demonstrate heightened susceptibility of Terc-KO mice to S. aureus-induced pneumonia, perform gene expression analysis from the infected lungs, find an elevated inflammatory (NLRP3) signature in some Terc-KO but not control mice, and some reduction in T cell signatures. Based on that, They conclude that dysregulated inflammation and T-cell dysfunction play a major role in these phenomena.*

##### *Strengths:*

*The strengths of the work include a problem not previously addressed (the role of the Terc component of the telomerase complex) in certain aspects of resistance to bacterial infection and innate (and maybe adaptive) immune function.*

We would like to thank the reviewer for the positive feedback regarding our aim to investigate the impact of *Terc* deletion on the pulmonary immune response to *S. aureus*.

##### *Weaknesses:*

*The weaknesses outweigh the strengths, dominantly because conclusions are plagued by flaws in experimental design, by lack of rigorous controls, and by incomplete and inadequate approaches to testing immune function. These weaknesses are as follows*

*(1) Terc-KO mice are a genomic knockout model, and therefore the authors need to carefully consider the impact of this KO on a wide range of tissues. This, however, is not the case. There are no attempts to perform cell transfers or use irradiation chimera or crosses that would be informative.*

We thank the reviewer for bringing up this important point. The aim of our study, however; was to investigate the impact of *Terc* deletion in the lung and on the response to bacterial pneumonia, rather than to provide a comprehensive characterization of the Tercko/ko model itself. This characterization of different tissues and cell types has already been conducted by previous studies. For instance, studies that characterize the general phenotype of the model (Herrera et al., 1999; Lee et al., 1998; Rudolph et al., 1999) but also investigations that shed light on the impact of *Terc* deletion on specific cell types such as microglia (Khan et al., 2015) or T cells (Matthe et al., 2022). The impact of *Terc* deletion on T cells is also discussed in our manuscript in lines 89 to 105. Furthermore, a section about the general phenotype of the *Terc* deletion model is included in the introduction in lines 126 to 138. Thus we discussed the relevant literature regarding Tercko/ko mice in our manuscript and attempted to provide a more in-depth characterization of the lung by investigating the inflammatory response to infection as well as changes in the gene expression (Figure 2-4).

*(2) Throughout the manuscript the authors invoke the role of telomere shortening in aging, and according to them, their Terc-KO mice should be one potential model for aging. Yet the authors consistently describe major differences between young Terc-KO and naturally aging old mice, with no discussion of the implications. This further confuses the biological significance of this work as presented.*

Thank you for mentioning this relevant point. We want to apologize for the confusion regarding this matter. While Tercko/ko mice are a well-established model for premature aging, these effects become more apparent with increasing generations (G) and thus, G5 and 6 mice are the most affected by *Terc* deletion (Lee et al., 1998; Wong et al., 2008).

Thus, while Tercko/ko mice are a common model for premature aging, this accelerated aging phenotype is predominantly apparent in later-generation Tercko/ko (G5 and 6) or aged Tercko/ko mice (Lee et al., 1998; Wong et al., 2008). Since the aim of this study was to analyze the impact of *Terc* deletion on the lung and its immune response to bacterial infections instead of the impact of telomere shortening and telomerase dysfunction, young G3 Tercko/ko mice (8 weeks) were used in this study. This is also mentioned in the lines 131-134. In this study, Tercko/ko mice were used not as a model of aging, but rather as a model specifically for *Terc* deletion. The old WT mice function as a control cohort to observe possible common but also deviating effects between aging and *Terc* deletion. In our sequencing data, we observe that uninfected young WT mice are very similar to uninfected Tercko/ko mice. Other studies have also reported this lack of major differences between uninfected WT and Tercko/ko mice in the G3 knockout mice (Kang et al., 2018). Conversely, uninfected young WT and Tercko/ko mice exhibited great differences, for instance, regarding the numbers of differentially expressed genes (Supplemental Figure 1H). Thus, differences between naturally aged mice and young G3 Tercko/ko mice are not surprising. To clarify this aspect we reconstructed the paragraph discussing the Tercko/ko mice (lines 126-134). Additionally we added a paragraph explaining the purpose of the naturally aged mice to the lines 134 to 138:

“As control cohort age-matched young WT mice were utilized. To investigate whether *Terc* deletion, beyond critical telomere shortening, impacts the pulmonary immune response, we used young Tercko/ko mice. Additionally, naturally aged mice (2 years old) were infected to explore the potential link to a fully developed aging phenotype.”

*(3) Related to #2, group design for comparisons lacks a clear rationale. The authors stipulate that TerckO will mimic natural aging, but in fact, the only significant differences seen between groups in susceptibility to *S. aureus* are, contrary to the authors' expectation, between young Terc-KO and naturally old mice (Figures 1A and B, no difference between young Terc-KO and young wt); or there are no significant differences at all between groups (Figures 1, C, D.).*

We thank the reviewer for this essential comment. As mentioned above the Tercko/ko mice in this study are not selected to model natural aging. To model telomerase dysfunction and accelerated aging selection of later generation or aged Tercko/ko mice would have been more suitable.

The lack of statistical significance in some figures is likely due to the heterogeneity of disease phenotype of *S. aureus* infection in mice, which is a limitation of our study that we discuss in our discussion section in lines 576-582. The phenotype of *S. aureus* infection can vary greatly within a mouse population, highlighting the limitations of mice as a model for *S. aureus* infections. To account for this heterogeneity we divided the infected Tercko/ko mice cohort into different degrees of severity based on the clinical score and the presence of bacteria in organs other than the lung (mice with systemic infection).

Despite the heterogeneity especially within the Tercko/ko mice cohort the differences between the knockout and young as well as old WT mice were striking. Including the fatal infections, 80% of the Tercko/ko mice had a severe course of disease, while none of the WT mice displayed a severe course (Figure 1A, B and Supplemental Figure 1A, B). This hints towards a clear role of *Terc* in the response to *S. aureus* infection in mice. Thus while in some figures the differences are not significant, strong trends towards a more severe phenotype of *S. aureus* infection in the Tercko/ko mice regarding bacterial load, score and inflammatory response could be observed in our study.

*Another example of inadequate group design is when the authors begin dividing their Terc-KO groups by clinical score into animals with or without "systemic infection" (the condition where a bacterium spreads uncontrollably across the many organs and via blood, which should be properly called sepsis), and then compare this sepsis group to other groups (Supplementary Figures 1G; Figure 2; lines 374-376 and 389391). This gives them significant differences in several figures, but because they did not clearly indicate where they applied this stratification in the figure legends, the data are somewhat confusing. Most importantly, methodologically it is highly inappropriate to compare one mouse with sepsis to another one without. If Terc-KO mice with sepsis are a comparator group, then their controls have to be wild-type mice with sepsis, who are dealing with the same high bacterial load across the body and are presumably forced to deploy the same set of immune defenses.*

We sincerely appreciate the significant time and effort you have invested in reviewing our manuscript. However, with all due respect, we must point out that the definition of sepsis you have referenced is considered outdated. According to the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3), sepsis is defined as "a life-threatening organ dysfunction caused by a dysregulated host response to infection" (Marvin Singer, 2016, JAMA). Given this fundamental misunderstanding of our findings, we find the comment regarding the inadequacy of our groups to be both dismissive and lacking in scientific merit. We would like to emphasize that the group size used in our study is consistent with accepted standards in infection research. We strongly reject any insinuations of inadequacy that have been repeatedly mentioned throughout the review.

In order to provide a nuanced investigation of disease severity in Tercko/ko mice, we added the term "systemic infection" to the figures whenever the mice were divided into groups of mice with and without systemic infection. This is the case for Figure 2A and Supplemental Figure 1C-E. The division into mice with and without systemic infection is also mentioned in the figure legend of Figure 2A in lines 932 to 935 and for Supplemental Figure 1 in lines 1052-1053. We agree that Supplemental Figure 1G is somewhat confusing as the mice with systemic infection are highlighted in this graph but not included as a separate group within our sequencing analysis. We added a sentence to the figure legend clarifying this (lines 1042-1044):

"Nevertheless, the infected Tercko/ko mice were considered one group for the expression analysis and not split into separate groups for the subsequent analysis."

Additionally, we revised the section regarding this grouping in different degrees of severity in our Material and Methods section to clarify that this division was only performed for specific analysis (line 191):

"...for the indicated analysis."

Furthermore, the mice which were classified as systemically infected mice were not septic mice, as mentioned above. Those mice were classified by us as systemically infected based on their clinical score and the presence of bacteria in other organs than the lung as stated in the



lines 188-191 and 377-381. Bacteremia is a symptom of very severe cases of hospital-acquired pneumonia with a very high mortality (De la Calle et al., 2016).

Therefore, the systemically infected mice or rather mice with bacteremia display an especially severe pneumonia phenotype, which is distinct from sepsis. The presence of this symptom in our *Terc*ko/ko mice further highlights the clinical relevance of our study. This aspect was added to the manuscript in the lines 568-570.

“The detection of bacteria in extra pulmonary organs is of particular interest, as bacteremia is a symptom of severe pneumonia and is associated with high mortality (De la Calle et al., 2016).”

*(4) The authors conclude that dysregulated inflammation and T-cell dysfunction play a major role in S. aureus susceptibility. This may or may not be an important observation, because many KO mice are abnormal for a variety of reasons, and until such reasons are mechanistically dissected, the physiological importance of the observation will remain unclear.*

*Two points are important here. First, there is no natural counterpart to a *Terc*-KO, which is a complete loss of a key non-enzymatic component of the telomerase complex starting in utero.*

*Second, the authors truly did not examine the key basic features of their model, including the features of basic and induced inflammatory and immune responses. This analysis could be done either using model antigens in adjuvants, defined innate immune stimuli (e.g. TLR, RLR, or NLR agonists), or microbial challenge. The only data provided along these lines are the baseline frequencies of total T cells in the spleen of the three groups of mice examined (not statistically significant, Figure 4B). We do not know if the composition of naïve to memory T cell subsets may have been different, and more importantly, we have no data to evaluate whether recruitment of the immune response (including T cells) to the lung upon microbial challenge is similar or different. So, what are the numbers and percentages of T cells and alveolar macrophages in the lung following *S. aureus* challenge and are they even comparable or are there issues in mobilizing the T cell response to the site of infection? If, for example, *Terc*-KO mice do not mobilize enough T cells to the lung during infection, that would explain the paucity in many T-cell-associated genes in their transcriptomic set that the authors report. That in turn may not mean dysfunction of T cells but potentially a whole different set of defects in coordinating the response in *Terc*-KO mice.*

We thank the reviewer for highlighting these important aspects. Regarding the first point, indeed there is no naturally occurring deletion of *Terc* in humans. However, studies reported reduced expression of *Terc* and *Tert* in the tissues of aged mice and rats (Tarry-Adkins et al., 2021; Zhang et al., 2018). *Terc* itself has been found to have several important immunomodulatory functions such as the activation of the NFκB or PI3-kinase pathway (Liu et al., 2019; Wu et al., 2022). As those aforementioned pathways are relevant for the immune response to *S. aureus* infections, the authors were interested in exploring the impact of *Terc* deletion on the pulmonary immune response. The potential immunomodulatory functions of *Terc* are discussed in lines 106-121. To further clarify our rationale we added a sentence to the introduction in lines 121-125.

“Interestingly, downregulation of *Terc* and *Tert* expression in tissues of aged mice and rats has been found (Tarry-Adkins, Aiken, Dearden, Fernandez-Twinn, & Ozanne, 2021; Zhang et al., 2018). Therefore, as a potential immunomodulatory factor reduced *Terc* expression could be connected to age-related pathologies.”

Regarding the second point, as we focused on the effect of *Terc* deletion in the lung and its role in *S. aureus* infection, we investigated inflammatory and immune response parameters relevant to this setting. For instance, inflammation parameters in the lungs of all three mice cohorts were measured to investigate differences in the inflammatory response in the non-infected and infected mice (Figure 2A). Those measurements showed no baseline difference in key inflammatory parameters between young WT and *Tercko/ko* mice, which is consistent with previous findings (Kang et al., 2018). The inflammatory response to infection with *S. aureus* in the *Tercko/ko* mice cohort differed significantly from the other cohorts (Figure 2A), hinting towards a dysregulated inflammatory response due to *Terc* deletion. Furthermore, we investigated general immune cell frequencies such as dendritic cells, macrophages, and B cells in the spleen of all three mice cohorts to gather a baseline understanding of the general immune cell populations. In our manuscript only total T cell frequencies were included due to its relevance for our data regarding T cells (Figure 4B). This data could show that there was no difference of total amount of T cells in the spleen of all three mice cohorts. For a more detailed insight into our analysis we added the frequencies of the other immune cell populations analyzed in the spleen as a Supplemental Figure 3B-F. Additionally, a figure legend for the graphs was added to lines 1075-1094.

Therefore, while we did not analyze baseline frequencies of specific populations of T cells, we analyzed and characterized the inflammatory and immune response of our model in a way relevant to our research question.

The differences observed in T cell marker and TCR gene expression was also partly present between the uninfected and infected *Tercko/ko* mice such as the complete absence of *CD247* expression in infected *Tercko/ko*, which is however expressed in uninfected mice of this cohort (Figure 4A, C and D). Thus, this effect cannot be solely attributed to an inadequate mobilization of T cells to the lung after infectious challenge. However, we agree that a more detailed insight into recruited immune cells to the lung or frequencies of different T cell populations could contribute to a better understanding of the proposed mechanism and would be an interesting experiment to conduct in further studies. We accept this as a limitation of our study and included it in our discussion section in lines 719-723:

“As total CD4<sup>+</sup> T cells were analyzed in this study, it would be useful to investigate specific T cell populations such as memory and effector T cells to elucidate the potential mechanism leading to T cell dysfunctionality in further detail. Additionally, analysis of differences in immune cell recruitment to the lungs between young WT and *Tercko/ko* mice would be relevant.”

*(5) Related to that, immunological analysis is also inadequate. First, the authors pull signatures from the total lung tissue, which is both imprecise and potentially skewed by differences, not in gene expression but in types of cells present and/or their abundance, a feature known to be affected by aging and perhaps by Terc deficiency during infection. Second, to draw any conclusions about immune responses, the authors would have to track antigen-specific T cells, which is possible for a wide range of microbial pathogens using peptide-MHC multimers. This would allow highly precise analysis of phenomena the authors are trying to conclude about. Moreover, it would allow them to confirm their gene expression data in populations of physiological interest*

We thank the reviewer for highlighting this important and relevant point. In our study, we aimed to investigate the role of *Terc* expression in modulating inflammation and the immune response to *S. aureus* infection in the lung. To address this, we examined the overall impact of age, genotype, and infection on lung inflammation and gene expression. Therefore, sequencing of total lung tissue was essential for addressing the research question posed. Our findings demonstrate that *Tercko/ko* mice exhibit a more severe phenotype following *S.*

*aureus* infection, characterized by an increased bacterial load and heightened lung inflammation (Figures 1 and 2). Furthermore, our data suggest that *Terc* plays a role in regulating inflammation through activation of the NLRP3 inflammasome, along with the dysregulation of several T cell marker genes (Figures 2, 4, and 5). However, this study lacks a detailed analysis of distinct T cell populations, including antigen-specific T cells, as noted earlier. Investigating these aspects in future studies would be valuable to validate and expand upon our findings. We have incorporated these suggestions into the discussion section (lines 719-723)

“As total CD4<sup>+</sup> T cells were analyzed in this study, it would be useful to investigate specific T cell populations such as memory and effector T cells to elucidate the potential mechanism leading to T cell dysfunctionality in further detail. Additionally, analysis of differences in immune cell recruitment to the lungs between young WT and *Tercko/ko* mice would be relevant.”

Nevertheless, our study provides first evidence of a potential connection between T cell functionality and *Terc* expression.

*Third, the authors co-incubate AM and T cells with S. aureus. There is no information here about the phenotype of T cells used. Were they naïve, and how many S. aureus-specific T cells did they contain? Or were they a mix of different cell types, which we know will change with aging (fewer naïve and many more memory cells of different flavors), and maybe even with a Terc-KO? Naïve T cells do not interact with AM; only effector and memory cells would be able to do so, once they have been primed by contact with dendritic cells bringing antigen into the lymphoid tissues, so it is unclear what the authors are modeling here. Mature primed effector T cells would go to the lung and would interact with AM, but it is almost certain that the authors did not generate these cells for their experiment (or at least nothing like that was described in the methods or the text).*

Thank you for bringing up this important question. For the co-cultivation experiment of T cells and alveolar macrophages, total CD4<sup>+</sup> T cells of both young WT and *Tercko/ko* were used. We did not select for a specific population of T cells. Our sequencing data indicated the complete downregulation of *CD247* expression, which is an important part of the T cell receptor, in the lungs of infected *Tercko/ko* mice (Figure 4A, C and D). Given that this factor is downregulated under chronic inflammatory conditions, we investigated the impact of the inflammatory response in alveolar macrophages on the expression of various T cell-derived cytokines, as well as *CD247* expression (Figure 5D, E) (Dexiu et al., 2022). This aspect is also highlighted in the discussion in lines 622-636. Therefore, a co-cultivation model of T cells and alveolar macrophages was established and confronted with heat-killed *S. aureus* to elicit an inflammatory response of the macrophages. To emphasize this purpose, we have revised our statement about the model setup in lines 516-518 of the manuscript:

“An overactive inflammatory response could be a potential explanation for the dysregulated TCR signaling.”

The authors hope this will clarify the intent behind the model setup.

*(6) Overall, the authors began to address the role of Terc in bacterial susceptibility, but to what extent that specifically involves inflammation and macrophages, T cell immunity, or aging remains unclear at present.*

We thank the reviewer for the helpful and relevant comments. The authors accept the limitations of the presented study such as the reduced number of *Tercko/ko* mice and the limitations of murine models for *S. aureus* infection itself and discuss those in the discussion

section in the lines 558-560; 576-582; 688-690 and 719-725. However, we hope that our responses have provided sufficient evidence to convince the reviewer that our data supports a clear role for *Terc* expression in regulating the immune response to bacterial infections, particularly with respect to inflammation and its potential connection to T cell functionality.

**Recommendations for the authors:**

**Reviewer #1 (Recommendations For The Authors):**

*The good element first:*

*I read this paper with genuine interest and applaud the authors for investigating the posited question. I consider it by all means scientifically relevant in the context of physiological/pathophysiological aging and reaction to a disease (here pneumonia). The *Terc* deletion model looks very appropriate for the question and the methodology is very advanced/in-depth. The data flow/selection of endpoints and assays is very logical to me. Moreover, I like the breakdown of pneumonia into varying levels of severity.*

We thank the reviewer for their time and effort taken to revise our manuscript. Additionally, we are grateful to receive your positive feedback regarding our study design and research question.

*The weaknesses:*

*(1) I cannot help but notice that the study is heavily underpowered. As such, it is inadmissible. The key reason is that it is the first of its kind and seminal findings must be strongly propped by the evidence. It is apparent to me that the data scatter presented in the figures tends to be abnormally distributed (e.g. obvious bimodal distribution in some groups). Therefore, the presented comparisons (even if stat. sign) can be heavily misleading in terms of: i) the true magnitude of the observed effects and ii) possible type 2 error in some cases of  $p$  value  $>0.05$ . Solution: repeat the study to ensure reasonable power/reliability. This will also make it stronger as it will immediately demonstrate its reproducibility (or lack of it).*

Thank you for bringing up this extremely relevant point. We acknowledge the issue of the small sample size of *Tercko/ko* mice as a major limitation of our study. This limitation is also included in our discussion section in the lines 558-560. Thus we fully agree with this limitation and transparently discuss this in our manuscript. However, due to the strict German animal welfare regulations it is not possible to obtain more *Tercko/ko* mice, as mentioned above. Furthermore, since fatal infections occurred in the *Tercko/ko* mice cohort we had a reduced number of mice available.

However, the differences between the *Tercko/ko* and WT mice were striking. Including the fatal infections 80% of the *Tercko/ko* mice had a severe course of disease, while none of the WT mice displayed a severe course. This hints towards a clear role of *Terc* in the response to *S. aureus* infection in mice.

*(2) In the stat analysis section of M&Ms, the authors feature only 1 sentence. This cannot be. A detailed stats workup needs to be included there. This is very much related to the above weakness; e.g. it is impossible to test for normality (to choose an appropriate post-hoc test) with  $n=3$ . Back to square one: study underpowered.*

We thank the reviewer for highlighting this important aspect. We carefully revised the method section in lines 357-360 to include all relevant information:

“Data are presented as mean  $\pm$  SD, or as median with interquartile range for violin and box plots, with up to four levels of statistical significance indicated. P-values were calculated using Kruskal-Wallis test. Individual replicates are represented as single data points.”

*(3) Pneumonia severity. While I noted that as a strength, I also note it as weakness here. It looks to me like the authors stopped halfway with this. I totally support testing a biological effect(s) such as the one investigated here across a spectrum of a given disease severity. The authors mention that they had various severity phenotypes produced in their model but this is not visible in the data figs. I strongly suggest including that as well; i.e., to study the posited question in the severe and mild pneumonia phenotype. This is a very smart path and previous preclinical research clearly demonstrated that this severe/mild distinction is very relevant in the context of the observed responses (their presence/absence, longevity, dynamics, etc). I realize this is challenging, thus, I would probably use this approach in the Terc k/o model as sort of a calibrator to see whether the exacerbation observed in the current setup (severe?) will be also present in a mild pneumonia phenotype. S. aureus can be effectively titrated to produce pneumonia of varying severity.*

We thank the reviewer for bringing up this relevant point.

In our study, we could observe heterogeneity within the infected Tercko/ko cohort. Therefore as pointed out by the reviewer we assigned different degrees of severity to those groups based on clinical scores, the fatal outcome of the disease (fatal subgroup), and the presence of bacteria in organs other than the lungs (systemic infection subgroup) as stated in our materials and methods part in the lines 188-191 (Supplemental Figure 1A and B). Moreover, we highlighted this difference in a number of our figures. For example, when categorizing the mice into groups with and without systemic infection, we noticed that the mice with systemic infection demonstrated a higher bacterial load, significant body weight loss, and increased lung weight (see Supplemental Figure 1C-E). Interestingly, the two mice with systemic infection clustered separately from the other mice, indicating that the mice with systemic infection are transcriptomically distinct from the other mice cohorts (Supplemental Figure 1G). Additionally, the inflammatory response was exclusively elevated in the lungs of mice with systemic infection (Figure 2C). Thus, we included this distinction in several figures and attempted to study the differences between those subgroups but also their similarities. For instance, we could observe that some changes in the transcriptome were present in all three infected Tercko/ko mice such as the complete absence of *CD247* expression at 24 hpi (Figure 4D). This distinction therefore provided a more detailed insight into the underlying mechanisms of disease severity in Tercko/ko mice and is lacking in other studies. We agree with the reviewer, that a study investigating mild and severe pneumonia phenotypes would be clinically relevant. However, as noted above, due to ethical considerations related to animal welfare and sustainability, as well as compliance with German animal welfare regulations, it is not possible to obtain additional Tercko/ko mice to carry out the proposed experiment.

*(4) Please read ARRIVE guidelines and note the relevant info in M&Ms as ARRIVE guidelines point out.*

Thank you for emphasizing this crucial aspect. We revised our materials and methods section according to the ARRIVE guidelines (lines 179-206).

“Tercko/ko mice aged 8 weeks, were used for infection studies (n = 8; non-infected = 3; infected = 5). Female young WT (age 8 weeks) and old WT (age 24 months) C57Bl/6 mice (both n = 10; non-infected = 5; infected = 5) were purchased from Janvier Labs (Le Genest-Saint-Isle, France). All infected mouse cohorts were compared to their respective non-infected controls,

as well as to the infected groups from other cohorts. Additionally, comparisons were made between the non-infected cohorts across all groups.

All mice were anesthetized with 2% isoflurane before intranasal infection with *S. aureus* USA300 (1x10<sup>8</sup> CFU/20µl) per mouse. After 24 hours, the mice were weighed and scored as previously described (Hornung et al., 2023). Infected Tercko/ko mice were grouped into different degrees of severity based on their clinical score, fatal outcome of the disease (fatal) and the presence of bacteria in organs other than the lung (systemic infection) for the indicated analysis. Mice with fatal infections were excluded from subsequent analyses, with only their final scores being reported. The mice were sacrificed via injection of an overdose of xylazine/ketamine and bleeding of axillary artery after 24 hpi. BAL was collected by instillation and subsequent retrieval of PBS into the lungs. Serum and organs were collected. Bacterial load in the BAL, kidney and liver was determined by plating of serially diluted sample as described above. For this organs were previously homogenized in the appropriate volume of PBS. Gene expression was analyzed in the right superior lung lobe. Lobes were therefore homogenized in the appropriate amount of TriZol LS reagent (Thermo Fisher Scientific, Waltham, MA, US) prior to RNA extraction. The left lung lobe was embedded into Tissue Tek O.C.T. (science services, Munich, Germany) and stored at 80°C until further processing for histological analysis. Cytokine measurements were performed using the right inferior lung lobe. Lobes were previously homogenized in the appropriate volume of PBS. Remaining organs were stored at -80°C until further usage. Mouse studies were conducted without the use of randomization or blinding.“

*(5) There are also some other descriptive deficits but they are of a much smaller caliber so I do not list them.*

We thank the reviewer for their valuable and insightful suggestions for improving our manuscript. We hope that our responses and the corresponding revisions address these suggestions satisfactorily.

*Concluding: the investigative idea is great/interesting and the methodological flow is adequate but the low power makes this study of low reliability in its current form. I strongly urge the authors to walk the extra mile with this work to make it comprehensive and reliable. Best of luck!*

#### **Reviewer #2 (Recommendations For The Authors):**

*(1) Many legends are uninformative and do not contain critical information about the experiments. For example, Figure 2A with cytokine measurements (in lung homogenates?) is likely showing data from an ELISA or Luminex test, but there is no mention of that in the legend. It stands next to Figure 2B, which is a gene expression map, again, likely from the lung (prepared how, normalized how, etc?) lacking even the most basic information. Further, Figure 2D has no information on the meaning/effect size of gene ratios on the x-axis. Figures 3 and 4 are presumably the subsets of their transcriptome data set (whole lung, harvested on d ?? post-infection), but that is just a guess on my part. Even in the main text, the timing and the controls for the transcriptomic study are not stated (ln. 398 and onwards). The authors really need to revise the figure legends and provide all the details that an average reader would need to be able to interpret the data.*

We thank the reviewer for bringing up this important point. The figure legends of all figures including supplemental figures were revised to ensure they include all relevant data necessary for accurate interpretation of the graphs. Additionally, we clarified the sequenced samples in lines 427-429:



“We performed mRNA sequencing of the murine lung tissue of infected and non-infected mice at 24 hpi to elucidate potential differentially expressed genes that contribute to the more severe illness of Tercko/ko mice.”

*(2) Telomere shortening affects differentially different cells and its role in aging is nuanced - different in mesenchymal cells with no telomerase induction, in non-replicating cells, and in hematopoietic cells that can readily induce telomerase. The authors should be mindful of that in setting up their introduction and discussion.*

Thank you for mentioning this essential aspect. We revised our introduction and discussion to reflect the nuanced role of telomerase shortening in different tissues (lines 83-92 and 690-695):

“Telomerase activity is restricted to specific tissues and cell types, largely dependent on the expression of *Tert*. While *Tert* is highly expressed in stem cells, progenitor cells, and germline cells, its expression is minimal in most differentiated cells (Chakravarti, LaBella, & DePinho, 2021). Consequently, the impact of telomerase dysfunction on tissues varies according to their self-renewal rate. (Chakravarti et al., 2021). One important aspect of telomere dysfunction is the impact of telomere shortening on the immune system as well as the hematopoietic system. Tissues or organ systems that are highly replicative, such as the skin or the hematopoietic system, are affected first by telomere shortening (Chakravarti et al., 2021).”

“It is important to note that telomere shortening has a significant impact on the immune system. Although young Tercko/ko mice were used in this study, telomere shortening is still likely to be a contributing factor. Therefore, further experiments investigating the role of T cell senescence in this model should therefore be conducted.”

*(3) Syntax and formulations need to be improved and made more scientifically precise in several spots. Specifically, in 62-63, the authors say that the aged immune system "is also discussed to be more irritable", please change to reflect the common notion that the reaction to infection is dysregulated; in many cases inflammation itself is initially blunted, misdirected, and of different type (e.g. for viruses, the key IFN-I responses are not increased but decreased). In lines 114-117, presumably, the two sentences were supposed to be connected by a comma, although some editing for clarity is probably needed regardless. Line 252, please change "unspecific" to "non-specific". Line 264, please capitalize German.*

We thank the reviewer for bringing these important points to our attention. We revised our introduction regarding the aged immune response in lines 61-69:

“Age-related dysregulation of the immune response is also characterized by inflammaging, defined as the presence of elevated levels of pro-inflammatory cytokines in the absence of an obvious inflammatory trigger (Franceschi et al., 2000; Mogilenko, Shchukina, & Artyomov, 2022). Additionally, immune cells, such as macrophages, exhibit an activated state that alters their response to infection (Canan et al., 2014). In contrast, the immune response of macrophages to infectious challenges has been shown to be initially impaired in aged mice (Boe, Boule, & Kovacs, 2017). Thus aging is a relevant factor impacting the pulmonary immune response.”

Sentences were edited to provide more clarity in lines 131-134:

“Although G3 Tercko/ko mice with shortened telomeres were used in this study, they were infected at a young age (8 weeks). This approach allowed for the investigation of *Terc* deletion effects rather than telomere dysfunction.”

“Unspecific was changed to “non-specific” in line 282 and “German” was capitalized in line 293 and 558.

We appreciate and thank you for your time spent processing this manuscript and look forward to your response.

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