1 Murine ex vivo cultured alveolar macrophages provide a novel tool to
2 study tissue-resident macrophage behavior and function

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24 KEYPOINTS

- 25 • A novel method to culture and expand primary alveolar macrophages
- 26 over several months ex vivo
- 27 • Murine ex vivo cultured alveolar macrophages (mexAMs) restore lung
- 28 function in a murine pulmonary alveolar proteinosis model

30 SUMMARY

31 Tissue-resident macrophages are of vital importance as they preserve tissue
 32 homeostasis in all mammalian organs. Nevertheless, appropriate cell culture
 33 models are still limited. Here, we propose a novel culture model to study and
 34 expand murine primary alveolar macrophages (AMs), the tissue-resident
 35 macrophages of the lung, in vitro over several months. By providing a
 36 combination of GM-CSF, TGF β and the PPAR γ activator rosiglitazone, we
 37 maintain and expand mouse ex vivo cultured AMs, short mexAMs, over
 38 several months. MexAMs maintain typical morphologic features and stably
 39 express primary AM surface markers throughout in vitro culture. They respond
 40 to microbial ligands and exhibit an AM-like transcriptional profile, including the
 41 expression of AM specific transcription factors. Furthermore, when transferred
 42 into AM deficient mice, mexAMs efficiently engraft in the lung and fulfill key
 43 macrophage functions leading to a significantly reduced surfactant load in
 44 those mice. Altogether, mexAMs provide a novel, simple and versatile tool to
 45 study AM behavior in homeostasis and disease settings.

47 INTRODUCTION

48 Tissue-resident macrophages (TRMs) are capable of self-renewal under
 49 homeostatic conditions in many organs including the lung^{1,2}. By continuous
 50 sensing of the surrounding milieu, TRMs adapt to microenvironmental signals,
 51 resulting in distinct, tissue-specific macrophage identities³⁻⁵.
 52 Alveolar macrophages (AMs), the TRMs of the lung, reside in the alveoli, the
 53 air-liquid interface of the lung, where they perform organ-specific functions

54 such as the clearance of surfactant proteins and cell debris. AMs arise from
 55 fetal liver-derived monocytes that differentiate via granulocyte-macrophage
 56 colony-stimulating factor (GM-CSF)¹ and transforming growth factor (TGFβ)⁶
 57 induced expression of the transcription factor peroxisome proliferator-
 58 activated receptor gamma (PPARγ) around postnatal day 3^{7,8}. The absence of
 59 autocrine TGFβ signaling in AMs of young mice resulted in reduced AM
 60 numbers, together with increased protein content in the bronchoalveolar
 61 lavage⁶. The accumulation of surfactant and subsequent development of
 62 pulmonary alveolar proteinosis (PAP) is also observed in mice and humans
 63 that lack mature AMs due to a loss of GM-CSF receptor subunits^{9,10}. In
 64 patients, PAP is a rare lung disease associated increased susceptibility to
 65 infections and pulmonary fibrosis, which requires regular bronchoscopic
 66 removal of the protein-rich liquid^{11,12}. To fully appreciate the functional
 67 versatility and therapeutic potential of AMs, appropriate cell culture models
 68 are required.

69 In vitro research on macrophages is essentially limited to the use of bone
 70 marrow-derived macrophages that can be expanded and cultured in sufficient
 71 numbers. In contrast, TRMs such as AMs must be isolated from mice and can
 72 only be kept in culture for a few days. In this study, we established a protocol
 73 for the ex vivo expansion and culture of primary AMs, which we termed
 74 mexAMs, by providing AMs with culture conditions that mimic lung
 75 microenvironmental factors. These cultured mexAMs expand rapidly and can
 76 be maintained and stored for several months, while continuously exhibiting
 77 characteristic features of primary AMs, including typical cell surface markers
 78 such as CD11c and Siglec-F and an AM-like transcriptional profile. Adoptively
 79 transferred mexAMs efficiently engraft in the lung and fulfill AM functions. This
 80 includes the reduction of surfactant and protein accumulation upon transfer
 81 into AM deficient mice. Taken together, mexAMs represent a valuable and
 82 versatile tool to study primary AM functions in health and disease.

83 **METHODS**

84 **Mice.** C57BL/6J, CD45.1¹³ and UBI-GFP¹⁴ mice were originally obtained from
 85 Jackson Laboratory. *CD169^{Cre/+}STAT5ab^{fl/fl}* mice (STAT5ΔCD169) or
 86 *STAT5^{fl/fl}* littermate controls were obtained by crossing CD169-Cre¹⁵ provided
 87 by the RIKEN BRC, Japan, and floxed STAT5ab¹⁶ mice provided by R. Morigl
 88 (University of Veterinary Medicine, Vienna). *Csf2rb^{-/-}Csf2rb2^{-/-}* mice^{10,17} were
 89 provided by M. Busslinger (Research Institute of Molecular Pathology,
 90 Vienna). All mice were maintained on a C57BL/6 background and bred and
 91 housed under specific pathogen-free conditions. Mice of both sexes, aged 7-
 92 14 weeks were used. All animal experiments were approved by the Austrian
 93 Federal Ministry of Sciences and Research (BMBWF-66.009/0340-
 94 V/3b/2019).

96 **Culture of murine postnatal liver cells.** Livers were taken between
 97 postnatal day 0.5 and 5 and squeezed through a sterile 70 μm cell strainer.
 98 Red blood cells were lysed using ammonium chloride lysis buffer. Single cell
 99 suspensions (0.2x10⁶/ml) were cultured in RPMI 1640 containing 10% FCS
 100 and 1% pen/strep (“experiment medium”) supplemented with indicated
 101 combinations of murine GM-CSF (30 ng/ml), human TGFβ (10 ng/ml) and
 102 rosiglitazone (1 μM). After 6 days, adherent cells were detached and replated
 103 at 4x10⁴ cells/cm². Subsequently, cells were passaged when 70-90%
 104 confluency was reached.

106 **Maintenance of mouse ex-vivo cultured alveolar macrophages (mexAM).**
 107 Bronchoalveolar lavage from single or a pool of three to four mice was used.
 108 Cells were counted and 0.2x10⁶ cells/ml were cultured in experiment medium
 109 supplemented with murine GM-CSF (30 ng/ml), human TGFβ (10 ng/ml) and
 110 rosiglitazone (1 μM). After 2 h non-adherent cells were washed off and fresh
 111 medium was added. Cells were passaged every 5-6 days when 70-90%
 112 confluency was reached.

113

114 **Isolation of murine bone marrow-derived macrophages and peritoneal**
 115 **macrophages.** Femurs and tibias were flushed with PBS and mouse bone
 116 marrow cells were differentiated for 5 days in experiment medium
 117 supplemented with 10% L929 conditioned medium. Peritoneal lavage was
 118 performed using sterile PBS and cells were plated in experiment medium for 3
 119 h and then washed twice to remove non-adherent cells. Peritoneal
 120 macrophages were immediately used for flow cytometry or RNA-seq
 121 protocols.

122

123 **Flow cytometry.** Single cell suspensions were incubated with anti-mouse
 124 CD16/CD32 monoclonal antibody for 10 min at 4°C. A mix of fluorescently
 125 labeled monoclonal antibodies was added for 30 min at 4°C (Resource table).
 126 Sample acquisition was performed on a LSR Fortessa equipped with
 127 FACSDiva software (BD Biosciences). Singlets were gated using FSC-A
 128 versus FSC-H, followed by a FSC-A/SSC-A gate. Dead cells and erythrocytes
 129 were removed from analysis, using a fixable viability dye eFluor780 and anti-
 130 mouse Ter119. Next, CD45.1 and CD45.2 positive cells were used for further
 131 analysis. Alveolar macrophages were defined as SiglecF^{high}CD11c⁺ cells.

132

133 **Seahorse measurement.** Extracellular acidification rate (ECAR) and oxygen
 134 consumption rate (OCR) were analysed using a XF-96 Extracellular Flux
 135 Analyzer (Seahorse Bioscience) according to manufacturer's instructions.
 136 Cells were plated in XF-96 cell culture plates (1x10⁵ cells/well). To remove
 137 non-adherent cells, cells were washed twice with Seahorse XF RPMI medium
 138 supplemented with 10 mM Glucose, 1 mM Pyruvate, 2mM L-Glutamine and
 139 3% FCS. For real-time analysis of ECAR and OCR, cells were pre-incubated
 140 for 1 h under non-CO₂ conditions. To assess mitochondrial function, 1 μM
 141 oligomycin, 1.5 μM fluoro-carbonyl cyanide phenylhydrazone (FCCP) and 100
 142 nM rotenone plus 1 μM antimycin A (all Sigma-Aldrich) were injected where
 143 indicated. Data analysis was performed on obtained OCR values after
 144 subtraction of respective non-mitochondrial respiration values from all data

145 points. ATP production was represented as OCR difference to baseline after
146 injection of oligomycin.

147

148 **Cytospin.** Cells were spun onto glass slides using the Shandon Cytospin 4
149 and air-dried. Staining was performed using Giemsa solution.

150

151 **Tissue sampling and processing.** Mice were sacrificed by isoflurane
152 inhalation (3.5% isoflurane, 2 l/min oxygen) followed by intraperitoneal
153 injection of 450 mg/kg ketamine and 37.5 mg/kg Rompun in sterile saline. A
154 bronchoalveolar lavage with 1 ml saline was performed. Afterwards, lungs
155 were mechanically disrupted by GentleMACS dissociation (Miltenyi Biotec) in
156 RPMI 1640 containing 5% FCS, 165 U/ml Collagenase I and 12 U/ml Dnase I,
157 followed by digestion for 30 min at 37°C and a final homogenization step. Cell
158 suspension was passed through a 70 µm cell strainer and red blood cells
159 were lysed using ammonium chloride buffer.

160 **Phagocytosis assay.** Cells were plated for 3 h, followed by an incubation
161 with FITC-labeled heat-inactivated *S. pneumoniae* (MOI 100) for 45 min at
162 37°C or 4°C (negative control). Uptake of bacteria was assessed via FITC
163 expression using flow cytometry. Phagocytosis index was calculated as (MFI
164 × % positive cells at 37°C) minus (MFI × % positive cells at 4°C).

165

166 **Electron microscopy.** Cells were fixed in Karnovsky fixative (2% PFA, 2.5%
167 GA in 0.1M cacodylate buffer), washed with cacodylate buffer and stored
168 overnight at 4°C. Next, they were embedded in 1% agarose type IV and
169 treated with 1% osmium tetroxide for 1 h and dehydrated through an ethanol
170 series. After embedding in resin, ultrathin sections were placed on copper
171 mesh grids and stained with 2% uranyl acetate and lead citrate. Samples
172 were examined with a JEM-1400 Plus transmission electron microscope
173 (JEOL).

174

175 **Cell stimulations.** Cell types were stimulated in 96-well plates (5x10⁵
176 cells/ml) with heat-inactivated *S. pneumoniae* (MOI 100) or LPS E.coli O55:B5

177 (10 ng/ml). The levels of secreted cytokines were determined in supernatants
178 after 16 h. For polarization experiments cells were seeded for 2.5 h and
179 afterwards treated with LPS (100 ng/ml) and IFN γ (200 U/ml), IL-4 (10 ng/ml)
180 and IL-13 (10 ng/ml) or IL-10 (10 ng/ml) for 1.5 h (qPCR) or 16 h (nitrite
181 measurement).

182

183 **Cytokine analysis.** Indicated mouse cytokines were measured using the
184 LEGENDplex Mouse Macrophage/Microglia Panel (BioLegend). Samples
185 were prepared according to manufacturer's instructions and analysed by flow
186 cytometry. Data analysis was performed using the LEGENDplex data analysis
187 software.

188

189 **Proliferation assay.** To assess cell proliferation, intracellular ATP levels were
190 measured according to the CellTiter-Glo $\text{\textregistered}$ assay (Promega) instructions.
191 Luminescence was expressed as fold change compared to time-point of
192 seeding.

193

194 **Immunocytochemistry.** Lungs of untreated *Csf2rb*^{-/-}*Csf2rb2*^{-/-} mice or *Csf2rb*^{-/-}
195 *Csf2rb2*^{-/-} that received GFP mexAMs intranasally 4 weeks earlier were fixed
196 in 4% PFA for 48 h, dehydrated in 15% and 30% sucrose for 24 h each as
197 described earlier¹⁸. Sections were stained with anti-pro + mature Surfactant
198 Protein B antibody and DAPI.

199

200 **qPCR.** Total mRNA was isolated using the NucleoSpin kit (Macherey-Nagel)
201 according to manufacturers' instructions. Real-time PCR was performed using
202 the Perfecta SYBR Green Master Mix (Quant Bio). Following primers were
203 used: *mMrc1* (TCTGGGCCATGAGGCTTCTC, CACGCAGCGCTTGTGATC
204 TT), *mYm1* (TCTGGGTACAAGATCCCTGAACTG, GCTGCTCCATGGTCC
205 TTCCA). Gene expression was normalized to *mHprt* and expressed as fold
206 change to indicated control.

207

208 **Nitrite measurement.** Nitrite in cell culture supernatants was measured using
209 the Griess Reagent System (Promega) according to manufacturers'
210 instructions.

211

212 **Adoptive cell transfer.** Mice were anesthetized with isoflurane and $0.4-1 \times 10^6$
213 cells per mouse were intranasally administered. Mice were sacrificed at
214 indicated time-points. The bronchoalveolar lavage was centrifuged at 300 g
215 and the optical density at 600 nm was recorded using an Ultrospec 10
216 (Amersham Biosciences). Cells were manually counted using a Neubauer
217 chamber or a Z2 cell counter (Beckman Coulter).

218

219 **RNA-sequencing.** Total RNA from indicated cell types was isolated using
220 RNeasy Micro kit (Qiagen). Libraries were prepared from 150 ng total RNA
221 input using the QuantSeq 3' mRNA-Seq Library Prep Kit and UMI Second
222 Strand Synthesis Module (Lexogen), according to the manufacturer's
223 instructions. Pooled libraries were 65 bp single-end sequenced on the
224 HiSeq4000 (Illumina). Sequencing was performed at the Biomedical
225 Sequencing Facility (CeMM and Medical University of Vienna). Demultiplexing
226 of raw data and mapping to the mouse genome GRCm38 (mm10) was done
227 using the Bluebee® software (version Quantseq 2.3.6 FWD UMI).

228

229 **DEG and GO enrichment.** Differential expression analysis was performed
230 using functions from the Bioconductor package DESeq2¹⁹. All macrophage
231 populations derived from three independent biological replicates (mouse 1-3)
232 or from two independent mexAM cultures taken at passage 8 and 13 (Pool 1)
233 or passage 5 and 10 (Pool 2). DEGs were defined as absolute log2 fold
234 change >2 and adjusted p-value <0.01 in comparisons between primary AMs
235 and any other cell type (BMDM, PM, mexAM). Heatmaps were generated
236 using the pheatmap function. The most significantly enriched GO terms were
237 assessed using the enrichGO function of clusterprofiler²⁰. The AM specific
238 gene list was curated from two publications^{3,21} and unpublished data.

239

240

241 **Statistical analysis**

242 Data are presented as mean $\pm$ SEM. Comparisons were performed using
 243 unpaired two-tailed Student's *t*-test for two groups or one-way ANOVA for
 244 more than two groups. Statistical significance was defined as $p < 0.05$.
 245 Number of animals is indicated as "n". Sizes of tested animal groups were
 246 dictated by availability of the transgenic strains and litter sizes, allowing
 247 littermate controls.

248 RESULTS

249 Murine alveolar macrophage-like cells can be derived from postnatal 250 liver cells

251 To identify the optimal culture conditions for the expansion of AMs, we
252 considered a previously published protocol, where murine fetal liver cells were
253 cultured in the presence of GM-CSF²². Aiming for a setting that more closely
254 resembles the lung microenvironment, we cultured postnatal murine liver cells
255 in the presence of indicated combinations of GM-CSF, TGF β and
256 rosiglitazone, an activator of the AM transcription factor PPAR γ ²³ (Fig. 1A).
257 Already after six days, all cells treated with GM-CSF plus TGF β or the triple
258 combination had a round shape, closely resembling primary AMs (Fig. 1B,
259 S1A). In contrast, two microscopically distinct cell populations, round and
260 elongated, were observed when cells were grown in the presence of GM-CSF
261 alone or GM-CSF plus rosiglitazone (Fig. 1B, S1A). In addition, we noticed
262 that postnatal liver cells expanded very slowly in the presence of GM-CSF
263 alone. This was reflected in significantly lower intracellular ATP levels in cells
264 treated with GM-CSF alone (Fig. 1C, S1B).

265 Next, we assessed the expression of AM specific as well as pan-macrophage
266 surface markers to define the differentiation profile by flow cytometry. AMs
267 typically express high levels of Siglec-F and CD11c²⁴ (Fig. 1D, S1C). From
268 day six onwards, CD11c⁺ Siglec-F⁺ cells emerged in the fetal liver cell cultures
269 and the relative proportion of AM-like cells in culture gradually increased over
270 time in all conditions (Fig. 1E, S1C). While CD11c was not expressed on
271 freshly isolated postnatal liver cells, it increased over time, being highest on
272 cells treated with GM-CSF only (Fig. 1F). Siglec-F expression was
273 upregulated within 26 days in all conditions (Fig. 1G). Of note, cells treated
274 with the combination of GM-CSF, TGF β and rosiglitazone reached 100% of
275 primary AM Siglec-F expression levels by differentiation day 77 (Fig. S1D,
276 S1E). Mer tyrosine kinase (MerTK), a receptor involved in the engulfment of
277 apoptotic cells, is highly expressed on various macrophage populations
278 including AMs²⁵. Already on differentiation day 6, MerTK expression was
279 comparable to primary AM levels when cells were treated with GM-CSF plus

280 rosiglitazone or the triple combination (Fig. 1H). As AMs develop from Ly-
281 6C⁺CD11b⁺ fetal liver monocytes, we analyzed the expression of CD11b and
282 Ly-6C over time, and observed a gradual downregulation of Ly-6C and
283 consistently very low levels of CD11b (Fig. S1F, S1G).

284 These results show the ability of murine postnatal liver cells, cultured in the
285 presence of GM-CSF, TGFβ and rosiglitazone, to progressively transition from
286 a monocyte phenotype to an AM-like morphology and marker profile, while
287 maintaining their proliferative capacity.

288

289 **Murine ex vivo cultured alveolar macrophages are functionally similar to** 290 **primary alveolar macrophages**

291 TRMs are terminally differentiated immune cell populations that retain self-
292 renewing capacities^{2,26,27}. Being able to generate AM-like cells from murine
293 fetal liver cells, we continued to test our optimized protocol on mature, primary
294 murine AMs (Fig. 2A). Primary AMs expanded and maintained their CD11c⁺
295 Siglec-F⁺ cell expression profile in culture over six months when treated with
296 the combination of GM-CSF, TGFβ and rosiglitazone (Fig. 2B, S2A). These
297 murine ex vivo cultured AMs (mexAMs) appeared strikingly similar to primary
298 AMs, but distinct from BMDMs (Fig. 2C, S2B).

299 As innate immune cells, macrophages play a key role in the defense against
300 pathogens by initiating a pro-inflammatory response. To test their functional
301 properties, we exposed mexAMs, AMs and BMDMs to heat-inactivated *S.*
302 *pneumoniae* (HI *S. pneu*, Fig. 2D, Table S1) and lipopolysaccharide (LPS,
303 Fig. 2E, Table S1). With a few exceptions, mexAMs responded like primary
304 AMs and showed a less vigorous release of cytokines and chemokines than
305 BMDMs (Fig. 2D, 2E). We also tested if a freeze-thaw cycle affects the
306 responsiveness of mexAMs and discovered that IL-6 (Fig. S2C) and CXCL1
307 (Fig. S2D) levels did not differ between HI *S. pneu* stimulated thawed and
308 continuously cultured mexAMs.

309 A main function of AMs in situ pertains to the phagocytosis of surfactant
310 proteins and cellular debris in the alveoli. To test the phagocytic activity of
311 mexAMs compared to primary AMs, we incubated them with FITC-labeled HI
312 *S. pneu*, and observed an efficient and comparable uptake of bacteria by both

cell types (Fig. 2F). Another key characteristic of macrophages is the plasticity in their response to stimuli they are exposed to, while constantly surveying the surrounding tissue²⁸. To assess this, we polarized mexAMs with classically activating M1 (IFN- γ and LPS), alternatively activating M2 (IL-4 and IL-13), as well as deactivating (IL-10) stimuli. MexAMs maintained their plasticity, illustrated by the nitrite release upon M1-polarization (Fig. 2G) and induction of mannose receptor, C type I (*Mrc1*, Fig. 2H) and chitinase-like 3 (*Ym1*, Fig. 2I) upon M2-polarization. To investigate if mexAMs can be maintained without the combination of GM-CSF, TGF β and rosiglitazone, we removed all trophic factors from the medium, which resulted in cell death of mexAMs within one week (Fig. S2E).

Collectively, these data demonstrate that mexAMs, in contrast to BMDMs, exhibit phenotypic and functional properties of primary AMs.

326

MexAMs are phenotypically and transcriptionally closest to primary murine alveolar macrophages

Having established that mexAMs are functionally similar to primary AMs, we next compared their surface marker and transcriptional profile to different macrophage populations. These included BMDMs, as the macrophage type predominantly used for in vitro studies, arising from myeloid bone marrow progenitor cells, as well as peritoneal macrophages (PMs), a mature TRM population exposed to a different tissue microenvironment. Cell surface expression levels of the pan-macrophage marker F4/80 were comparable between all macrophage types, with slightly higher levels in mexAMs and PMs (Fig. 3A). In contrast, the AM surface markers Siglec-F (Fig. 3B) and CD11c (Fig. 3C) were exclusively expressed on mexAMs and AMs, but not BMDMs and PMs.

By examining differences in the transcriptional profile of these four macrophage types, principal component analysis conclusively revealed that mexAMs and AMs clustered tightly together, whereas BMDMs and PMs showed distinct transcriptional profiles (Fig. 3D). Consistently, hierarchical clustering of 2273 differentially expressed genes (DEG) revealed that mexAM samples clustered next to AMs, whereas BMDM samples were closest to PMs

(Fig. 3E). We identified nine different gene clusters (Fig. 3E, Table S2) with cluster IV and V consisting of genes upregulated in BMDMs and PMs. Cluster VIII comprised PM-specific genes such as *Klf2* and *Naip1*. The BMDM specific cluster I contained genes previously shown to be highly expressed in BMDMs such as *Trem2*²⁹. Most interesting were two prominent clusters of genes, cluster II and VI, because they were upregulated in AMs and mexAMs but downregulated in BMDMs and PMs. When we compiled a list of 133 AM specific transcription factors and genes^{3,21} (Table S3), we found 97 to be included in the DEG list shown in Fig. 3E. Most of these genes including *Ear2*, *Marco*, *Fabp1* as well as the transcription factor *Klf4* were part of the two clusters (II and VI) shared between mexAMs and AMs (Fig. 3F). Similarly, the transcription factor *Car4*, which is uniquely expressed in AMs⁴, was elevated in all mexAM and AM samples (cluster II). *Itgax*, the gene underlying CD11c, was highly upregulated in AMs but less expressed on a transcriptional level in mexAMs, whereas *Siglec f* was highly expressed in mexAM and AM samples, coinciding with the flow cytometry results shown before (Fig. 3B, 3C). In the mexAM specific cluster (cluster III), many metabolic genes such as *Acly*, *Pdk1* or *Fasn* were upregulated (Fig. S3A). This was also reflected in the enriched GO terms, which included different metabolic processes (Fig. S3B). Seahorse experiments confirmed highly elevated basal OCR, ATP production and basal ECAR levels in actively expanding mexAMs when compared to primary AMs and terminally differentiated BMDMs (Fig. S3C-G). These data led us to conclude that mexAMs and primary AMs share a common transcriptional signature that differs from BMDMs and PMs.

MexAMs engraft efficiently in a partially depleted alveolar macrophage niche in vivo

To test whether mexAMs engraft in the physiological AM niche in vivo, we transferred CD45.1⁺ mexAMs by intranasal administration into CD45.2 expressing STAT5ΔCD169 mice or littermate controls (Fig. 4A). STAT5 is required for the development of lung dendritic cells (DC) and AMs³⁰. The loss of STAT5 in CD169 expressing cells, which include AMs but not monocytes or DCs³¹, led to a partially emptied AM niche, indicated by a significantly reduced

number of AMs in the bronchoalveolar lavage fluid (BALF) (Fig. S4A). Four weeks after transfer, a pronounced CD45.1⁺ mexAM population was found in the BALF of STAT5ΔCD169 and control mice (Fig. 4B, upper and lower right panel). In STAT5ΔCD169 mice we observed up to 50% of CD11c⁺Siglec-F⁺ cells to be of CD45.1⁺ mexAM origin, while in littermate controls about 9% of BALF (Fig. 4C) or lung (Fig. S4B) CD11c⁺Siglec-F⁺ cells expressed CD45.1. When expressed in absolute numbers, mexAM transfer sufficiently restored the AM niche in STAT5ΔCD169 animals after four weeks (Fig. 4D). To test if transferred mexAMs are capable to long-term repopulate the alveolar niche², we transferred CD45.1⁺ mexAMs into the partially empty AM niche of STAT5ΔCD169 mice and analyzed the BALF eight weeks later. We found a prominent CD45.1⁺ mexAM population, comprising up to 73% of the total AM population in STAT5ΔCD169 mice. This indicates that transferred mexAMs self-renew and repopulate the AM niche long-lasting (Fig. 4E, S4C). Finally, we tested if mexAMs possess the ability to settle in and repopulate the lungs of newborn mice. Thus, we transferred CD45.1⁺ mexAMs into two weeks old STAT5ΔCD169 animals. Consistent with the results in adult STAT5ΔCD169 mice, we found a significant increase in BALF cell numbers 14 weeks after mexAM transfer into young mice (Fig. 4F). These findings demonstrate that mexAMs can efficiently engraft and replenish the AM niche in vivo.

MexAMs restore lung function in a murine pulmonary alveolar proteinosis model

To understand if mexAMs can home to the AM niche as efficiently as primary AMs, we transferred CD45.1⁺ mexAMs and GFP⁺ AMs in a 1:1 ratio into the partially depleted AM niche of STAT5ΔCD169 mice (Fig. 5A). Using flow cytometry we could clearly distinguish these two populations after the transfer (Fig. 5B) and on average 27% of BALF cells consisted of transferred cells after four weeks (Fig. S5A). Analysis of the transferred CD11c and Siglec-F expressing population revealed that the ratio between CD45.1⁺ mexAMs and GFP⁺ primary AMs remained unchanged and that mexAMs and primary AMs contributed equally to the AM population (Fig. 5C). Twelve weeks after

transfer, BALF cells in STAT5 Δ CD169 mice mainly comprised of transferred cells (Fig. S5C), consistent with data shown in Fig. 4E. However, at this time GFP⁺ primary AMs outnumbered CD45.1⁺ mexAMs (Fig. S5B). To compare the proliferation and homing potential of two in vitro cultured macrophage types, we repeated the experimental set-up described in Fig. 5A and transferred GFP⁺ BMDMs and CD45.1⁺ mexAMs in a 1:1 ratio (Fig. S5D). Already after four weeks, BMDMs made up a higher proportion of the transferred cells in the immune cell population of the BALF (Fig. S5E-F).

One of the essential housekeeping functions of AMs is the clearance of lipids and proteins from the alveolar space³². Impaired GM-CSF signaling leads to the absence of mature and functional AMs, increased accumulation of surfactant proteins and subsequent development of pulmonary alveolar proteinosis (PAP)¹². To assess if mexAMs are capable of lipid and protein clearance, we transferred CD45.1⁺ mexAMs into lungs of GM-CSF receptor knock out mice (*Csf2rb*^{-/-}*Csf2rb2*^{-/-}, GM-CSFR KO). Four weeks thereafter, we detected a CD11c⁺ Siglec-F⁺ AM population in the lavage (Fig. 5D). Development of PAP and surfactant accumulation was significantly reduced upon mexAM transfer, as illustrated by a significantly reduced BALF turbidity (Fig. 5E), and a decreased surfactant protein B content in the lungs of GM-CSFR KO mice (Fig. 5F).

These data show that mexAMs engraft the alveolar niche and take over AM functions in vivo, thereby preventing PAP development in GM-CSFR KO mice. Collectively, these data support the usefulness of mexAMs to study AM behavior in homeostatic and disease settings.

436 DISCUSSION

437 The tissue environment shapes the identity of macrophage subsets³⁻⁵, making
438 it almost mandatory to use specific, tissue derived macrophages for in vitro
439 studies. Furthermore, most TRM populations are of embryonic origin² and are
440 thereby quite different in their development compared to the widely used,
441 adult hematopoietic stem cell derived BMDMs.

442 By mimicking the lung microenvironment using GM-CSF, TGF β and the
443 PPAR γ activator rosiglitazone we first used fetal liver cells to generate AM-like
444 cells. Supplementing the medium with GM-CSF induced expression of the
445 integrin molecule CD11c in accordance with established protocols that use
446 GM-CSF to generate CD11c expressing DCs from hematopoietic
447 progenitors^{33,34}. The addition of TGF β to the culture medium induced constant
448 expansion of fetal liver derived AM-like cells, confirming the importance of
449 TGF β in the early AM differentiation⁶. Notably, only the use of the combination
450 of GM-CSF, TGF β and rosiglitazone induced high Siglec-F expression levels,
451 a hallmark of primary AMs. We propose to use these fetal liver-derived AM-
452 like cells as a tool to study AM development in vitro.

453 In a next step we cultured mature, fully differentiated primary AMs ex vivo.
454 MexAMs expand rapidly while keeping their phenotypic and functional AM-like
455 profile over several months in culture. In line with published results^{1,8} we
456 found that expansion of mexAMs is dependent on GM-CSF and cannot be
457 accomplished by supplementing medium with TGF β or rosiglitazone alone
458 (data not shown). While limited GM-CSF concentrations regulate the
459 population of the AM niche in vivo²³, excessive GM-CSF concentrations used
460 under culture conditions allow constant mexAM proliferation and expansion.

461 A main advantage of any in vitro culture system is the unrestricted number of
462 cells that can be utilized for high-throughput assays like whole genome
463 CRISPR screens. We used a common transfection reagent and could confirm
464 that mexAMs can be efficiently transfected with established protocols (data
465 not shown), supporting their usability for high-throughput screening
466 experiments.

467 Analysis of the transcriptional profile of mexAMs revealed that these cells
468 clustered most closely with AM samples and maintained an AM specific gene

expression profile including the transcription factors Klf4³⁵ and Car4⁴. The fact that these mexAM samples derived from single biological replicates or pooled lavages as well as from different passaging numbers, confirms the reproducibility and stability of the AM transcriptomic profile over time. Yet, it is important to note that mexAMs, as an actively proliferating population, exhibited features of expanding cells in their metabolic profile. We therefore recommend additional optimization strategies, when using mexAMs for metabolic assays.

In the next step, we demonstrated that transferred mexAMs replenished the partially depleted AM niche of STAT5 Δ CD169 mice and could be detected up to 14 weeks later in the lavage. Remarkably, even when transferred to WT mice mexAMs settled in the filled niche, albeit to a much lower extent than in STAT5 Δ CD169 mice, supporting the idea that macrophage niches are self-regulating systems that contain a stable macrophage number²³. When performing competitive transfers with 1:1 ratio of mexAMs and AMs, we detected equal numbers of both populations after four weeks. Surprisingly though, when we repeated the competitive transfer assay, mixing mexAMs and BMDMs, we saw a higher proliferative capacity of BMDMs as compared to mexAMs. The high proliferative capacity of transferred BMDMs is in line with data showing that adult bone marrow monocytes display a competitive advantage when re-filling an emptied AM niche, as compared to the remaining donor derived AM population^{1,2,36}.

We envision a wide range of potential applications for mexAMs including co-culture models with immune and structural cells, to better understand disease settings associated with macrophages including lung fibrosis³⁶ or cancer³⁷. Here, we provide evidence that mexAMs can substitute for primary AMs as they restored impaired alveolar surfactant cleaning and prevented the development of PAP in mice lacking primary AMs in vivo.

In summary, our study highlights a previously underappreciated ability to culture and expand fully differentiated TRMs ex vivo over several months by maintaining their intrinsic, tissue-resident macrophage profile.

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509

510 **AUTHOR CONTRIBUTIONS**

511 A.-D.G., D.S., S.Z., K.L. and A.H. performed experiments and analyzed data;
 512 A.-D.G. analyzed bioinformatic data; B.L. and R.K. performed electron
 513 microscopy experiments; B.M. and V.S. provided valuable reagents and
 514 technical advice. A.-D.G. and S.K. wrote the manuscript with input from co-
 515 authors and conceptualized the study.

516 FIGURE LEGENDS

517 **Fig 1. Murine alveolar macrophage-like cells can be derived from** 518 **postnatal liver cells.**

519 (A) Experimental set-up. (B) Primary AMs after 3h in culture and postnatal
520 liver cells treated with murine GM-CSF (30ng/ml) or murine GM-CSF
521 (30ng/ml) + human TGF β (10ng/ml) + rosiglitazone (1 μ M) (Triple) after 6 days
522 (D6) and 26 days (D26) in culture; 40x magnification. (C) Cell proliferation of
523 postnatal liver cells under indicated conditions over 5 days compared to time
524 of seeding. (D) FACS analysis of Siglec-F and CD11c expression on primary
525 AMs and postnatal liver cells grown under indicated conditions on D26. (E)
526 Percentage of CD11c⁺Siglec-F⁺ cells in postnatal liver cell cultures at day of
527 seeding, D6 and D26. (F) CD11c, (G) Siglec-F and (H) MerTK mean
528 fluorescence intensity levels of postnatal liver cell cultures as fold change to
529 primary AMs at indicated days. (D-H) Pre-gated on single, viable CD45⁺ cells.
530 Graphs show means $\pm$ SEM of 3-4 biological replicates. Data are
531 representative of at least two independent experiments. *p < 0.05, **p<0.01
532 (Student's t test). AM= alveolar macrophages, D= day, PN= postnatal, Rosi=
533 Rosiglitazone.

534

535 **Fig 2. Murine ex vivo cultured alveolar macrophages (mexAMs) are** 536 **functionally similar to primary alveolar macrophages.**

537 (A) Experimental set-up. (B) FACS analysis of Siglec-F and CD11c
538 expression on primary AMs and mexAMs on day 13. Pre-gated on viable
539 CD45⁺ cells. (C) Electron microscopy pictures of primary AMs, mexAMs or
540 BMDMs. Magnification: 3000x, scale bar: 3 μ m. (D and E) Measurement of
541 indicated cytokines upon heat-inactivated *S. pneumoniae* (MOI 100) (D) or
542 LPS (10ng/ml) (E) stimulation of AMs, mexAMs or BMDMs for 16 h,
543 expressed as fraction of maximal secretion. (F) Phagocytosis index of primary
544 AMs and mexAMs. (G) Nitrite concentration in supernatants of polarized
545 mexAMs after 16 h. (H and I) M2 polarization markers m*Mrc1* (H) and m*Ym1*
546 (I) assessed by RT-PCR in polarized mexAMs after 1.5 h. Graphs show
547 means $\pm$ SEM of 3-4 biological replicates (D, E) or technical quadruplicates
548 (F-I). Data are representative of at least two independent experiments. *p <

0.05, **p<0.01, ***p<0.001, ****p<0.0001 (one-way ANOVA followed by Dunnett's multiple comparison test). AM= alveolar macrophages, BAL= bronchoalveolar lavage, BMDM= bone marrow-derived macrophages, D= day, HI= heat-inactivated, mexAM= mouse ex vivo cultured alveolar macrophages.

554

Fig 3. MexAMs are phenotypically and transcriptionally closest to primary murine alveolar macrophages.

(A) F4/80, (B) Siglec-F and (C) CD11c cell surface expression on mexAMs, primary AMs, primary PMs and BMDMs measured by FACS. Pre-gated on viable CD45⁺ cells. (D) PCA analysis of indicated cell types of three biological replicates (mouse 1-3) plus mexAM samples derived from different passages of mexAM cultures (Pool). (E) Heatmap of genes differentially expressed (absolute log₂fc value >2, p-adj <0.01) between primary AMs and any other cell type (PM, BMDM, mexAM). n indicates total number of genes per cluster. Raw counts were rlog transformed, followed by z-score scaling. (F) Heatmap of AM specific genes found in indicated clusters. AM= alveolar macrophages, BMDM= bone marrow-derived macrophages, mexAM= mouse ex vivo cultured alveolar macrophages, PC= principal component, PM= peritoneal macrophages.

569

Fig 4. MexAMs engraft efficiently in a partially depleted alveolar macrophage niche in vivo.

(A) Experimental set-up. Intranasal transfer of CD45.1⁺ mexAMs into CD45.2 expressing control (STAT5fl/fl) or STAT5ΔCD169 mice. (B) FACS analysis of CD45.1 and CD11b expression of control (upper panel) and STAT5ΔCD169 (lower panel) BALF cells untreated, or 4 weeks after transfer of CD45.1⁺ mexAMs. Pregated on viable Siglec-F⁺/CD11c⁺ cells. (C) Percentage of resident (CD45.2⁺, grey) and transferred (CD45.1⁺, orange) cells in BALF of control (STAT5fl/fl, n=4) and STAT5ΔCD169 mice (n=4) 4 weeks post CD45.1⁺ mexAM transfer. (D) CD11c⁺Siglec-F⁺ lung cell number in STAT5fl/fl and STAT5ΔCD169 mice untreated and 4 weeks post transfer of CD45.1⁺ mexAMs. (E) Percentage of resident (CD45.2⁺, grey) and transferred

(CD45.1⁺, orange) cells in BALF of STAT5ΔCD169 mice (n=4-5) 8 weeks post CD45.1⁺ mexAM transfer. (F) BALF cell count per ml in STAT5fl/fl and STAT5ΔCD169 mice 14 weeks post transfer of CD45.1⁺ mexAMs into young (14 d) old mice. Graphs show means ± SEM of 2-5 biological replicates. *p < 0.05, **p<0.01, ***p<0.001 (one-way ANOVA followed by Sidak's multiple comparison test). BALF= bronchoalveolar lavage, i.n.= intranasal, mexAM= mouse ex vivo cultured alveolar macrophages, ns= non significant.

Fig 5. MexAMs restore lung function in a murine pulmonary alveolar proteinosis model.

(A) Experimental set-up. Intranasal transfer of CD45.1⁺ mexAMs and GFP⁺ AMs in a 1:1 ratio into CD45.2 expressing STAT5ΔCD169 mice. (B) FACS analysis of GFP and CD45.1 expression in BALF of STAT5ΔCD169 mice 4 weeks after transfer of 50% GFP⁺ AMs and 50% CD45.1⁺ mexAMs. Pre-gated on viable Siglec-F⁺/CD11c⁺ cells. (C) Percentage of AMs (GFP⁺, green) and mexAMs (CD45.1⁺, orange) Siglec-F⁺/CD11c⁺ cells in BALF of STAT5ΔCD169 (n=4) mice 4 weeks post transfer. (D) FACS analysis of Siglec-F and CD11c expression on cells in BALF of GM-CSF receptor knock-out mice with and without transferred CD45.1⁺ mexAMs. (E) Representative picture and quantification of BALF turbidity of GM-CSF receptor knock-out mice control and after transfer of CD45.1⁺ mexAMs (4 weeks). (F) Immunofluorescent picture of surfactant protein B accumulation (SFB, red) in GM-CSF receptor knock-out mice without (left) and 4 weeks after transfer of GFP⁺ mexAMs (green). Magnification: 20x, scalebar: 50 μm. Graphs show means ± SEM of 4-5 biological replicates or representative pictures. *p < 0.05 (Student's t test). BALF= bronchoalveolar lavage, mexAM= mouse ex vivo cultured alveolar macrophages.

Supplemental figure legends

Fig. S1

(A) Primary AMs after 3 h in culture and postnatal liver cells treated with murine GM-CSF (30ng/ml)+ human TGFβ (10ng/ml) or GM-CSF (30ng/ml)+ Rosiglitazone (1μM) after 6 d (D6) and 26 d (D26) in culture; 40x

614 magnification. **(B)** Cell proliferation of postnatal liver cells under indicated
 615 conditions over 5 days compared to time of seeding. **(C)** FACS analysis of
 616 Siglec-F and CD11c expression in primary AMs and postnatal liver cells
 617 grown under indicated conditions on D26. **(D)** FACS analysis of Siglec-F and
 618 CD11c expression in primary AMs and postnatal liver cells grown with murine
 619 GM-CSF+ human TGF β + Rosiglitazone (Triple) on D77. **(E)** Siglec-F
 620 expression in primary AMs and postnatal liver cells grown with murine GM-
 621 CSF+ human TGF β + Rosiglitazone (triple) on D77. **(F)** CD11b and **(G)** Ly-6C
 622 mean fluorescence intensity levels of postnatal liver cell cultures as fold
 623 change to primary alveolar macrophages at indicated days. **(C-G)** Pre-gated
 624 on single, viable CD45⁺ cells. Graphs show means $\pm$ SEM of 3-4 biological
 625 replicates. Data are representative of at least two independent experiments.
 626 AM= alveolar macrophages, D= day, PN= postnatal, Rosi= Rosiglitazone.

627

628 **Fig. S2**

629 **(A)** FACS analysis of Siglec-F and CD11c expression in primary AMs and
 630 mexAMs after 170 days in culture. Pre-gated on viable CD45⁺ cells. **(B)**
 631 Cytospin pictures of Giemsa-stained primary AMs or mexAMs cultured for 102
 632 days. Magnification: 40x, scale bar: 15 μ m. **(C and D)** IL-6 **(C)** and CXCL-1 **(D)**
 633 levels in supernatants of continuously cultured and thawed mexAMs
 634 stimulated with heat-inactivated *S. pneumoniae* (MOI 100) for 16 h. **(E)** Cell
 635 number of mexAMs cultured in murine GM-CSF+ human TGF β +Rosiglitazone
 636 (Triple) containing medium or in medium without trophic factors over time.
 637 Graphs show means $\pm$ SEM of technical quadruplicates. Data are
 638 representative of at least two independent experiments. AM= alveolar
 639 macrophages, D= day, h= hours, mexAM= mouse ex vivo cultured alveolar
 640 macrophages, nd= non detectable, MOI= multiplicity of infection.

641

642 **Fig. S3**

643 **(A)** Heatmap of genes found in cluster III (Fig. 3E). Raw counts were rlog
 644 transformed, followed by z-score scaling. **(B)** GO pathway enrichment of
 645 cluster III. **(C-G)** Oxygen consumption rate (OCR, **C**) and extracellular

acidification rate (ECAR, **F**) of mexAMs, primary AMs or BMDMs (d5) were measured by Seahorse extracellular flux analysis at baseline and after injection of oligomycin (O), FCCP (F) and rotenone/antimycin A (R&A). Basal OCR (**D**), ATP production (**E**) and basal ECAR (**G**) of indicated cell types. (**C-G**) data are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA followed by Dunnett's multiple comparison test). AM= alveolar macrophages, BMDM= bone marrow-derived macrophages, ECAR= extracellular acidification rate, GO= gene ontology, mexAM= mouse ex vivo cultured alveolar macrophages, ns= non significant, OCR= oxygen consumption rate, PM= peritoneal macrophages.

Fig. S4

(**A**) BALF cell count per ml in STAT5fl/fl (control) and STAT5 Δ CD169 mice. (**B**) Percentage of resident (CD45.2⁺, grey) and transferred (CD45.1⁺, orange) cells in lungs of control (STAT5fl/fl, n=4) and STAT5 Δ CD169 mice (n=4) 4 weeks post CD45.1⁺ mexAM transfer. (**C**) BALF cell count per ml in STAT5fl/fl and STAT5 Δ CD169 mice 8 weeks post transfer of CD45.1⁺ mexAMs. Graphs show means $\pm$ SEM of 4-5 biological replicates. * $p < 0.05$, ** $p < 0.01$ (Student's t test (A) or one-way ANOVA followed by Sidak's multiple comparison test (C)). BALF= bronchoalveolar lavage, mexAM= mouse ex vivo cultured alveolar macrophages.

Fig. S5

(**A**) Percentage of resident (CD45.2⁺, grey) and transferred (CD45.1⁺ mexAMs and GFP⁺ AMs, red) cells in BALF of STAT5 Δ CD169 mice (n=4) 4 weeks post transfer. (**B**) Percentage of AMs (GFP⁺, green) and mexAMs (CD45.1⁺, orange) Siglec-F⁺CD11c⁺ cells in BALF of STAT5 Δ CD169 (n=3) mice 12 weeks post transfer. (**C**) Percentage of resident (CD45.2⁺, grey) and transferred (CD45.1⁺ mexAMs and GFP⁺ AMs, red) cells in BALF of STAT5 Δ CD169 mice (n=3) 12 weeks post transfer. (**D**) FACS analysis of GFP⁺ BMDM and CD45.1⁺ mexAM ratio on day of transfer. (**E**) BMDM (GFP⁺, green) and mexAM (CD45.1⁺, orange) percentage of Siglec-F⁺/CD11c⁺ cells in BALF of STAT5 Δ CD169 (n=4) mice 4 weeks post transfer. (**F**) Percentage

678 of resident (CD45.2⁺, grey) and transferred (CD45.1⁺ mexAMs and GFP⁺
679 BMDMs, red) cells in BALF of STAT5ΔCD169 mice (n=4) 4 weeks post
680 transfer. Graphs show means ± SEM of 3-4 biological replicates. AM=
681 alveolar macrophages, BALF= bronchoalveolar lavage, BMDM= bone
682 marrow-derived macrophages, i.n.= intranasal, mexAM= mouse ex vivo
683 cultured alveolar macrophages.

684

685 **Supplemental Tables**

686 Resource table

687 Table S1 (Legendplex data for AM, mexAM and BMDM samples, related to
688 Figure 2)

689 Table S2 (DEGs rlog transformed including cluster annotation and gene
690 symbol, related to Figure 3)

691 Table S3 (List of AM specific genes, related to Figure 3)

692

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Figure 5

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Figure S1

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Figure S3

Figure S5

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