**1Adenoviral vector-mediated GM-CSF gene transfer improves anti-
2mycobacterial immunity in mice – role of regulatory T cells**

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18

19Running title: Role of AdGM-CSF in anti-mycobacterial immunity

20

21**Abstract**

22Granulocyte macrophage-colony stimulating factor (GM-CSF) is a hematopoietic
23growth factor involved in differentiation, survival and activation of myeloid and non-
24myeloid cells with important implications for lung antibacterial immunity. Here we
25examined the effect of pulmonary adenoviral vector-mediated delivery of GM-CSF
26(AdGM-CSF) on anti-mycobacterial immunity in *M. bovis* BCG infected mice.
27Exposure of *M. bovis* BCG infected mice to AdGM-CSF either applied on 6 h, or 6 h
28and 7 days post-infection substantially increased alveolar recruitment of iNOS and IL-
2912 expressing macrophages, and significantly increased accumulation of IFN γ^{pos} T
30cells and particularly regulatory T cells (Tregs). This was accompanied by
31significantly reduced mycobacterial loads in the lungs of mice. Importantly, diphtheria
32toxin-induced depletion of Tregs did not influence mycobacterial loads, but
33accentuated immunopathology in AdGM-CSF-exposed mice infected with *M. bovis*
34BCG. Together, the data demonstrate that AdGM-CSF therapy improves lung
35protective immunity against *M. bovis* BCG infection in mice independent of co-
36recruited Tregs, which however critically contribute to limit lung immunopathology in
37BCG-infected mice. These data may be relevant to the development of
38immunomodulatory strategies to limit immunopathology-based lung injury in
39tuberculosis in humans.

40

41**Key words:** GM-CSF, tuberculosis, Regulatory T cells, FoxP3

42Introduction

43*Mycobacterium tuberculosis* (*M. tuberculosis*) is the main causative pathogen in
44pulmonary tuberculosis (TB), which causes major morbidity and mortality worldwide .
45Lung infection with *M. tuberculosis* occurs via inhalative routes, eventually resulting
46in uptake of the bacteria by professional phagocytes, primarily alveolar macrophages
47(AM), as well as dendritic cells (DC) of the lung . While resident AM are the cellular
48reservoir of mycobacteria, classical lung DCs are considered to shuttle the
49mycobacteria towards the draining lymph nodes to facilitate adaptive immune
50responses, but this simultaneously promotes their dissemination .

51 Uncontrolled mycobacterial replication in the lung is typically inhibited in
52immunocompetent individuals by formation of granulomas, consisting of epithelioid
53macrophages surrounded by lymphocyte subsets . Both CD4^{pos} and CD8^{pos} T cells
54are recruited to sites of infection, and IFN- γ from T cells is necessary to inhibit
55mycobacterial replication in macrophages. At the same time, this triggers robust CD8
56T cell responses, thereby contributing to maintenance of granuloma formation .
57However, initiation of lung anti-mycobacterial immunity develops relatively late after
58infection, with onset of effector lymphocyte recruitment to infected lungs usually
59starting not before 2–3 weeks post-infection . This calls for novel anti-mycobacterial
60strategies aiming to accelerate and enhance lung protective immunity against this
61serious lung pathogen .

62 The hematopoietic growth factor granulocyte macrophage colony stimulating
63factor (GM-CSF) is a pleiotropic cytokine regulating differentiation, proliferation and
64survival of myeloid and non-myeloid cells . GM-CSF is predominantly released in the
65lungs by epithelial cells and macrophages and is critical to surfactant homeostasis as

66well as macrophage-mediated protective immunity , which is mediated by
67JAK/STAT5 signaling pathways, induction of the myeloid transcription factor PU.1
68and interferon-regulating factor 5 (IRF5) . Lack of GM-CSF or its endogenous
69neutralization results in pulmonary alveolar proteinosis (PAP), characterized by
70accumulation of surfactant lipids and proteins within alveoli which strongly impairs
71host defense in humans and mice . Particularly, absence of GM-CSF renders mice
72more susceptible to systemic and pulmonary infections with mycobacteria .
73Mycobacterial disease progression in GM-CSF KO mice is comparable to that of IFN-
74 γ KO mice, as in the absence of granuloma formation, both mouse strains exhibited
75fatal disease courses . *In vitro* studies showed improved anti-mycobacterial
76responses of macrophages against *M. tuberculosis* through stimulation with AdGM-
77CSF . However, congenital overexpression of GM-CSF in the lungs of mice did not
78improve lung anti-mycobacterial resistance against *M. tuberculosis* due to lack of
79appropriate granuloma formation, suggesting the necessity of GM-CSF release
80regulation .

81 After infection with *M. tuberculosis*, the onset of adaptive immunity is
82substantially delayed as evidenced by lack of antigen-specific CD4^{pos} T cell reactivity
83and delayed DC recruitment to mediastinal lymph nodes within the first two weeks of
84infection . We recently demonstrated that prophylactic adenoviral delivery of GM-CSF
85protected mice against lethal pneumococcal pneumonia . Mechanistically, GM-CSF
86activation of macrophages has been reported to result in sequestration of Zn to
87metallothioneins, thereby shuttling Zn away from the phagosomal compartment,
88finally resulting in increased killing of phagocytosed pathogens via oxidative burst .
89However, there are only limited data showing the therapeutic effect of pulmonary
90GM-CSF release on T cell and regulatory T cell dependent lung protective immunity
91against mycobacterial infections in mice. Therefore, we analyzed the effect of
92transient adenoviral GM-CSF gene transfer on lung anti-mycobacterial immunity in
93mice infected with *M. bovis* BCG and characterized the role of newly recruited
94FoxP3^{pos} Tregs herein.

95

96**Materials and methods**

97**Animals**

98C57BL/6J mice were purchased from Janvier (Sulzfeld, Germany). DERE mice
99(C57BL/6J background) bearing the human diphtheria toxin receptor (DTR) coupled
100to enhanced GFP (eGFP) under control of the forkhead box P3 (*foxp3*) locus allowing
101specific diphtheria toxin-induced depletion of regulatory T cells were generated as
102previously described . Animals were used for experiments at 8-12 weeks of age,
103following the European Council Directive 2010/63/EU as well as the German Animal

104Welfare Act, and were approved by the Lower Saxony State Office for Consumer
105Protection and Food Safety.

106

107**Reagents**

108Antibodies anti-CD3 FITC (clone 145-2C11, Armenian Hamster IgG1), anti-CD3 PE
109(clone 145-2C11, Armenian Hamster IgG1), anti-CD3e APC-Cy7 (clone 145-2C11,
110Armenian Hamster IgG1), anti-CD4 PerCP-Cy5.5 (clone RM4-5, rat IgG2a), or anti-
111CD8 PE-Cy7 (clone 53-6.7, rat IgG2a), anti-CD8a APC (clone 53-6.7, rat IgG2a),
112anti-CD11b BV 510 (clone M1/70, rat IgG2b, κ), anti-CD11b PE-Cy7 (clone M1/70,
113rat IgG2b), anti-CD11c APC (clone HL3, Armenian Hamster IgG₁, λ 2), anti-CD19 PE
114(clone 1D3, rat IgG2a), anti CD45 (clone 30-F11, rat IgG2b) anti-CD45 PE-Cy7
115(clone 30F11, rat IgG2b), anti-CD45 APC (clone 30F11, rat IgG2b), anti-IFN- γ -APC
116(clone XMG1.2, IgG1), anti-Ly6G PE (clone 1A8, rat IgG2a) and anti-MHC II PE I-A /
117I-E (clone M5/114.15.2, rat IgG2b) were purchased from BD Biosciences
118(Heidelberg, Germany). Antibody anti-F4/80 APC (clone Cl:A3-1, rat IgG2b) was
119obtained from Serotec (Düsseldorf, Germany). Anti-CD4 FITC (clone RM4-5, rat
120IgG2a), anti-CD11c PE-Cy5.5 (clone N418, Armenian Hamster IgG), anti-CD25 APC
121(clone PC61.5, rat IgG1), anti-CD103 APC (clone 2E7, Armenian Hamster IgG), anti-
122F4/80 PE (clone C1:3A-1, rat IgG2b), anti-IgG2a PE (clone FJK-16s), anti-FoxP3 PE
123(clone FJK-16s, rat IgG2a) and anti-Ly6G eFluor 450 (clone 1A8, rat IgG2a) were
124purchased from eBioscience (San Diego, USA). For purification of CD11c-positive
125and CD45-positive cells from lung parenchymal tissue, magnetic anti-CD11c and
126anti-CD45 antibodies were obtained from Miltenyi Biotec (Bergisch Gladbach,
127Germany). Diphtheria toxin was purchased from Sigma (Deisenhofen, Germany).

128 **Culture of *M. bovis* BCG and infection of mice**

129 *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) (strain Pasteur) was cultured
130 in Middlebrook 7H9 medium enriched with oleic acid-albumine-dextrose-catalase
131 supplement (BD Biosciences, Heidelberg, Germany) until mid-log phase and was
132 then frozen in 1 ml aliquots at -80°C until use. For quantification, mycobacteria were
133 plated in ten-fold serial dilutions in Middlebrook 7H9 medium on Middlebrook 7H10
134 agar plates (BD Biosciences, Heidelberg, Germany), and after 3 weeks of incubation
135 at 37°C, CFU were determined .

136 Mice were infected with *M. bovis* BCG (2-3 x 10⁵ CFU/mouse) via intratracheal
137 routes, while mock-infection of mice was achieved by intratracheal instillation of PBS
138 (50 µl per mouse) . Subsequently, mice were kept in individually ventilated cages
139 (IVC) with free access to autoclaved food and water.

140

141 **Exposure of mice to adenoviral vectors**

142 Adenoviral vectors encoding the cDNA of murine GM-CSF (AdGM-CSF), or empty
143 control vector (Addl70-3) were constructed as previously described . Mice were
144 anesthetized with desflurane (Baxter, Unterschleissheim, Germany) and were then
145 instilled intratracheally with AdGM-CSF or empty control vector at 10⁸ PFU/mouse
146 diluted in 50 µl PBS, as recently described . Mice previously infected with *M. bovis*
147 BCG received either a single (6 h post-infection), or repetitive AdGM-CSF treatment,
148 or empty control vector administration (6 h and 7 days post-infection).

149

150 **Diphtheria toxin induced depletion of FoxP3^{pos} regulatory T cells**

151 To examine the role of FoxP3^{pos} regulatory T cells (Tregs) in *M. bovis* BCG infected
152 mice, both BCG challenged WT mice (serving as DT treatment controls) and DERE
153 mice received i.p. injections of diphtheria toxin (DT) (20 ng/g body weight (b.w.)
154 dissolved in PBS) at day -1, followed by i.p. injections of DT (10 ng/g b.w.) every 48 h
155 for 14 days.

156

157 **Bronchoalveolar lavage**

158 Bronchoalveolar lavage of mice of the various treatment groups was performed
159 essentially as described elsewhere .

160

161 **Immunophenotypic analysis of leukocytes in lung tissue and lung draining** 162 **lymph nodes**

163 Lungs were perfused *in situ* with Hank's balanced salt solution (HBSS) via the right
164 ventricle for removal of erythrocytes and then carefully dissected from large airways
165 while avoiding contamination with lung dLNs and then teased in small pieces and
166 subsequently digested in RPMI 1640 supplemented with collagenase A (5 mg/ml)
167 and DNase I (1 mg/ml), as recently described in detail . Subsequently, CD11c^{pos}
168 leukocytes or CD45^{pos} leukocytes were purified from lung tissue digests using a
169 MACS purification kit (Miltenyi Biotec) according to our previously published reports .

170 Immunophenotypic analysis of lung and draining LN cells was performed as
171 described in detail recently . Analysis of intracellular FoxP3 expression in lung and
172 lung dLN lymphocyte subsets was performed using a mouse regulatory T Cell

173 Staining Kit (eBioscience) following the manufacturer's instructions. Analysis of
174 intracellular IFN- γ expression in lung and lung dLN lymphocyte subsets was
175 performed using a Fixation/Permeabilization Kit, according to the manufacturer's
176 instructions for multicolor staining for cell surface antigens and intracellular cytokines
177 (BD Cytofix/Cytoperm™, BD Bioscience).

178 Flow cytometric analysis was performed using a FacsCanto flow cytometer or
179 BD LSR Fortessa (BD Biosciences, Heidelberg, Germany) equipped with a blue laser
180 operating at 488 nm excitation wavelengths, a red laser operating at 633 nm
181 wavelength, a violet laser operating at 450 nm and a green/yellow laser operating at
182 561 nm (BD LSR Fortessa). Lung CD11c^{pos} mononuclear phagocyte subsets were
183 gated according to their forward scatter area (FSC-A) versus side scatter area (SSC-
184 A) characteristics. Lung macrophages were further sub-gated according to their FSC-
185 A versus green autofluorescence properties, and their F4/80^{pos}, CD11c^{pos}, CD11b^{neg}
186 cell surface expression (lung macrophages), and their F4/80^{pos}, CD11c^{pos}, CD11b^{pos}
187 cell surface expression profile (lung exudate macrophages). Neutrophils were gated
188 according to their FSC-A/SSC-A characteristics and Ly6G^{pos} cell surface expression
189 profile. CD4^{pos} T cells in lung tissue homogenates and lung dLN (analyzed
190 immediately prior to isolation of CD11c^{pos} cells) were gated according to their FSC-
191 A/SSC-A, CD19^{neg}, CD3^{pos}, CD4^{pos}, CD8^{neg} expression profiles, and Tregs were
192 identified according to their FSC-A/SSC-A and CD3^{pos}, CD4^{pos}, CD25^{pos}, FoxP3^{pos}
193 antigen expression, as previously reported. IFN- γ expressing T cells were gated
194 based on their FSC-A/SSC-A characteristics and CD3^{pos}, CD4^{pos}, CD8^{neg}, IFN- γ ^{pos}
195 expression profiles. Post-acquisition compensation of spectral overlaps of different

196fluorescence channels and data analysis was performed using BD FACSDiva
197software.

198

199**Flow sorting of lung macrophages and exudate macrophages**

200Lungs of mice previously infected with *M. bovis* BCG followed by therapeutic
201treatment with AdGM-CSF or control vector were processed for enrichment of
202CD11c^{pos} cells as described above, and were then briefly incubated with octagam
203and stained with anti-CD11c-PE-Cy5.5 and anti-CD11b-PE-Cy-7 Ab for 20 min at
2044°C. After washing the cells twice with FACS buffer at 1200 rpm (10 min, 4°C),
205populations of lung macrophages and lung exudate macrophages were gated, as
206outlined above and were then sorted at a flow rate of ~ 10.000 events/s with an 85
207µm nozzle. Cell-sorting was performed at 4°C and resulted in purities of sorted
208populations exceeding >90 %, with overall cell viabilities of >90 %, as judged by
209propidium iodide staining .

210

211**Real-time RT-PCR**

212Analysis of target genes in flow-sorted lung macrophages and exudate macrophages
213was done essentially as previously described . PCR primers for β-actin (forward
214primer: 5'-CCACAGCTGAGAGGGAAATC-3', reverse primer 5'-TCTCCAGGGA
215GGAAGAGG AT-3'; NM_007393.3), iNOS (forward primer: 5'-
216GCCACCAACAATGGC AACA-3', reverse primer 5'-CGTACCGGATGACTGTG
217AATT-3'; NM_010927.3) and IL-12 (forward primer: 5'- CTTTGATGATGACCCTG
218TGC-3', reverse primer 5'- GTGATT CTGAAGTGCTGCGT -3'; NM_001159424.2)

219were designed using Primer Express software (Applied Biosystems, Warrington, UK),
220based on the gene sequence data retrieved from GenBank. For normalization, β -
221actin was used as the housekeeping gene. For calculation of mean fold-changes, the
222 $2^{-\Delta\Delta C_t}$ method was used . Samples were all tested in duplicate and for each
223amplification run a non-template control was included.

224

225**Determination of mycobacterial loads in BAL, lung tissue, and lung draining LN**

226After bronchoalveolar lavage, lung lobes were isolated and homogenized in HBSS
227using a tissue homogenizer (IKA, Staufen, Germany), and the lung tissue
228homogenate was filtered through a 40 μ m cell strainer. In parallel, lung dLN were
229carefully isolated with sterile forceps and incubated for 10 min in an ultrasonic bath,
230after which lung dLN were crushed with a pistil. Subsequently, BAL cells, lung tissue
231homogenates and lung dLN cells were lysed in HBSS containing 0,1 % saponin for
23210 min at 37°C and ten-fold serial dilutions of each sample were then processed for
233determination of CFU on 7H10 agar plates for three weeks .

234

235**Quantification of cytokines in BAL fluids**

236BAL fluids of mice of the various treatment groups were centrifuged at 1400 rpm, 4°C
237for 9 min, and aliquots of cell-free supernatants were stored at -20°C until use.
238Quantification of cytokines was done using commercially available ELISA kits
239(TNF- α , MIP-2 and GM-CSF), according to the manufacturer's instructions (R&D
240Systems). Lower detection limits were 10,9 pg/ml for TNF- α , 7,6 pg/ml for MIP-2, and
2417,8 pg/ml for GM-CSF.

242

243 **Lung histopathology of *M. bovis* BCG infected mice**

244 Untreated or *M. bovis* BCG infected WT and DEREK mice were euthanized at
245 different time points after vector treatment. Lungs were removed and immediately
246 fixed in 4 % neutral buffered formaldehyde solution (Histofix, Roth, Karlsruhe,
247 Germany) for at least 24 h at room temperature. After routine paraffin-embedding,
248 lung tissue sections of 3 μ m were prepared and stained with hematoxylin and eosin
249 (HE). Histopathological examination was done at 2,5x and 40x original magnification
250 using a Zeiss Axiovert 200 M microscope (Carl Zeiss).

251

252 **Statistics**

253 The data are presented as mean $\pm$ SD. Comparisons between groups were
254 performed using Mann-Whitney U test using GraphPad Prism Software, version 6.07.
255 Statistically significant differences between treatment groups were assumed when *P*
256 values were < 0.05 .

257 **Results**

258 **Kinetics of GM-CSF protein release in Ad-GM-CSF exposed mice either or not** 259 **infected with *M. bovis* BCG**

260 In the first set of experiments, we analyzed the kinetics of GM-CSF release in mock-
261 or *M. bovis* BCG-infected WT mice exposed to control vector or AdGM-CSF at 6 h
262 post-infection, as illustrated in Fig. 1A. As shown in Fig. 1 B, mock-infected or *M.*
263 *bovis* BCG infected mice exposed to control vector exhibited basal GM-CSF release

264similar to untreated control mice (CL), demonstrating that BCG itself did not trigger
265GM-CSF release in the lungs of mice during an observation period of 7 days.
266However, GM-CSF protein levels were significantly and transiently increased in BAL
267fluids of both mock- and BCG-infected mice after exposure to AdGM-CSF until day 7,
268relative to control vector treatment. Repetitive AdGM-CSF exposure of BCG-infected
269mice triggered sustained elevated GM-CSF protein levels in BAL fluids on day 10
270until 14 post-infection (data not shown). These data illustrate successful adenoviral
271gene transfer of GM-CSF in BCG-infected mice, and confirm that a previous BCG
272infection did not affect AdGM-CSF induced GM-CSF release in the lungs of mice.

273

Fig. 1

274

Figure 1. GM-CSF protein release in mock-infected or *M. bovis* BCG infected mice exposed to adenoviral vectors. WT mice were either exposed to control vector or AdGM-CSF (1×10^8 pfu/mouse) at 6 h after mock- or *M. bovis* BCG-infection (2×10^5 CFU/mouse) and were then subjected to BAL on day 3, 5 or 7 post-infection compared to untreated WT mice (CL), as illustrated in (A). (B) GM-CSF protein release in bronchoalveolar lavage (BAL) of mice. Values are shown as mean $\pm$ SD of $n = 5$ mice (0 h time point) or $n = 3-4$ mice per treatment group and time point. * $p < 0.05$ relative to control vector treated mice after *M. bovis* BCG infection. + $p < 0.05$, relative to control vector treated mice (Mann-Whitney U test).

284

Effect of AdGM-CSF therapy on lung phagocyte recruitment and expression of iNOS and IL-12 genes in sorted alveolar macrophages of BCG-infected mice

We previously found that exposure of mice to AdGM-CSF time-dependently increased alveolar accumulation of inflammatory exudate macrophages, neutrophils and lymphocytes in BAL fluids of mice, which was accompanied by upregulated PU.1 and phosphorylated STAT5 (pSTAT5) activity in alveolar macrophages and lung tissue of AdGM-CSF exposed mice, thus indicating that adenoviral delivery of GM-CSF directly activates resident AM. Capitalizing on these findings, we here examined the effect of therapeutic treatment of *M. bovis* BCG infected mice with AdGM-CSF on recruitment and activation of professional phagocyte subsets in their lungs, according to the protocol outlined in Fig. 2A. As shown in Fig. 2B-G, exposure of BCG-infected mice to single or repetitive AdGM-CSF triggered recruitment of resident (CD11c^{pos}/CD11b^{neg}) and inflammatory exudate macrophages (CD11c^{pos}/CD11b^{pos}) and neutrophils in lung tissue of mice, compared to control vector exposed mice.

Inducible nitric oxide synthase (iNOS, NOS2) plays an important role in intracellular generation of nitric oxide, which is an important reactive nitrogen intermediate (RNI) involved in mycobacterial killing. Macrophage-derived interleukin 12 (IL-12) plays an important role in induction of TH1 immune responses to intracellular pathogens. As shown in Fig. 2H, I, both iNOS and IL-12 gene expression were significantly upregulated in flow-sorted CD11c^{pos}/CD11b^{neg} lung macrophages but not CD11c^{pos}/CD11b^{pos} lung exudate macrophages collected from the lungs of *M. bovis* BCG-infected mice after exposure to single or, even more so, repetitive AdGM-CSF, relative to control vector treatment of BCG-infected mice. These data show that transient adenoviral delivery of GM-CSF to the

309bronchoalveolar space enhances anti-mycobacterial mediator expression particularly
 310in resident rather than newly recruited pulmonary macrophage subsets.

311

Fig 2

312

313**Figure 2. Effect of pulmonary overexpression of GM-CSF on lung leukocyte**
 314**subsets and gene expression profiles in flow-sorted lung macrophages after**

315 ***M. bovis* BCG infection.** (A) *M. bovis* BCG-infected WT mice were exposed either to
316 control vector or AdGM-CSF at 6 h p.i., or at 6 h and 7 d p.i. At the indicated time
317 points, lungs of mice were processed for quantification of alveolar macrophages
318 (CD11c^{pos}/CD11b^{neg}) (B, E) and newly recruited exudate macrophages
319 (CD11c^{pos}/CD11b^{pos}) (C, F), and neutrophils (PMN) (D, G). Real-time RT-PCR
320 analysis of iNOS mRNA (H), or IL-12 mRNA levels (I) in flow-sorted alveolar
321 macrophages (white bars), or exudate macrophages (black bars) collected at day 21
322 p.i.. Values are shown as mean $\pm$ SD of n = 3-6 mice per time point and experimental
323 group. Real-time RT-PCR values are expressed relative to rAM and ExMacs
324 collected from *M. bovis* BCG-infected WT mice exposed to control vector. p* < 0.05
325 relative to control vector treated mice (Mann-Whitney U test), as indicated (B-G). p* <
326 0.05, p** < 0.01 relative to ExMacs (H, I).

327

328 **Pulmonary AdGM-CSF treatment accelerates lung lymphocyte subset** 329 **recruitment and improves anti-mycobacterial immunity in mice**

330 In the next set of experiments, we examined the effect of single as compared to
331 repetitive intratracheal AdGM-CSF application on recruitment of CD4^{pos} T cell
332 subsets, including FoxP3^{pos} and IFN- γ ^{pos} effector CD4^{pos} T cells in lungs and lung
333 dLNs of mock and *M. bovis* BCG infected WT mice. As shown in Fig. 3, AdGM-CSF
334 but not control vector exposure triggered accumulation of CD4^{pos} T cells and Tregs in
335 the lungs and draining lymph nodes of mock-infected mice (Fig. 3B, C, E, F).
336 Similarly, Ad-GMCSF treatment evoked an increased accumulation of IFN- γ ^{pos}

337effector CD4^{pos} T cells in lungs and draining LNs of mock-infected mice, relative to
 338control vector treatment (Fig. 3D, G).

339

Fig. 3

340

341Figure 3. Effect of adenoviral GM-CSF transfer on lung lymphocyte recruitment

342in mice. (A) Lung lymphocyte subset recruitment in untreated (CL), mock-infected

343and control vector or AdGM-CSF (6 h p.i.) exposed mice, as indicated. (B-G)

344Numbers of CD4^{pos} T cells, CD4^{pos}, CD25^{pos}, FoxP3^{pos} T cells and CD4^{pos}, IFN-γ^{pos} T

345cells in lung (B-D) and lung dLN of mice (E-G), as indicated. Values are shown as

346mean ± SD (n = 3-5 mice) per time point and treatment group. p* < 0.05, relative to

347control vector treated mice.

348

349 We next examined the effect of single or repetitive AdGM-CSF application on
350lung lymphocyte subset accumulation in mice infected with *M. bovis* BCG, according
351to the protocol outlined in Fig. 4A, H. *M. bovis* BCG infected mice exposed to AdGM-
352CSF showed significantly increased numbers of CD4^{pos} T cells and CD4^{pos} FoxP3^{pos}
353Tregs in lungs and dLNs, relative to control vector exposed, *M. bovis* BCG infected
354mice during an observation period of 28 days (Fig. 4B, C, E, F). AdGM-CSF therapy
355of *M. bovis* BCG infected mice also evoked increased accumulation of IFN- γ ^{pos}
356effector CD4^{pos} T cells in lungs (day 14) and draining LNs (day 28), relative to control
357vector treatment (Fig. 4D, G). Notably, AdGM-CSF given repetitively at 6 h and 7
358days post *M. bovis* BCG infection (Fig. 4H) led to increased accumulation of CD4^{pos} T
359cells and CD4^{pos} FoxP3^{pos} Tregs in lung and dLNs of mice, similar to lymphocyte
360counts observed in mice exposed to single AdGM-CSF (Fig. 4I, J, L, M). However,
361numbers of CD4^{pos} IFN- γ ^{pos} effector T cell subsets were robustly elevated only in lung
362dLNs but not in lung tissue of repetitively AdGM-CSF exposed, BCG-infected mice,
363when compared to control vector exposed, *M. bovis* BCG infected mice (Fig. 4K, N).
364

Fig 4

365

366**Figure 4. Effect of adenoviral GM-CSF transfer on lung lymphocyte subset**
 367**recruitment in mice challenged with *M. bovis* BCG. (A, H) Experimental setup.**

Mice were orotracheally infected with *M. bovis* BCG and were then exposed to control vector or AdGM-CSF at 6 h or 6 h and 7 days p.i.. Lungs and lung dLNs of infected mice were processed for quantification of CD4^{pos} T cells (B, E, I, L), CD4^{pos} CD25^{pos} FoxP3^{pos} T cells (C, F, J, M), and IFN- γ ^{pos} T cells (D, G, K, N) at the indicated time points. Values are shown as mean $\pm$ SD of n = 4-5 mice per time point and treatment group. The data are representative of two independently performed experiments. p* < 0.05, p** < 0.01, relative to control vector treated mice.

375

Importantly, repetitive administration of AdGM-CSF given at 6 h and 7 days post-infection significantly reduced mycobacterial loads in BAL fluids, lung tissue and partially also in lung dLNs of BCG-infected mice during an observation period of 28 days, relative to BCG-infected mice repetitively exposed to control vector (Fig. 5A-C).

380

Fig.5

381

Figure 5. Effect of repetitive adenoviral GM-CSF transfer on mycobacterial loads in mice challenged with *M. bovis* BCG. Mice were orotracheally infected with *M. bovis* BCG followed by exposure to control vector or AdGM-CSF at 6 h and 7 days p.i.. Mycobacterial loads were determined in BAL fluids (A), lung (B) and lung

386dLNs (C), as indicated. Data are shown as mean $\pm$ SD of n = 4-5 mice per time point
387and experimental group, and are representative of two independently performed
388experiments. $p^* < 0.05$, $p^{**} < 0.01$, relative to control vector treated mice.

389

390**Effect of diphtheria toxin-induced depletion of regulatory T cells on lung anti-** 391**mycobacterial immunity in mice**

392The observation that *M. bovis* BCG-infected mice responded with a significant
393expansion of Tregs in their lungs after single or repetitive exposure to AdGM-CSF
394prompted us to examine the role of Tregs during mycobacterial infections. To exclude
395any side-effects of prolonged diphtheria toxin administration on anti-mycobacterial
396immunity, we initially analyzed the effect of DT application on mycobacterial
397pathogen elimination in mice, according to the experimental profile provided in
398Fig. S1A. Prolonged DT application did not influence mycobacterial loads in BAL
399fluids, lung tissue or dLNs of BCG-infected WT mice relative to PBS-treated, BCG-
400infected WT mice exhibiting similar CFU counts in their lungs as compared to BCG-
401infected DEREg mice at day 14 post-infection (Fig. S1B-D).

402 We then examined the effect of Treg depletion on mycobacterial loads in
403diphtheria toxin treated, BCG-infected WT mice (serving as DT treatment controls)
404and DEREg mice (Treg depleted) exposed to AdGM-CSF at 6 h and 7 days post-
405infection, as outlined in Fig. 6A. FACS-based determination of Treg depletion
406revealed ~ 75 % reduced Treg counts (gated as shown in Fig. S2) in lungs and ~60
407% reduced Treg counts in lung dLNs of AdGM-CSF exposed, *M. bovis* BCG-infected
408DEREG mice, relative to *M. bovis* BCG-infected, AdGM-CSF exposed WT mice on
409day 14 post-infection (Fig. 6B-E). Nevertheless, the observed reduction in absolute

410Treg counts did not affect mycobacterial loads in BAL fluids, lungs and dLNs of BCG-
 411infected DEREg mice compared to DT-treated, BCG-infected WT mice repetitively
 412exposed to AdGM-CSF (Fig. 6F-H), demonstrating that AdGM-CSF evoked Treg
 413accumulation appears to be dispensable for lung anti-mycobacterial immunity in
 414BCG-infected mice.

Fig. 6

415
 416**Figure 6. Effect of Treg depletion on the course of mycobacterial infection in**
 417**mice.** (A) Experimental setup. WT mice (white bars) and DEREg mice (black bars)

418 were orotracheally infected with *M. bovis* BCG (2×10^5 CFU/mouse). At the indicated
419 time points (arrows in A), mice were treated with diphtheria toxin (DT). Lungs and
420 lung dLNs of infected mice were processed for quantification of CD4^{pos} (B, D) and
421 CD4^{pos} CD25^{pos} FoxP3^{pos} T cells (C, E) at 14 days p.i.. (F-H) Mycobacterial loads were
422 determined in bronchoalveolar lavage (BAL) fluids (F), lungs (G) and lung dLNs (H)
423 of mice. Data are shown as mean $\pm$ SD of $n = 4-5$ mice per time point and treatment
424 group, and are representative of two independently performed experiments. * $p < 0.05$
425 (** $p < 0.01$) relative to WT mice.

426

427 **Depletion of Tregs accentuates *M. bovis* BCG induced immunopathology in** 428 **mice**

429 Tregs are considered to protect the host from excessive inflammatory responses and
430 autoimmunity . Therefore, we next evaluated the effect of DT-induced Treg depletion
431 on inflammatory responses in *M. bovis* BCG infected mice exposed to AdGM-CSF at
432 6 h and 7 days post-infection. As shown in Fig. 7A-C, *M. bovis* BCG infected, DT-
433 treated DEREg mice showed significantly increased levels of pro-inflammatory
434 cytokines TNF- α and MIP-2 together with increased neutrophil counts in their BAL
435 fluids, when compared to DT-treated, BCG-infected WT mice exposed to AdGM-
436 CSF.

437 Histopathological examination of lung tissue of untreated WT and DEREg
438 mice revealed a normal bronchial and alveolar architecture in both experimental
439 groups (Fig. 7E, F). As expected, *M. bovis* BCG infected WT and DEREg mice
440 exposed to control vector at 6 h and 7 days post infection were found to develop
441 typical lung granulomatous lesions at day 14 post-infection, with no apparent

442 differences between groups (Fig. 7G, H). Repetitive exposure of *M. bovis* BCG
443 infected WT and DERE mice to AdGM-CSF triggered peribronchial and
444 perivascular lymphocyte cuffs at day 14 post-infection, together with hyperplastic
445 type II epithelial cells and widened alveolar septa (Fig. 7D, I, J). However, depletion
446 of Tregs in *M. bovis* BCG infected DERE mice repetitively exposed to AdGM-CSF
447 at 6 h and 7 days post-infection led to significantly increased lung immunopathology,
448 as evidenced by subpleural pneumonic infiltrates and alveolar and interstitial edema
449 that was not observed in DT-treated, *M. bovis* BCG infected WT mice repetitively
450 exposed to AdGM-CSF (Fig. 7D, K-N).

451

Fig. 7

452

453 **Figure 7. Effect of Treg depletion on lung immunopathology in *M. bovis* BCG**
 454 **infected mice exposed to AdGM-CSF.** (A-D) WT mice and DERE mice were
 455 orotracheally infected with *M. bovis* BCG (2×10^5 CFU/mouse) followed by exposure
 456 to AdGM-CSF at 6 h and 7 d post-infection, and then received diphtheria toxin (DT)

457i.p., as outlined in Fig. 6A. (A) TNF- α and (B) MIP-2 protein levels and (C) numbers
458of neutrophils in BAL fluids of mice at day 14 p.i.. (D) Percent inflamed lung tissue in
459PBS- or DT-treated, BCG-infected and AdGM-CSF (6 h, 7 d) exposed WT or DERE
460mice at day 14 p.i.. (E, F) Lung histopathology of untreated WT mice and DERE
461mice, or *M. bovis* BCG (2×10^5 CFU/mouse) infected, control vector exposed WT and
462DEREG mice at day 14 p.i. (G, H), or PBS-treated WT and DERE mice infected
463with *M. bovis* BCG (2×10^5 CFU/mouse) followed by exposure to AdGM-CSF at 6 h
464and 7 days p.i. at day 14 p.i (I, J). (K-N) Lung histopathology of DT-treated WT mice
465and DERE mice infected with *M. bovis* BCG followed by repetitive exposure to
466AdGM-CSF at day 14 p.i. (K-L; M, N, insert magnifications of panels K, L, as
467indicated). Data in (A-D) are shown as mean $\pm$ SD of n = 3-5 mice per treatment
468group, and are representative of two independently performed experiments. *p< 0.05
469(** p< 0.01) relative to WT mice + DT. De-paraffinized lung tissue sections (3 μ m)
470were stained with hematoxylin/eosin. Scale bar, 200 μ m (E-L), and 10 μ m (M, N).

471

472 Discussion

473 The current study aimed to evaluate the efficacy of intratracheally delivered GM-CSF
474 to improve lung anti-mycobacterial immunity against *M. bovis* BCG in mice and to
475 decipher the role of regulatory T cells in this process, using adenoviral vector
476 mediated gene transfer of murine GM-CSF.

477 We found repetitive therapeutic GM-CSF gene transfer at 6 h and 7 days p.i.
478 to exhibit greater anti-mycobacterial efficacy as compared to single AdGM-CSF
479 exposure of mice, though overall pathogen elimination remained incomplete in the
480 employed BCG infection model. A previous report suggested that even a single
481 prophylactic AdGM-CSF treatment one day before infection with high-dose *M.*
482 *tuberculosis* (H37Rv) was sufficient to substantially reduce mycobacterial burden and
483 mortality in BALB/c mice . The apparent discrepancies in efficacy profiles between
484 these two studies are currently unclear. Possibly, the prophylactic as compared to
485 the currently employed therapeutic treatment of mice with AdGM-CSF exerted a
486 greater ‘priming’ of lung sentinel cells prior to pathogen encounter.

487 It is well known that mycobacterial infection triggers increased recruitment of
488 Tregs in mice and humans . In the current study, we found that Ad-GM-CSF
489 exposure further significantly increased numbers of Tregs in lungs and lung dLNs of
490 *M. bovis* BCG-infected mice, representing a novel aspect in the pathobiology of GM-
491 CSF therapy. Basically, regulatory T cells play a crucial role in self-tolerance and
492 Treg deficiency results in autoimmunity in mice and humans (reviewed in). Previous
493 reports demonstrated that mycobacterial antigen-specific Tregs may limit the amount
494 of effector T cell priming and expansion in dLNs (reviewed in). At the same time,
495 various studies showed that anti-CD25 antibody dependent Treg depletion did not

496 affect mycobacterial clearance in mice . However, anti-CD25 mAbs may also deplete
497 other cell types, such as activated CD25^{pos} effector T cells . Therefore, in the current
498 study, we depleted Tregs in transgenic DERE^G mice by prolonged DT application to
499 avoid rapid recovery of the Treg compartment, as observed after single or two-fold
500 DT application, which is not sufficient for long-lasting Treg depletion in chronic
501 infection models . We found that the currently achieved Treg depletion did not affect
502 mycobacterial loads, but was sufficient to cause TH1 driven immunopathology in
503 AdGM-CSF exposed mice, characterized by increased TNF- α and MIP-2 levels as
504 well as severe pneumonic infiltrates typical of a dysregulated Treg compartment .
505 Since Treg deficiency inevitably results in immunopathology in mice and men, it is
506 currently difficult to define the relative contribution of Treg depletion itself versus Treg
507 depletion-induced immunopathology on anti-mycobacterial immunity in the current
508 model.

509 We and others have shown previously that Tregs of BCG-infected mice show
510 typical activation markers such as CTLA-4 and CD103 to maintain their suppressive
511 character towards effector T cells . Another study reported that Tregs would delay
512 priming and arrival of effector T cell subsets at the site of infection in a mouse model
513 of pulmonary tuberculosis, thereby affecting anti-mycobacterial immunity . However,
514 in AdGM-CSF exposed mice, we found that significantly elevated numbers of Tregs
515 were accompanied by similarly increased T cell subset responses in lungs and lung
516 LNs of BCG-infected mice. It is possible that differences in virulence profiles
517 between employed pathogens (*M. tuberculosis* versus *M. bovis* BCG) and the
518 currently employed prolonged GM-CSF transgene expression may have accounted
519 for the divergent roles of Tregs reported in the previous work and our study.

520Nevertheless, our findings suggest that GM-CSF therapy may even be suitable to
521shorten the time gap between infection and the following natural onset of anti-
522mycobacterial immunity.

523 Collectively, the data show that adenoviral GM-CSF therapy improved lung
524protective immunity against *M. bovis* BCG in mice, characterized by increased
525alveolar availability of iNOS and IL-12 expressing macrophages and increased
526pulmonary accumulation of effector T cells and Tregs. Moreover, specific depletion of
527regulatory T cells did not affect anti-mycobacterial immunity, but rather accentuated
528lung immunopathology in BCG-infected mice exposed to AdGM-CSF. These data
529may be relevant for the evaluation of inhaled GM-CSF therapy as a reasonable asset
530to existing antibiotic therapies to fight this destructive lung infectious disease in
531humans.

532

533**Conflicts of interest**

534The authors declare no financial or commercial conflict of interest.

535

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544**References**

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