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

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

Granulocyte macrophage-colony stimulating factor (GM-CSF) is a hematopoietic growth factor involved in differentiation, survival and activation of myeloid and non-myeloid cells with important implications for lung antibacterial immunity. Here we examined the effect of pulmonary adenoviral vector-mediated delivery of GM-CSF (AdGM-CSF) on anti-mycobacterial immunity in *M. bovis* BCG infected mice. Exposure of *M. bovis* BCG infected mice to AdGM-CSF either applied on 6 h, or 6 h and 7 days post-infection substantially increased alveolar recruitment of iNOS and IL-12 expressing macrophages, and significantly increased accumulation of IFN-γ<sup>pos</sup> T cells and particularly regulatory T cells (Tregs). This was accompanied by significantly reduced mycobacterial loads in the lungs of mice. Importantly, diphtheria toxin-induced depletion of Tregs did not influence mycobacterial loads, but accentuated immunopathology in AdGM-CSF-exposed mice infected with *M. bovis* BCG. Together, the data demonstrate that AdGM-CSF therapy improves lung protective immunity against *M. bovis* BCG infection in mice independent of co-recruited Tregs, which however critically contribute to limit lung immunopathology in BCG-infected mice. These data may be relevant to the development of immunomodulatory strategies to limit immunopathology-based lung injury in tuberculosis in humans.

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

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

Uncontrolled mycobacterial replication in the lung is typically inhibited in immunocompetent individuals by formation of granulomas, consisting of epithelioid macrophages surrounded by lymphocyte subsets. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are recruited to sites of infection, and IFN-γ from T cells is necessary to inhibit mycobacterial replication in macrophages. At the same time, this triggers robust CD8 T cell responses, thereby contributing to maintenance of granuloma formation.

However, initiation of lung anti-mycobacterial immunity develops relatively late after infection, with onset of effector lymphocyte recruitment to infected lungs usually starting not before 2–3 weeks post-infection. This calls for novel anti-mycobacterial strategies aiming to accelerate and enhance lung protective immunity against this serious lung pathogen.

The hematopoietic growth factor granulocyte macrophage colony stimulating factor (GM-CSF) is a pleiotropic cytokine regulating differentiation, proliferation and survival of myeloid and non-myeloid cells. GM-CSF is predominantly released in the lungs by epithelial cells and macrophages and is critical to surfactant homeostasis as
well as macrophage-mediated protective immunity, which is mediated by JAK/STAT5 signaling pathways, induction of the myeloid transcription factor PU.1 and interferon-regulating factor 5 (IRF5). Lack of GM-CSF or its endogenous neutralization results in pulmonary alveolar proteinosis (PAP), characterized by accumulation of surfactant lipids and proteins within alveoli which strongly impairs host defense in humans and mice. Particularly, absence of GM-CSF renders mice more susceptible to systemic and pulmonary infections with mycobacteria. Mycobacterial disease progression in GM-CSF KO mice is comparable to that of IFN-\(\gamma\) KO mice, as in the absence of granuloma formation, both mouse strains exhibited fatal disease courses. In vitro studies showed improved anti-mycobacterial responses of macrophages against \textit{M. tuberculosis} through stimulation with AdGM-CSF. However, congenital overexpression of GM-CSF in the lungs of mice did not improve lung anti-mycobacterial resistance against \textit{M. tuberculosis} due to lack of appropriate granuloma formation, suggesting the necessity of GM-CSF release regulation.
After infection with *M. tuberculosis*, the onset of adaptive immunity is substantially delayed as evidenced by lack of antigen-specific CD4\(^{+}\) T cell reactivity and delayed DC recruitment to mediastinal lymph nodes within the first two weeks of infection. We recently demonstrated that prophylactic adenoviral delivery of GM-CSF protected mice against lethal pneumococcal pneumonia. Mechanistically, GM-CSF activation of macrophages has been reported to result in sequestration of Zn to metallothioneins, thereby shuttling Zn away from the phagosomal compartment, finally resulting in increased killing of phagocytosed pathogens via oxidative burst. However, there are only limited data showing the therapeutic effect of pulmonary GM-CSF release on T cell and regulatory T cell dependent lung protective immunity against mycobacterial infections in mice. Therefore, we analyzed the effect of transient adenoviral GM-CSF gene transfer on lung anti-mycobacterial immunity in mice infected with *M. bovis* BCG and characterized the role of newly recruited FoxP3\(^{+}\) Tregs herein.

**Materials and methods**

**Animals**

C57BL/6J mice were purchased from Janvier (Sulzfeld, Germany). DEREG mice (C57BL/6J background) bearing the human diphtheria toxin receptor (DTR) coupled to enhanced GFP (eGFP) under control of the forkhead box P3 (*foxp3*) locus allowing specific diphtheria toxin-induced depletion of regulatory T cells were generated as previously described. Animals were used for experiments at 8-12 weeks of age, following the European Council Directive 2010/63/EU as well as the German Animal
Welfare Act, and were approved by the Lower Saxony State Office for Consumer Protection and Food Safety.

Reagents

Antibodies anti-CD3 FITC (clone 145-2C11, Armenian Hamster IgG1), anti-CD3 PE (clone 145-2C11, Armenian Hamster IgG1), anti-CD4 PerCP-Cy5.5 (clone RM4-5, rat IgG2a), or anti-CD8 PE-Cy7 (clone 53-6.7, rat IgG2a), anti-CD8a APC (clone 53-6.7, rat IgG2a), anti-CD11b BV 510 (clone M1/70, rat IgG2b, κ), anti-CD11b PE-Cy7 (clone M1/70, rat IgG2b), anti-CD11c APC (clone HL3, Armenian Hamster IgG1, λ2), anti-CD19 PE (clone 1D3, rat IgG2a), anti CD45 (clone 30-F11, rat IgG2b), anti-CD45 PE-Cy7 (clone 30F11, rat IgG2b), anti-IFN-γ-APC (clone XMG1.2, IgG1), anti-Ly6G PE (clone 1A8, rat IgG2a) and anti-MHC II PE I-A/I-E (clone M5/114.15.2, rat IgG2b) were purchased from BD Biosciences (Heidelberg, Germany). Antibody anti-F4/80 APC (clone CI:A3-1, rat IgG2b) was obtained from Serotec (Düsseldorf, Germany). Anti-CD4 FITC (clone RM4-5, rat IgG2a), anti-CD11c PE-Cy5.5 (clone N418, Armenian Hamster IgG), anti-CD25 APC (clone PC61.5, rat IgG1), anti-CD103 APC (clone 2E7, Armenian Hamster IgG), anti-F4/80 PE (clone C1:3A-1, rat IgG2b), anti-IgG2a PE (clone FJK-16s), anti-FoxP3 PE (clone FJK-16s, rat IgG2a) and anti-Ly6G eFluor 450 (clone 1A8, rat IgG2a) were purchased from eBioscience (San Diego, USA). For purification of CD11c-positive and CD45-positive cells from lung parenchymal tissue, magnetic anti-CD11c and anti-CD45 antibodies were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Diphtheria toxin was purchased from Sigma (Deisenhofen, Germany).
Culture of *M. bovis* BCG and infection of mice

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

Mice were infected with *M. bovis* BCG (2-3 x 10^5 CFU/mouse) via intratracheal routes, while mock-infection of mice was achieved by intratracheal instillation of PBS (50 µl per mouse). Subsequently, mice were kept in individually ventilated cages (IVC) with free access to autoclaved food and water.

Exposure of mice to adenoviral vectors

Adenoviral vectors encoding the cDNA of murine GM-CSF (AdGM-CSF), or empty control vector (Addl70-3) were constructed as previously described. Mice were anesthetized with desflurane (Baxter, Unterschleissheim, Germany) and were then instilled intratracheally with AdGM-CSF or empty control vector at 10^8 PFU/mouse diluted in 50 µl PBS, as recently described. Mice previously infected with *M. bovis* BCG received either a single (6 h post-infection), or repetitive AdGM-CSF treatment, or empty control vector administration (6 h and 7 days post-infection).
Diphtheria toxin induced depletion of FoxP3\textsuperscript{pos} regulatory T cells

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

### Bronchoalveolar lavage

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

### Immunophenotypic analysis of leukocytes in lung tissue and lung draining lymph nodes

Lungs were perfused *in situ* with Hank’s balanced salt solution (HBSS) via the right ventricle for removal of erythrocytes and then carefully dissected from large airways while avoiding contamination with lung dLNs and then teased in small pieces and subsequently digested in RPMI 1640 supplemented with collagenase A (5 mg/ml) and DNase I (1 mg/ml), as recently described in detail. Subsequently, CD11c\textsuperscript{pos} leukocytes or CD45\textsuperscript{pos} leukocytes were purified from lung tissue digests using a MACS purification kit (Miltenyi Biotec) according to our previously published reports. Immunophenotypic analysis of lung and draining LN cells was performed as described in detail recently. Analysis of intracellular FoxP3 expression in lung and lung dLN lymphocyte subsets was performed using a mouse regulatory T Cell
Staining Kit (eBioscience) following the manufacturer’s instructions. Analysis of intracellular IFN-\(\gamma\) expression in lung and lung dLN lymphocyte subsets was performed using a Fixation/Permeabilization Kit, according to the manufacturer’s instructions for multicolor staining for cell surface antigens and intracellular cytokines (BD Cytofix/Cytoperm™, BD Bioscience).

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

Flow sorting of lung macrophages and exudate macrophages

Lungs of mice previously infected with *M. bovis* BCG followed by therapeutic treatment with AdGM-CSF or control vector were processed for enrichment of CD11c<sup>pos</sup> cells as described above, and were then briefly incubated with octagam and stained with anti-CD11c-PE-Cy5.5 and anti-CD11b-PE-Cy-7 Ab for 20 min at 4°C. After washing the cells twice with FACS buffer at 1200 rpm (10 min, 4°C), populations of lung macrophages and lung exudate macrophages were gated, as outlined above and were then sorted at a flow rate of ~10,000 events/s with an 85 µm nozzle. Cell-sorting was performed at 4°C and resulted in purities of sorted populations exceeding >90 %, with overall cell viabilities of >90 %, as judged by propidium iodide staining.

Real-time RT-PCR

Analysis of target genes in flow-sorted lung macrophages and exudate macrophages was done essentially as previously described. PCR primers for β-actin (forward primer: 5´-CCACAGCTGAGGGAAATC-3´, reverse primer 5´-TCTCCAGGGAAGAGGAT-3´; NM_007393.3), iNOS (forward primer: 5´-GCCACCAACAATGGCAACA-3´, reverse primer 5´-CGTACCGGATGACTGTGATT-3´; NM_010927.3) and IL-12 (forward primer: 5´-CTTTGATGATGACTGTGATT-3´; NM_001159424.2)
were designed using Primer Express software (Applied Biosystems, Warrington, UK), based on the gene sequence data retrieved from GenBank. For normalization, β-actin was used as the housekeeping gene. For calculation of mean fold-changes, the \(2^{-\Delta\Delta Ct}\) method was used. Samples were all tested in duplicate and for each amplification run a non-template control was included.

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

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

**Quantification of cytokines in BAL fluids**

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

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

Statistics

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

Results

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

In the first set of experiments, we analyzed the kinetics of GM-CSF release in mock- or *M. bovis* BCG-infected WT mice exposed to control vector or AdGM-CSF at 6 h post-infection, as illustrated in Fig. 1A. As shown in Fig. 1 B, mock-infected or *M. bovis* BCG infected mice exposed to control vector exhibited basal GM-CSF release
similar to untreated control mice (CL), demonstrating that BCG itself did not trigger GM-CSF release in the lungs of mice during an observation period of 7 days. However, GM-CSF protein levels were significantly and transiently increased in BAL fluids of both mock- and BCG-infected mice after exposure to AdGM-CSF until day 7, relative to control vector treatment. Repetitive AdGM-CSF exposure of BCG-infected mice triggered sustained elevated GM-CSF protein levels in BAL fluids on day 10 until 14 post-infection (data not shown). These data illustrate successful adenoviral gene transfer of GM-CSF in BCG-infected mice, and confirm that a previous BCG infection did not affect AdGM-CSF induced GM-CSF release in the lungs of mice.
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 (1x10^8 pfu/mouse) at 6 h after mock- or *M. bovis* BCG-infection (2x10^5 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 ± 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).
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 (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
bronchoalveolar space enhances anti-mycobacterial mediator expression particularly in resident rather than newly recruited pulmonary macrophage subsets.

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

Fig 2
M. bovis BCG infection. (A) M. bovis BCG-infected WT mice were exposed either to control vector or AdGM-CSF at 6 h p.i., or at 6 h and 7 d p.i. At the indicated time points, lungs of mice were processed for quantification of alveolar macrophages (CD11c<sup>pos</sup>/CD11b<sup>neg</sup>) (B, E) and newly recruited exudate macrophages (CD11c<sup>pos</sup>/CD11b<sup>pos</sup>) (C, F), and neutrophils (PMN) (D, G). Real-time RT-PCR analysis of iNOS mRNA (H), or IL-12 mRNA levels (I) in flow-sorted alveolar macrophages (white bars), or exudate macrophages (black bars) collected at day 21 p.i.. Values are shown as mean ± SD of n = 3-6 mice per time point and experimental group. Real-time RT-PCR values are expressed relative to rAM and ExMacs collected from M. bovis BCG-infected WT mice exposed to control vector. p* < 0.05 relative to control vector treated mice (Mann-Whitney U test), as indicated (B-G). p* < 0.05, p** < 0.01 relative to ExMacs (H, I).

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

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

Figure 3. Effect of adenoviral GM-CSF transfer on lung lymphocyte recruitment in mice. (A) Lung lymphocyte subset recruitment in untreated (CL), mock-infected and control vector or AdGM-CSF (6 h p.i.) exposed mice, as indicated. (B-G) Numbers of CD4\textsuperscript{pos} T cells, CD4\textsuperscript{pos}, CD25\textsuperscript{pos}, FoxP3\textsuperscript{pos} T cells and CD4\textsuperscript{pos}, IFN-\textgamma\textsuperscript{pos} T cells in lung (B-D) and lung dLN of mice (E-G), as indicated. Values are shown as mean ± SD (n = 3-5 mice) per time point and treatment group. p* < 0.05, relative to control vector treated mice.
We next examined the effect of single or repetitive AdGM-CSF application on lung lymphocyte subset accumulation in mice infected with *M. bovis* BCG, according to the protocol outlined in Fig. 4A, H. *M. bovis* BCG infected mice exposed to AdGM-CSF showed significantly increased numbers of CD4\(^{\text{pos}}\) T cells and CD4\(^{\text{pos}}\) FoxP3\(^{\text{pos}}\) Tregs in lungs and dLNs, relative to control vector exposed, *M. bovis* BCG infected mice during an observation period of 28 days (Fig. 4B, C, E, F). AdGM-CSF therapy of *M. bovis* BCG infected mice also evoked increased accumulation of IFN-\(\gamma\)^{\text{pos}} effector CD4\(^{\text{pos}}\) T cells in lungs (day 14) and draining LNs (day 28), relative to control vector treatment (Fig. 4D, G). Notably, AdGM-CSF given repetitively at 6 h and 7 days post *M. bovis* BCG infection (Fig. 4H) led to increased accumulation of CD4\(^{\text{pos}}\) T cells and CD4\(^{\text{pos}}\) FoxP3\(^{\text{pos}}\) Tregs in lung and dLNs of mice, similar to lymphocyte counts observed in mice exposed to single AdGM-CSF (Fig. 4I, J, L, M). However, numbers of CD4\(^{\text{pos}}\) IFN-\(\gamma\)^{\text{pos}} effector T cell subsets were robustly elevated only in lung dLNs but not in lung tissue of repetitively AdGM-CSF exposed, BCG-infected mice, when compared to control vector exposed, *M. bovis* BCG infected mice (Fig. 4K, N).
Figure 4. Effect of adenoviral GM-CSF transfer on lung lymphocyte subset 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 dLN s of infected mice were processed for quantification of CD4<sup>pos</sup> T cells, (B, E, I, L), CD4<sup>pos</sup>CD25<sup>pos</sup> FoxP3<sup>pos</sup> T cells (C, F, J, M), and IFN-γ<sup>pos</sup> T cells (D, G, K, N) at the indicated time points. Values are shown as mean ± 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.

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 dLN s of BCG-infected mice during an observation period of 28 days, relative to BCG-infected mice repetitively exposed to control vector (Fig. 5A-C).

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

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

We then examined the effect of Treg depletion on mycobacterial loads in diphtheria toxin treated, BCG-infected WT mice (serving as DT treatment controls) and DEREG mice (Treg depleted) exposed to AdGM-CSF at 6 h and 7 days post-infection, as outlined in Fig. 6A. FACS-based determination of Treg depletion revealed ~ 75% reduced Treg counts (gated as shown in Fig. S2) in lungs and ~60% reduced Treg counts in lung dLN of AdGM-CSF exposed, *M. bovis* BCG-infected DEREG mice, relative to *M. bovis* BCG-infected, AdGM-CSF exposed WT mice on day 14 post-infection (Fig. 6B-E). Nevertheless, the observed reduction in absolute
Treg counts did not affect mycobacterial loads in BAL fluids, lungs and dLNs of BCG-infected DEREG mice compared to DT-treated, BCG-infected WT mice repetitively exposed to AdGM-CSF (Fig. 6F-H), demonstrating that AdGM-CSF evoked Treg accumulation appears to be dispensable for lung anti-mycobacterial immunity in BCG-infected mice.

Figure 6. Effect of Treg depletion on the course of mycobacterial infection in mice. (A) Experimental setup. WT mice (white bars) and DEREG mice (black bars)
were orotracheally infected with *M. bovis* BCG (2 x 10⁵ CFU/mouse). At the indicated
time points (arrows in A), mice were treated with diphtheria toxin (DT). Lungs and
lung dLNs of infected mice were processed for quantification of CD4⁺ (B, D) and
CD4⁺CD25⁺FoxP3⁺ T cells (C, E) at 14 days p.i. (F-H) Mycobacterial loads were
determined in bronchoalveolar lavage (BAL) fluids (F), lungs (G) and lung dLNs (H)
of mice. Data are shown as mean ± SD of *n* = 4-5 mice per time point and treatment
group, and are representative of two independently performed experiments. *p* < 0.05
(** *p* < 0.01) relative to WT mice.

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

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

Histopathological examination of lung tissue of untreated WT and DEREG
mice revealed a normal bronchial and alveolar architecture in both experimental
groups (Fig. 7E, F). As expected, *M. bovis* BCG infected WT and DEREG mice
exposed to control vector at 6 h and 7 days post infection were found to develop
typical lung granulomatous lesions at day 14 post-infection, with no apparent
differences between groups (Fig. 7G, H). Repetitive exposure of *M. bovis* BCG infected WT and DEREG mice to AdGM-CSF triggered peribronchial and perivascular lymphocyte cuffs at day 14 post-infection, together with hyperplastic type II epithelial cells and widened alveolar septa (Fig. 7D, I, J). However, depletion of Tregs in *M. bovis* BCG infected DEREG mice repetitively exposed to AdGM-CSF at 6 h and 7 days post-infection led to significantly increased lung immunopathology, as evidenced by subpleural pneumonic infiltrates and alveolar and interstitial edema that was not observed in DT-treated, *M. bovis* BCG infected WT mice repetitively exposed to AdGM-CSF (Fig. 7D, K-N).
Figure 7. Effect of Treg depletion on lung immunopathology in *M. bovis* BCG infected mice exposed to AdGM-CSF. (A-D) WT mice and DEREG mice were orotracheally infected with *M. bovis* BCG (2 x 10⁵ CFU/mouse) followed by exposure to AdGM-CSF at 6 h and 7 d post-infection, and then received diphtheria toxin (DT)
as outlined in Fig. 6A. (A) TNF-α and (B) MIP-2 protein levels and (C) numbers of neutrophils in BAL fluids of mice at day 14 p.i.. (D) Percent inflamed lung tissue in PBS- or DT-treated, BCG-infected and AdGM-CSF (6 h, 7 d) exposed WT or DEREG mice at day 14 p.i.. (E, F) Lung histopathology of untreated WT mice and DEREG mice, or M. bovis BCG (2x10⁵ CFU/mouse) infected, control vector exposed WT and DEREG mice at day 14 p.i. (G, H), or PBS-treated WT and DEREG mice infected with M. bovis BCG (2x10⁵ CFU/mouse) followed by exposure to AdGM-CSF at 6 h and 7 days p.i. at day 14 p.i (I, J). (K-N) Lung histopathology of DT-treated WT mice and DEREG mice infected with M. bovis BCG followed by repetitive exposure to AdGM-CSF at day 14 p.i. (K-L; M, N, insert magnifications of panels K, L, as indicated). Data in (A-D) are shown as mean ± SD of n = 3-5 mice per treatment group, and are representative of two independently performed experiments. *p< 0.05 (** p< 0.01) relative to WT mice + DT. De-paraffinized lung tissue sections (3 µm) were stained with hematoxylin/eosin. Scale bar, 200 µm (E-L), and 10 µm (M, N).
Discussion

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

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

It is well known that mycobacterial infection triggers increased recruitment of Tregs in mice and humans. In the current study, we found that Ad-GM-CSF exposure further significantly increased numbers of Tregs in lungs and lung dLNs of *M. bovis* BCG-infected mice, representing a novel aspect in the pathobiology of GM-CSF therapy. Basically, regulatory T cells play a crucial role in self-tolerance and Treg deficiency results in autoimmunity in mice and humans (reviewed in ). Previous reports demonstrated that mycobacterial antigen-specific Tregs may limit the amount of effector T cell priming and expansion in dLNs (reviewed in ). At the same time, various studies showed that anti-CD25 antibody dependent Treg depletion did not
affect mycobacterial clearance in mice. However, anti-CD25 mAbs may also deplete other cell types, such as activated CD25^{pos} effector T cells. Therefore, in the current study, we depleted Tregs in transgenic DEREG mice by prolonged DT application to avoid rapid recovery of the Treg compartment, as observed after single or two-fold DT application, which is not sufficient for long-lasting Treg depletion in chronic infection models. We found that the currently achieved Treg depletion did not affect mycobacterial loads, but was sufficient to cause TH1 driven immunopathology in AdGM-CSF exposed mice, characterized by increased TNF-\(\alpha\) and MIP-2 levels as well as severe pneumonic infiltrates typical of a dysregulated Treg compartment.

Since Treg deficiency inevitably results in immunopathology in mice and men, it is currently difficult to define the relative contribution of Treg depletion itself versus Treg depletion-induced immunopathology on anti-mycobacterial immunity in the current model.

We and others have shown previously that Tregs of BCG-infected mice show typical activation markers such as CTLA-4 and CD103 to maintain their suppressive character towards effector T cells. Another study reported that Tregs would delay priming and arrival of effector T cell subsets at the site of infection in a mouse model of pulmonary tuberculosis, thereby affecting anti-mycobacterial immunity. However, in AdGM-CSF exposed mice, we found that significantly elevated numbers of Tregs were accompanied by similarly increased T cell subset responses in lungs and lung dLNs of BCG-infected mice. It is possible that differences in virulence profiles between employed pathogens (\textit{M. tuberculosis} versus \textit{M. bovis} BCG) and the currently employed prolonged GM-CSF transgene expression may have accounted for the divergent roles of Tregs reported in the previous work and our study.
Nevertheless, our findings suggest that GM-CSF therapy may even be suitable to shorten the time gap between infection and the following natural onset of anti-mycobacterial immunity.

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

**Conflicts of interest**

The authors declare no financial or commercial conflict of interest.

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