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

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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 IFNγ^{pos} 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.

1. Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) is the main causative pathogen in pulmonary tuberculosis (TB), which causes major morbidity and mortality worldwide (W.H.O., 2015). 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 (Marino et al., 2004). 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 (Marino et al., 2004; Bodnar et al., 2001).

Uncontrolled mycobacterial replication in the lung is typically inhibited in immunocompetent individuals by formation of granulomas, consisting of epithelioid macrophages surrounded by lymphocyte subsets (Ramakrishnan, 2012). Both CD4^{pos} and CD8^{pos} 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 (Green et al., 2013). 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 (Wolf et al., 2008). This calls for novel anti-mycobacterial strategies aiming to accelerate and enhance lung protective immunity against this serious lung pathogen (Gonzalez-Juarrero, 2012).

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 (Metcalf, 2008). 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 (Carey and Trapnell, 2010; Trapnell and Whitsett, 2002), which is mediated by JAK/STAT5 signaling pathways, induction of the myeloid transcription factor PU.1 and interferon-regulating factor 5 (IRF5) (Carey and

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Trapnell, 2010; Shibata et al., 2001; Krausgruber et al., 2011). 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 (Carey and Trapnell, 2010; Dranoff et al., 1994). Particularly, absence of GM-CSF renders mice more susceptible to systemic and pulmonary infections with mycobacteria (Gonzalez-Juarrero et al., 2005). Mycobacterial disease progression in GM-CSF KO mice is comparable to that of IFN-ɣ KO mice, as in the absence of granuloma formation, both mouse strains exhibited fatal disease courses (Gonzalez-Juarrero et al., 2005; Szeliga et al., 2008; Cooper et al., 1993). *In vitro* studies showed improved anti-mycobacterial responses of macrophages against *M. tuberculosis* through stimulation with AdGM-CSF (Vogt and Nathan, 2011). However, congenital overexpression of GM-CSF in the lungs of mice did not improve lung anti-mycobacterial resistance against *M. tuberculosis* due to lack of appropriate granuloma formation, suggesting the necessity of GM-CSF release regulation (Gonzalez-Juarrero et al., 2005; Szeliga et al., 2008).

After infection with *M. tuberculosis*, the onset of adaptive immunity is substantially delayed as evidenced by lack of antigen-specific $CD4^{pos}$ T cell reactivity and delayed DC recruitment to mediastinal lymph nodes within the first two weeks of infection (Garcia-Romo et al., 2004). We recently demonstrated that prophylactic adenoviral delivery of GM-CSF protected mice against lethal pneumococcal pneumonia (Steinwede et al., 2011). 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 (Subramanian Vignesh et al., 2013). 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 $FoxP3^{pos}$ Tregs herein.

2. Materials and methods

2.1. 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 (Lahl et al., 2007). 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.

2.2. Reagents

Antibodies anti-CD3 FITC (clone 145-2C11, Armenian Hamster IgG1), anti-CD3 PE (clone 145-2C11, Armenian Hamster IgG1), anti-CD3e APC-Cy7 (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 IgG_1, λ2), anti-CD19 PE (clone 1D3, rat IgG2a), anti CD45 (clone 30-F11, rat IgG2b) anti-CD45 PE-Cy7 (clone 30F11, rat IgG2b), anti-CD45 APC (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).

2.3. 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 (Schreiber et al., 2008).

Mice were infected with *M. bovis* BCG ($2–3 \times 10^5$ CFU/mouse) via intratracheal routes, while mock-infection of mice was achieved by intratracheal instillation of PBS (50 µl per mouse) (Herbold et al., 2010). Subsequently, mice were kept in individually ventilated cages (IVC) with free access to autoclaved food and water.

2.4. 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 (Xing et al., 1996). 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 (Maus et al., 2004). 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).

2.5. Diphtheria toxin induced depletion of $FoxP3^{pos}$ regulatory T cells

To examine the role of $FoxP3^{pos}$ 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.

2.6. Bronchoalveolar lavage

Bronchoalveolar lavage of mice of the various treatment groups was performed essentially as described elsewhere (Steinwede et al., 2011; Srivastava et al., 2007; Taut et al., 2008).

2.7. Immunophenotypic analysis of leukocytes in lung tissue and lung draining lymph nodes

Lungs were perfused *in situ* with Hanḱs 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 (Behler et al., 2012). Subsequently, $CD11c^{pos}$ leukocytes or $CD45^{pos}$ leukocytes were purified

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