Vaccine xxx (xxxx) xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Weaker protection against tuberculosis in BCG-vaccinated male 129 S2 mice compared to females

Natalie E. Nieuwenhuizen [a,b,*], Joanna Zyla [c], Ulrike Zedler [a], Silke Bandermann [a], Ulrike Abu Abed [d], Volker Brinkmann [d], Stefan H.E. Kaufmann [a,e,f]

[a] Department of Immunology, Max Planck Institute for Infection Biology, Chariteplatz 1, 10117 Berlin, Germany
[b] Institute for Hygiene and Microbiology, Julius-Maximilians-Universität Würzburg, Josef-Schneider-Str.2, 97080 Würzburg, Germany
[c] Department of Data Science and Engineering Silesian University of Technology, Akademicka 16, 44-100 Gliwice, Poland
[d] Microscopy Core Facility, Max Planck Institute for Infection Biology, Chariteplatz 1, 10117 Berlin, Germany
[e] Max Planck Institute for Biophysical Chemistry, Am Faßberg, 1137077 Göttingen, Germany
[f] Hagler Institute for Advanced Study, Texas A&M University, College Station, TX, United States

ARTICLE INFO

Article history:
Available online xxxx

Keywords:
BCG
Tuberculosis
Vaccination
Sex differences
T cells

ABSTRACT

BCG – the only available vaccine against tuberculosis (TB) - was first given to babies 100 years ago in 1921. While it is effective against TB meningitis and disseminated TB, its efficacy against pulmonary TB is variable, notably in adults and adolescents. TB remains one of the world's leading health problems, with a higher prevalence among men. Male sex is associated with increased susceptibility to *Mycobacterium tuberculosis* in mice, but sex-specific responses to BCG vaccination have not been examined. In this study we vaccinated TB-susceptible 129 S2 mice with BCG and challenged with low-dose *M. tuberculosis* H37Rv by aerosol infection. BCG was protective against TB in both sexes, as unvaccinated mice lost weight more rapidly than vaccinated ones and suffered from worse lung pathology. However, female mice were better protected than males, showing lower lung bacterial burdens and less weight loss. Overall, vaccinated female mice had increased numbers of T cells and less myeloid cells in the lungs compared to vaccinated males. Principal component analysis of measured features revealed that mice grouped according to timepoint, sex and vaccination status. The features that had the biggest impact on grouping overall included numbers of CD8 T cells, CD8 central memory T cells and CD4 T effector cells, with neutrophil and $CD11b^{+}GR\text{-}1^{-}$ cell numbers having a big impact at day 29. Hierarchical clustering confirmed that the main difference in global immune response was due to mouse sex, with only a few misgrouped mice. In conclusion, we found sex-specific differences in response to *M. tuberculosis* H37Rv -challenge in BCG-vaccinated 129 S2 mice. This highlights the need to include both male and female mice in preclinical testing of vaccine candidates.

1. Introduction

One hundred years ago (1921), Albert Calmette and Camille Guérin from the Pasteur Institute in Lille supervised the first vaccination of a newborn with their newly developed vaccine, Bacille Calmette Guérin (BCG) [1,2]. They had continuously passaged *Mycobacterium bovis* on ox bile-soaked potato slices over 230 times. *M. bovis* is the etiologic agent of cattle tuberculosis (TB), and at that time was particularly threatening to neonates, as contaminated milk from infected cows could cause infection orally. Accordingly, BCG was initially given orally, and only later by intradermal application. The first vaccinated neonate was born into a household containing a TB patient, and was considered highly at risk. The death rate of neonates born into such environments ranged from 15% to 50%. Over the following years, numerous neonates were vaccinated with BCG and retrospective analysis revealed that <1% of them died of TB [2]. Although these were not controlled clinical trials, it was obvious that the vaccine could reduce mortality of neonates markedly. Subsequently, BCG has been adminis-

Abbreviations: TB, tuberculosis; *Mtb*, *Mycobacterium tuberculosis*; BCG, *Mycobacterium bovis* Bacille Calmette-Guérin; CFUs, colony forming units; PBS, phosphate-buffered saline; T_{RM}, resident memory T cells; T_{EM}, effector memory T cells; T_{CM}, central memory T cells; PCA, principal component analysis; HC, Hierarchical clustering; MLR, monocyte-to-lymphocyte ratio; MTR, myeloid-cell-to-T-cell-ratio; PBMCs, peripheral blood mononuclear cells.

* Corresponding author at: Institute for Hygiene and Microbiology, Julius-Maximilians-Universität Würzburg, Josef-Schneider-Str.2, 97080 Würzburg, Germany.

E-mail address: natalie.nieuwenhuizen@uni-wuerzburg.de (N.E. Nieuwenhuizen).

https://doi.org/10.1016/j.vaccine.2021.09.039
0264-410X/

tered to over 4 billion individuals – mostly neonates – and has become the most widely used vaccine globally. Every year, 120–140 million neonates are immunized with BCG as part of the Expanded Program of Immunization (https://www.who.int/immunization/programmes_systems/en/). Numerous studies have shown that BCG protects neonates against TB, particularly extrapulmonary TB [3,4]. However, its value as vaccine against pulmonary TB remains questionable, especially in adolescents and adults. Meta-analyses suggest overall efficacy of approximately 50% for BCG against TB, but this varies greatly between regions [3,5]. Accordingly, today TB remains one of the deadliest infectious diseases worldwide. In 2019, around 10 million individuals developed active TB and 1.4 million died [6]. TB is a chronic disease primarily caused by the acid fast bacillus, *Mycobacterium tuberculosis* (*Mtb*). Factors that impact susceptibility to TB remain incompletely understood [7]. Gender and sex seem to influence the risk of TB, with males being more susceptible than females. Globally, more men become infected and die annually from TB than women [6]. A meta-analysis of TB prevalence surveys from 28 countries found that prevalence among men was more than twice as high as among women [8]. This study concluded that male sex should be considered a risk factor for TB disease. The increased prevalence in males was initially thought to be solely due to sociological factors; however, evidence suggests that biological differences contribute too [9–11]. Factors that may render men more susceptible to pulmonary TB include sex steroid hormones, genes on the X and Y chromosomes, and sex-specific metabolic features [9,12]. In childhood, TB prevalence in males and females is equal, but between 10 and 16 years the prevalence begins to rise in males, until from age 30 onwards they are more susceptible than females by twofold [13]. This indicates a role for sex steroid hormones in the increased susceptibility. The influence of sex steroid hormones on immune responses has been widely documented in humans and in animal models using castrated and hormone-reconstituted animals [7,9,14]. Castration of male mice causes androgen deprivation, leading to increased T cell numbers in peripheral lymph nodes and increased T cell proliferation after exposure to antigen [15]. Specific X and Y chromosome genes have also been identified that control molecular cascades leading to sex differences in disease [16,17].

Male mice of multiple strains have also demonstrated increased susceptibility to *Mtb* and other mycobacterial strains, including *M. avium*, *M. marinum* and *M. lepraemurium* [18–22]. In *M. avium* infection, ovariectomized mice suffered from increased lung bacterial loads [21]. This effect was reversed by administration of 17β-estradiol, which enhanced macrophage activity against *M. avium*. Conversely, castration reversed the increased susceptibility of male mice to *M. marinum* [20]. Male BALB/c mice have increased susceptibility to *Mtb*, with higher bacterial loads and increased mortality compared to females and castrated males [19]. Similarly, male C57BL/6 mice have increased susceptibility, with elevated bacterial loads and a strong, early inflammatory response in the lungs, coinciding with increased pathology and mortality [18]. Pulmonary pathology develops differently in C57BL/6 males and females in terms of immune cell recruitment and spatial organisation, with males having smaller lymphoid aggregates and increased myeloid cell infiltrates [18], as well as smaller B cell follicles in the lungs [23]. Little is known about the role of sex in BCG efficacy, although sex differences have been described in immunity to other vaccines [12].

The mouse has been used widely as an experimental model for TB. In the commonly used C57BL/6 and BALB/c mouse strains, males were found to be more susceptible than females [18,19]. Both of these mouse strains are relatively resistant to TB [24]. The less frequently used mouse strain 129 S2 is highly susceptible to TB and even low dose infection generally leads to death within weeks after challenge. Susceptibility is dependent on type I interferon responses and associated with infiltration of neutrophils and inflammatory monocytes into the lung [25], both characteristic of human TB [26,27]. Transcriptomic analysis revealed that compared to the gene expression profile of infected C56BL/6 mice, the expression profile of infected 129 S2 mice is more similar to that of humans with active TB disease [28]. At day 21 post infection, lung pathology of 129 S2 mice was characterized by large, necrotic lesions, whereas that of C57BL/6 mice comprised smaller, non-necrotic lesions with less inflammation. By analyzing concordant and discordant gene modules, the T-cell response was identified as the major discriminator between 129 S2 and C57BL/6 mice at day 21 post infection. Based on this information, we decided to test the 129 S2 mouse as a model for evaluating TB vaccine candidates. We analyzed the impact of sex on BCG vaccine efficacy in 129 S2 mice and found weaker protective efficacy against *Mtb* challenge in males. Our data indicate that male mice suffered from increased infiltration into the lung of myeloid cells as well as reduced T-cell responses.

2. Methods

2.1. Bacterial strains

The *Mtb* H37Rv (ATCC; catalogue no. 27294) and BCG Danish 1331 (BCG SSI) (ATCC; catalogue no. 35733) were grown in Middlebrook 7H9 broth (BD) supplemented with albumin-dextrose-catalase enrichment (BD), 0.2% glycerol, and 0.05% Tween 80 or on Middlebrook 7H11 agar (BD) containing 10% (vol/vol) oleic acid-albumin-dextrose-catalase enrichment (BD) and 0.2% glycerol. BCG was grown to mid-log phase, washed with phosphate-buffered saline (PBS) and stored at −80 °C in PBS/10% glycerol. Prior to vaccination, BCG were thawed, washed in PBS and prepared at a dose of 10^6 colony forming units (CFU) in 100 µl PBS.

2.2. Mouse model

Mice were bred in-house and housed in individually ventilated cages. All mouse experiments were ethically approved by the State Office for Health and Social Services, Berlin, Germany (project number G0120/16). Mice were handled in accordance with the European directive 2010/63/EU on Care, Welfare and Treatment of Animals. All efforts were taken to minimize their discomfort. 6–8 week old 129 S2 mice were vaccinated subcutaneously at the tail base with $1x10^6$ CFUs BCG or left unvaccinated as a control. At 60 days post vaccination, mice were aerosol-infected with a low dose of 20–50 CFU of *Mtb* H37Rv using a Glas-Col inhalation exposure system. Input CFUS were checked at 24 h post infection by plating whole lung homogenates from input control mice onto Middlebrook 7H11 agar plates, and counting colonies 3–4 weeks later. Mice were weighed weekly until day 14, and twice a week thereafter. Mice were euthanized if weight loss exceeded 15% or mice reached symptom score 2. Blood was collected for serum antibody measurements. Spleens were collected for CFUs. Lungs were collected and divided for CFUS (right lung) and flow cytometry (left lung). Right or left lungs were collected for histology from some of the mice.

2.3. Mycobacterial loads

Spleens and lungs were homogenized by manual mechanical disruption in Whirl-Pak sample collection bags (Roth) containing 1 ml PBS/0.05% Tween80 (PBS-T), prepared as 10-fold serial dilutions and plated on Middlebrook 7H11 agar containing ampicillin.

CFUs were counted 3–4 weeks after incubation of the plates at 37 °C.

2.4. Symptom score

0: Smooth coat, clear eyes, normal movement and behaviour, normal interactions with other mice, active movement when awake, no weight loss, adequate reaction to gentle stimuli, normal breathing

1: Unkempt coat, slightly delayed reaction to gentle stimuli, weight loss of 0–10%

2: Shaggy coat, secretions from eyes or nose, sunken eyes, poor interaction with other mice, delayed/ weak reaction to gentle stimuli, weight loss of 10–15%, increased breathing rate

3: Very shaggy coat, abnormal posture, avoiding movement, self-isolation, vocalisation, self-mutilation, lateral position, weight loss > 15%, increased breathing or obvious dyspnoea

We performed euthanasia if animals reached weight loss of 15% or score 2.

2.5. Histology

Lungs were fixed in 4% formaldehyde in PBS and embedded in paraffin wax. Tissue sections were stained with Giemsa and whole lung sections were scanned with a ZEISS Axioscan Z1 driven by ZEN. Lung pathology analysis was performed using Volocity (Perkin Elmer).

2.6. Flow cytometry

The left lung was cut into small pieces and digested for 1 h at 37 °C in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) containing 13 μg/mL DNase I (Sigma-Aldrich) and 50 U/mL collagenase IV (Sigma-Aldrich). Single-cell suspensions were prepared by passing digested tissue through 70 μM cell strainers (Corning Falcon). Isolated lung cells were stained with fluorescent antibodies in PBS/0.1% BSA containing Fc block (anti-FcγRII/III, clone 24G2, produced in-house) and 1% rat serum and detected by flow cytometry. Intracellular staining of transcription factors was performed using the Transcription Factor Buffer set (BD Pharmingen). Cells were acquired on a Cytoflex (Beckman Coulter). Data were analysed using Flowjo software (Tree Star Inc., Ashland, OR).

The antibody panels used and the gating strategy are shown in the Supplemental Material.

2.7. Specific IgG

Mycobacteria--specific antibodies were measured in serum collected at day 21 by indirect ELISA. Plates were coated with *Mtb* H37Rv lysate (BEI Resources), and IgG antibodies were detected with AP-labeled anti-mouse IgG (SouthernBiotech).

2.8. Statistical analyses

The comparison between four groups was performed using linear modelling (multicomp R package; glht function) with the following contrasts: differences between male mice (control vs BCG), differences between female mice (control vs BCG) separately and jointly between male and female (interaction), which is the equivalent contrast for two-way ANOVA. Correction for multiple testing was performed using the single-step approach (adjustment of p-values based on the joint t-distribution of the linear function). For comparison of two groups, the Mann-Whitney test was performed using GraphPad Prism version 9.0.0.

Principal Component Analysis (PCA) was performed for the complete set of measurements using the prcomp R function and the rotation vector of component 1 and 2 was investigated to reveal features impacting particular components. The hierarchical clustering (HC) was performed using the complete method on Euclidian distance (pheatmap R package). Both PCA and hierarchical clustering were performed in R programming language.

3. Results

3.1. Reduced efficacy of BCG vaccination in male mice

Male and female TB-susceptible 129 S2 mice were vaccinated subcutaneously with 1x10E6 CFUs BCG and challenged with 20-50CFUs *Mtb* H37Rv at 60 days post vaccination. Control mice were unvaccinated. Weights were monitored and the percentage weight change was calculated for each mouse (Fig. 1A). Unvaccinated female control mice showed weight loss starting shortly before day 21, while BCG-vaccinated mice did not lose weight. In contrast, both unvaccinated and BCG-vaccinated male mice showed weight loss shortly before day 21, although this was ameliorated in the vaccinated mice. By day 21, unvaccinated control male and female mice reached 15% body weight loss and had to be euthanized. BCG-vaccinated female mice showed no signs of illness up until the end of the experiment at day 29 (Fig. 1B), whereas the remaining male mice showed signs of illness such as reduced movement and ungroomed fur. We used linear modelling (see Methods) to to determine whether there was a difference between the effects of BCG vaccination in male and female mice. This method allowed us to contrast 1) unvaccinated and vaccinated female mice, 2) unvaccinated and vaccinated male mice, and 3) male and female (interaction). The results showed a significant difference between males and females in terms of symptom scores.

Bacterial burdens were measured in the lungs and spleens collected at the experimental endpoint (day 29 for vaccinated mice, or day 21 for unvaccinated mice, which had to be euthanized earlier) (Fig. 1C). BCG-vaccinated females had significantly lower bacterial burdens in the lungs and spleen at day 29 compared to the burdens in control unvaccinated mice at day 21. In contrast, there was no statistically significant reduction in *Mtb* load in the lungs of male BCG-vaccinated mice, although there was a trend to a reduction, with lower median values in vaccinated mice, and decreased bacterial loads in the spleen. In a separate experiment, we collected lung samples for histology at day 21. Image analysis of lung pathology indicated that BCG vaccination decreased the size of lesions in BCG-vaccinated male mice compared to unvaccinated controls (Fig. 2A and 2B). Female mice showed a similar tendency to decreased lesion size after BCG vaccination.

3.2. Lung cell populations in BCG-vaccinated male and female mice

We analysed lung cell populations in male and female BCG-vaccinated mice by flow cytometry at day 29 post *Mtb* challenge (Fig. 3A to J). The gating strategies are shown in Supplementary Fig. 1. Data were pooled from two experiments. We did not have sufficient cells to perform myeloid cell staining in one of the mice. Total numbers of lung cells were similar between male and female vaccinated mice (Supplementary Fig. 2A). Susceptibility of 129 S2 mice has been associated with increased infiltration of the lungs with inflammatory monocytes and neutrophils [25]. In our experiments, the percentages and numbers of neutrophils tended to be raised in male mice (Fig. 3A), and percentages and numbers of inflammatory monocytes (Fig. 3B) were increased in male mice compared to females. Macrophages are the main host cell for *Mtb* bacilli, therefore we also examined $CD11b^{+}GR\text{-}1^{-}$ cells (monocytes/macrophages) and alveolar macrophages. Percentages and numbers of $CD11b^{+}GR\text{-}1^{-}$ monocytes/macrophages were also

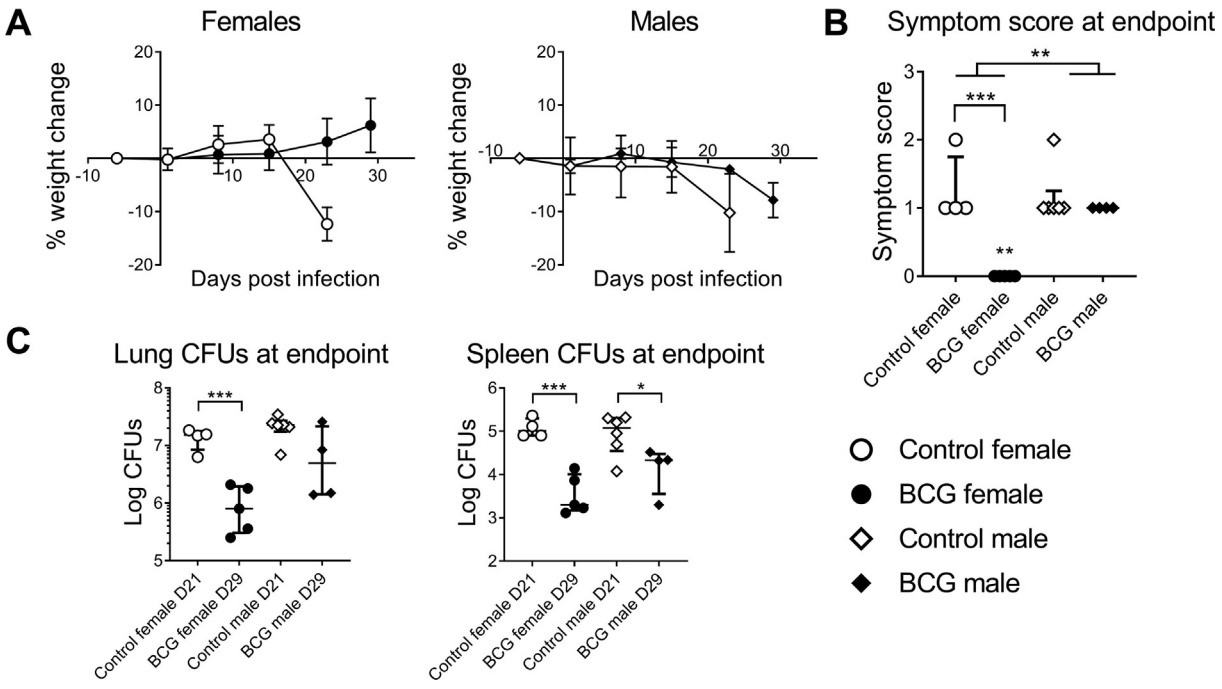

Fig. 1. Reduced efficacy of BCG vaccination in male compared to female mice. Mice were vaccinated with $1 \times 10E^6$ BCG and challenged by aerosol at day 60 post vaccination with 20–50 CFUs Mtb H37Rv. Unvaccinated control mice had to be euthanized at day 21 due to weight loss while vaccinated mice were analysed at day 29. A) Percentage weight change after infection. B) Symptom score at the endpoint. C) Bacterial burdens in lungs and spleen at the endpoint. CFUs: colony forming units. Statistical significance was calculated by linear modelling with contrast set within sex group and between groups and correction for multiple testing was performed. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Representative of two experiments, n = 4–6 per group.

increased in male mice compared to females (Fig. 3C), while alveolar macrophage numbers were similar in males and females (Fig. 3D). The myeloid-cell-to-T-cell ratio (MTR) was increased in male mice (Supplementary Fig. 2B).

T cell responses are considered critical in TB immunity. Effector memory T (T_{EM}) cells elicited by BCG can control acute infection, while long-term protection requires the generation of central memory T (T_{CM}) cells [29]. T_{CM} cells circulate between the blood and lymph nodes but can rapidly enter the tissues in response to infection or tissue injury [30,31]. In BCG-vaccinated 129 S2 mice challenged with *Mtb*, the populations of CD4 T_{EM}, CD8 T_{EM} and CD4 T_{CM} were similar between males and female lungs (Fig. 3E-G), but populations of CD8 T_{CM} cells were increased in females compared to males (Fig. 3H). The establishment of a resident memory T (T_{RM}) cell population in the lungs is considered an important component of immunity to pulmonary TB [32]. At day 29, percentages and numbers of CD4 T_{RM} were similar in males and females (Fig. 3I), while CD8 T_{RM} numbers were slightly increased in males (Fig. 3J). Numbers of CD4 and CD8 T cells and the CD4/CD8 T cell ratio were similar between males and females (Supplementary Fig. 2C-E). B cell populations were not significantly different between males and females (Supplementary Fig. 2F). In addition, mycobacteria-specific IgG levels were similar in males and females at day 21 (Supplementary Fig. 2G).

3.3. Pulmonary leukocyte populations in BCG-vaccinated and control mice at day 18

As differences in cell populations can reflect differences in bacterial burdens, we investigated lung cell populations at day 18 post challenge, prior to the onset of weight loss and signs of disease. At this timepoint, lung bacterial loads were decreased in both male and female BCG-vaccinated mice compared to unvaccinated controls, while spleen *Mtb* burdens were similar between all groups (Fig. 4A). Total numbers of isolated lung cells were not significantly different between the groups (Fig. 4B). Trends between mouse groups were the same in two experiments, but we did not pool the data as for some parameters the actual values varied. At this timepoint, populations of neutrophils, inflammatory monocytes, $CD11b^+GR\text{-}1^-$ monocytes/macrophages and alveolar macrophages were all similar between the groups (Fig. 4C-F). The MTR showed a tendency to be raised in both male groups (Supplementary Fig. 2H).

Male mice tended to have decreased proportions and numbers of both CD4 and CD8 T cells in lungs at day 18 compared to females (Fig. 5A and B). However, BCG vaccination tended to increase populations of CD4 T_{EM} in both male and female mice (Fig. 5C). In contrast, CD4 T_{CM} numbers tended to be lower in males (Fig. 5D). Similar to day 29, the numbers of CD8 T_{EM} tended to be lower in BCG-vaccinated males than in females (although percentages were raised) (Fig. 5E), and analysis by linear modelling indicated a difference between males and female responses in this cellular compartment. Also similar to day 29, populations of CD8 T_{CM} tended to be decreased in male BCG-vaccinated mice compared to females (Fig. 4F). CD4 T_{RM} and CD8 T_{RM} populations also tended to be lower in males than in females (Fig. 5G and H). Interestingly, in BCG-vaccinated male mice the CD4/CD8 ratio was increased compared to unvaccinated controls, whereas this was not the case in females (Supplementary Fig. 2I).

We performed additional experiments to examine the T cell phenotypes. The percentages of lung CD4 and CD8 T cells expressing the proliferation marker Ki-67 were increased in male mice only (Fig. 6A and B). Percentages of CD4 T cells expressing the transcription factors T-bet and RORγT, markers of Th1 cells and Th17 cells, respectively, were increased in both male and female mice (Fig. 6C and D). In contrast, BCG vaccination decreased percentages of lung $CD25^+Foxp3^+$ regulatory CD4 T cells (T_{reg}) in both males and females (Fig. 6E).

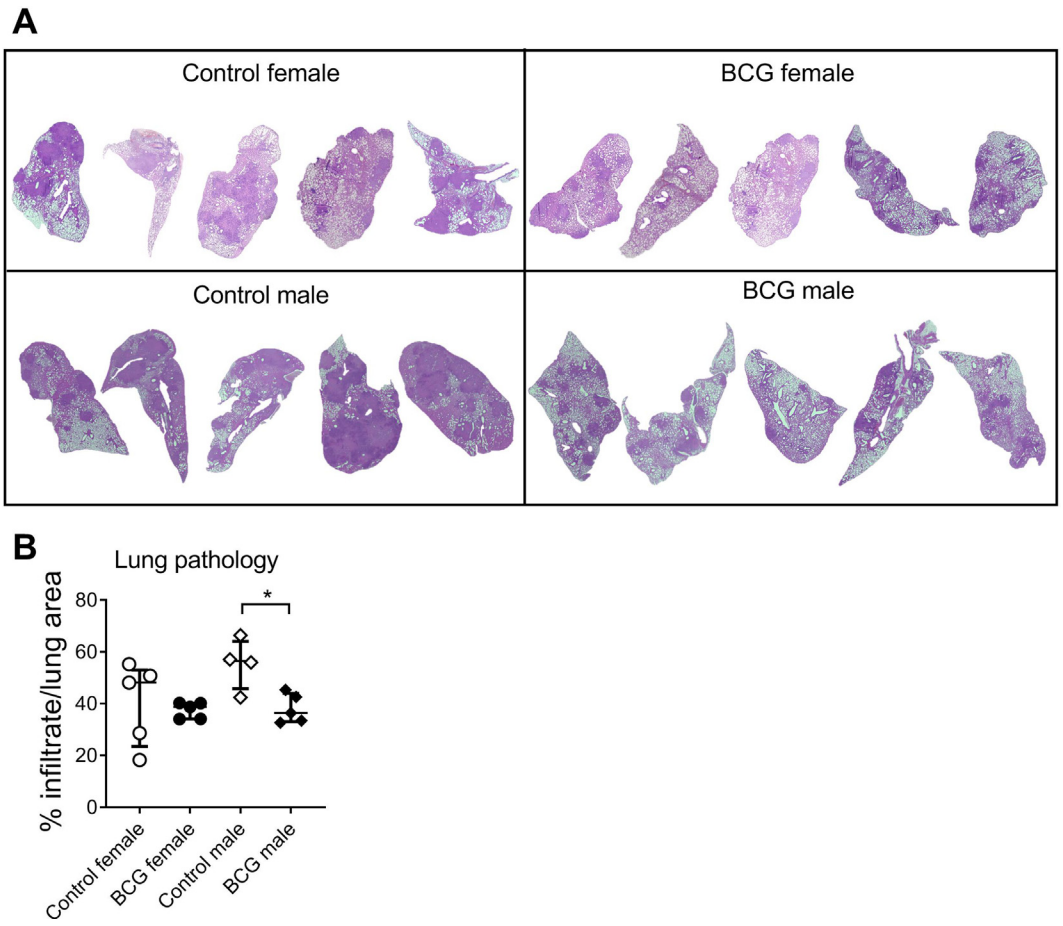

Fig. 2. Pulmonary pathology in BCG-vaccinated mice and unvaccinated controls. Mice were vaccinated with 1 × 10E6 BCG and challenged by aerosol at day 60 post vaccination with 20–50 CFUs Mtb H37Rv. Lungs were collected at day 21. A) Lung sections were scanned with a ZEISS Axioscan Z1 driven by ZEN. B) Scanned lung sections were analysed with Volocity for percentage of infiltrated area (Perkin Elmer). The % of infiltrated lung = infiltrated area/(normal tissue area) x100. Statistical significance was calculated by linear modelling with contrast set within sex group and between groups and correction for multiple testing was performed. *, $P < 0.05$. n = 5 per group.

3.4. Principal component analysis and hierarchical clustering

Principal component analysis (PCA) was performed on the measured features (immune cell populations, symptom scores, bacterial loads), to graphically represent the division between the analysed groups. Fig. 7A (Day 18) and Fig. 7B (Day 29) show that clusters of female and male mice can be distinguished by the most informative components 1 and 2 (details of levels of variance explained in PCA are included in Supplementary Fig. 3). Moreover, Fig. 7A shows that at day 18 the unvaccinated control mice of both sexes lie close together on the plot. A rotation matrix investigation was performed to extract the most influential measured features (Supplementary Fig. 4). At day 18, features that had the biggest impact on grouping included numbers of CD4 T_{EM}, percentages of CD4 T_{RM}, numbers of CD8 T cells, CD8 T_{CM} and CD8 T_{RM}, and numbers of T bet^{+} and ROR-γt CD4 T cells (Supplementary Fig. 4A), which is consistent with the single feature statistical analysis. At day 29, the neutrophils, CD8 T_{CM}, CD8 T cell numbers, and the CD11b^{+}GR-1^{-} cells had a big impact (Supplementary Fig. 4B). Principal component analysis of data from both days together showed that the timepoint had the biggest impact on grouping (Supplementary Fig. 5). Features that had a big impact on this grouping included numbers of CD4 and CD8 T cells, percentages of CD4 T_{RM}, CD4 T_{EM} and CD8 T_{CM}, the CD4/CD8 ratio, and numbers of inflammatory monocytes (Supplementary Fig. 6).

To confirm general differences between male and female mice, unsupervised clustering was performed (hierarchical clustering). Fig. 8A and 8B show that the main difference in global response was due to the mouse sex, with only a few misgrouped mice. At day 18, further grouping was related to vaccination status, and the similarity between unvaccinated controls can be observed, with 2 female mice having a similar global response profile to control males (Fig. 8A). Overall, this suggests differences between the sexes in the responses to BCG vaccination.

4. Discussion

In our experiments with TB-susceptible 129 S2 mice, BCG vaccination showed better protective efficacy against *Mtb* H37Rv challenge in female mice than it did in males. The efficacy of BCG vaccination in 129S2 mice has been previously assessed in two studies investigating BCG vaccination in different mouse strains [7,33]. In the study by Smith et al, only male mice were examined [7], and in the study by Zelmer et al., the sex of the mice was not reported [33]). Using only male mice, Smith et al. found that bacterial loads were only reduced in the spleen, and not in the lungs, of BCG-vaccinated mice at week 4 post infection with *Mtb* H37Rv [7], which agrees with our findings. In their study, mice were vaccinated with 1 × 10E5 BCG and challenged at 12 weeks post vaccination, while in ours mice were vaccinated with 1 × 10E6 BCG and challenged 2 months post vaccination. Zelmer et al. vaccinated mice with 2 × 10E5 CFUs of BCG and challenged with 30 CFUs of *Mtb* Erdman 6 weeks after vaccination [33]. They also did not find a significant effect of BCG vaccination on lung bacterial burdens at

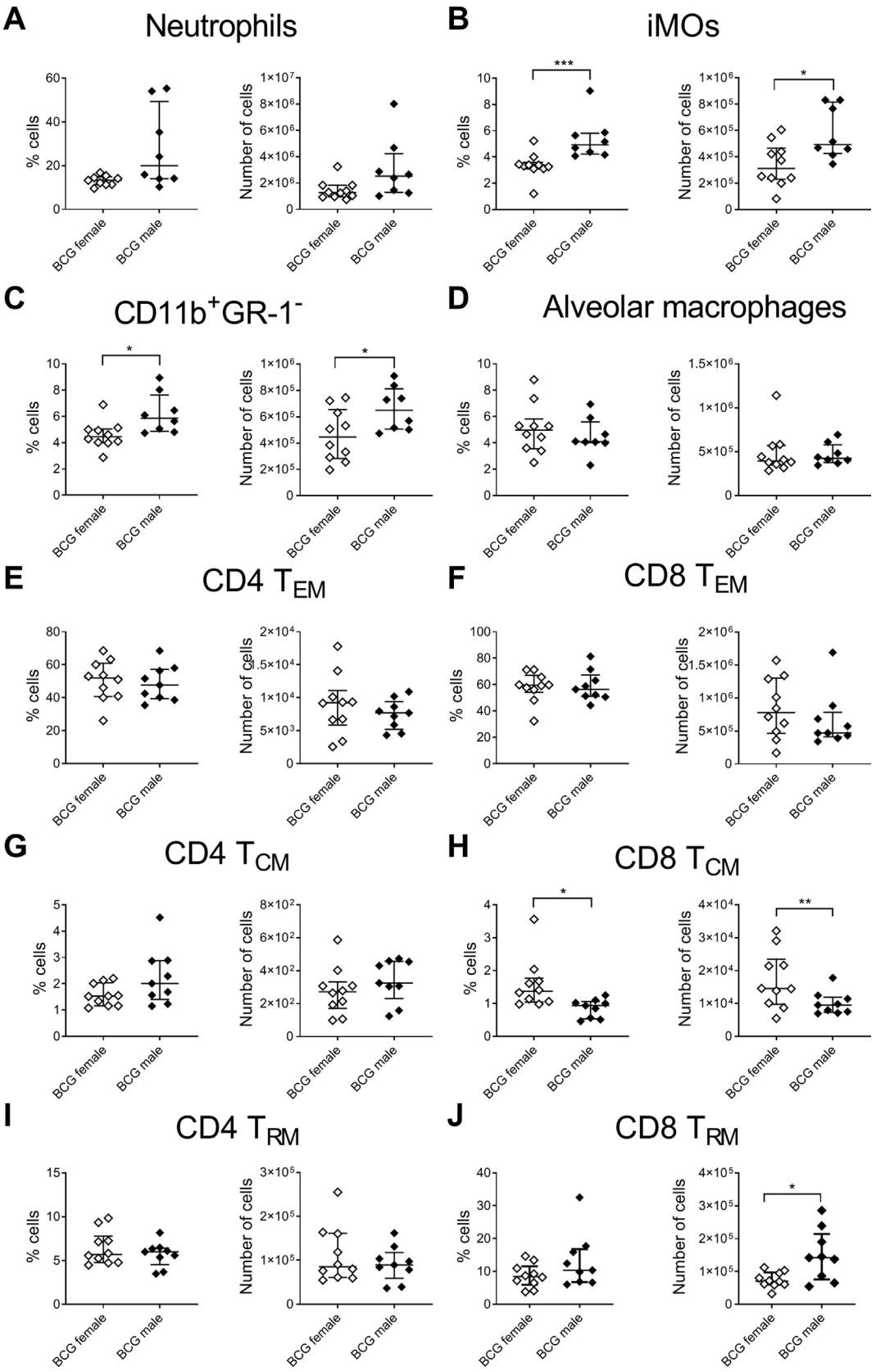

Fig. 3. Comparison of pulmonary immune cell populations in male and female BCG-vaccinated mice. Mice were vaccinated with 1 × 10E6 BCG and challenged by aerosol at day 60 post vaccination with 20–50 CFUs Mtb H37Rv. At day 29, lungs were harvested and cells were isolated by digestion with collagenase and DNAse. Lung cell populations were analysed by flow cytometry. A) Neutrophils ($Ly6G^{hi}$ $Ly6C^{hi}$ $CD11b^{+}$). B) Inflammatory monocytes (iMOs) ($Ly6C^{+}$ $Ly6G^{-}$ $CD11b^{+}CD11c^{-}$). C) $CD11b^{+}GR\text{-}1^{-}$ cells ($CD11c^{-}$) (monocytes/macrophages). D) Alveolar macrophages ($CD11c^{+}SiglecF^{+}$). E) CD4 T effector memory cells (T_{EM})($CD3^{+}CD4^{+}CD44^{+}CD62L^{-}$). F) CD8 T_{EM} ($CD3^{+}CD8^{+}CD44^{+}CD62L^{-}$). G) CD4 T central memory cells (T_{CM}) ($CD3^{+}CD4^{+}CD44^{+}CD62L^{+}$). H) CD8 T_{CM} ($CD3^{+}CD8^{+}CD44^{+}CD62L^{+}$). I) CD4 T resident memory cells (T_{RM}) ($CD3^{+}CD4^{+}CD103^{+}CD69^{+}$). J) CD8 T resident memory cells (T_{RM}) ($CD3^{+}CD8^{+}CD103^{+}CD69^{+}$). Statistical significance was calculated using the Mann Whitney. *, $P < 0.05$; **, $P < 0.01$. Pooled data from two individual experiments, n = 3–5 per group per experiment.

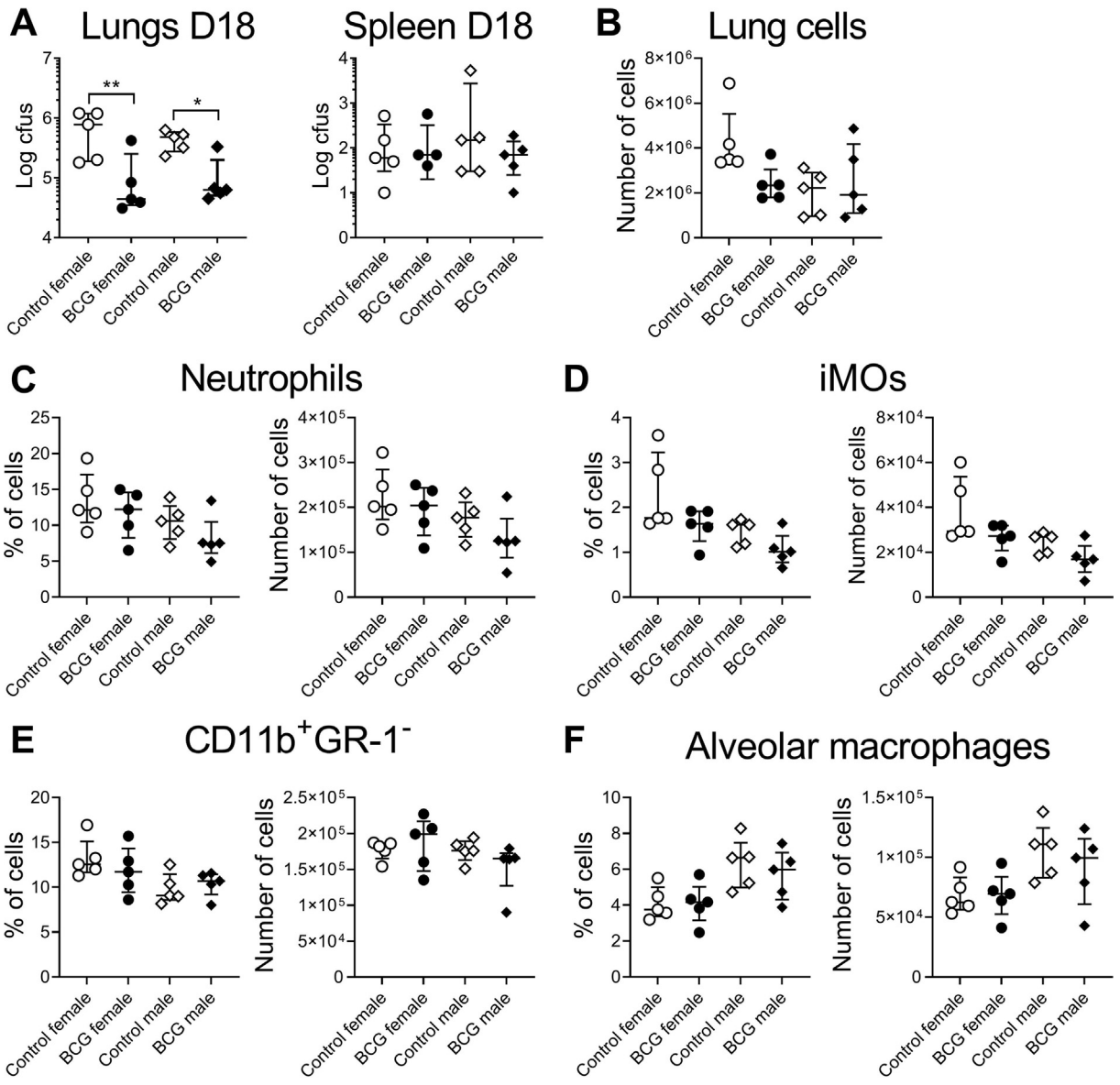

Fig. 4. Pulmonary myeloid cell populations of BCG-vaccinated and unvaccinated control mice at day 18. Mice were vaccinated with 1 × 10E6 BCG and challenged by aerosol at day 60 post vaccination with 20–50 CFUs Mtb H37Rv. At day 18, lungs were harvested and cells were isolated by digestion with collagenase and DNAse. Lung cell populations were analysed by flow cytometry. A) Bacterial loads in lungs and spleens at day 18 post-infection. B) Total lung cells isolated from one lung per mouse. C) Neutrophils ($Ly6G^{hi}$ $Ly6C^{hi}$ $CD11b^{+}$). D) Inflammatory monocytes (iMOs) ($Ly6C^{+}$ $Ly6G^{-}$ $CD11b^{+}CD11c^{-}$). E) $CD11b^{+}GR\text{-}1^{-}$ cells ($CD11c^{-}$)(monocytes/macrophages). F) Alveolar macrophages ($CD11c^{+}SiglecF^{+}$). Statistical significance was calculated using linear modelling with contrast set within sex group and between groups and correction for multiple testing was performed. *, $P < 0.05$; **, $P < 0.01$. Representative of two experiments, n = 4–5 per group per experiment.

7 weeks post challenge. Interestingly, Smith et al. found that susceptibility to TB and the efficacy of BCG vaccination in different mouse strains were independently controlled by the mouse genotype [7].

In our study, BCG vaccination reduced areas of lungs with lesions in both male and female mice after *Mtb* challenge. Yet, percentages of myeloid cells were increased among lung cells of male vaccinated and challenged mice, as previously observed in C57BL/6 mice [18]. Neutrophils contribute towards disease in 129 S2 mice [25], and are associated with active TB disease in humans [26,27]. Inflammatory monocytes and other myeloid cells serve as a niche for *Mtb* infection [34], and the monocyte-to-lymphocyte ratio (MLR) is increased during active TB in humans [35]. A recent study indicates that the MLR is higher in males than in females during TB [36]. As a substitute for MLR in humans, we calculated the MTR in our mice, and found that the MTR was increased in male mice.

At day 18 post challenge, there was a trend to increased percentages of CD4 T_{EM} and CD4 T_{RM} in both male and female BCG-vaccinated mice, but numbers of these cells tended to be lower in males. A limitation of our study is that we did not inject mice with labelled anti-CD45 prior to isolating the lungs, which can discriminate circulating cells and tissue-localized cells [37]. Therefore, some of the T_{EM} and T_{CM} we detected may have been circulating cells. However, T_{RM} were characterized by their expression of CD103 (alpha E integrin; αE) and CD69, functional markers that distinguish tissue-resident cells from those in circulation [38]. Adhesive interactions between αE/β7 and E-cadherin allow the retention of T_{RM} cells in the tissue, while CD69 limits the exit of the cells from the tissue [38]. In particular, CD103 is upregulated

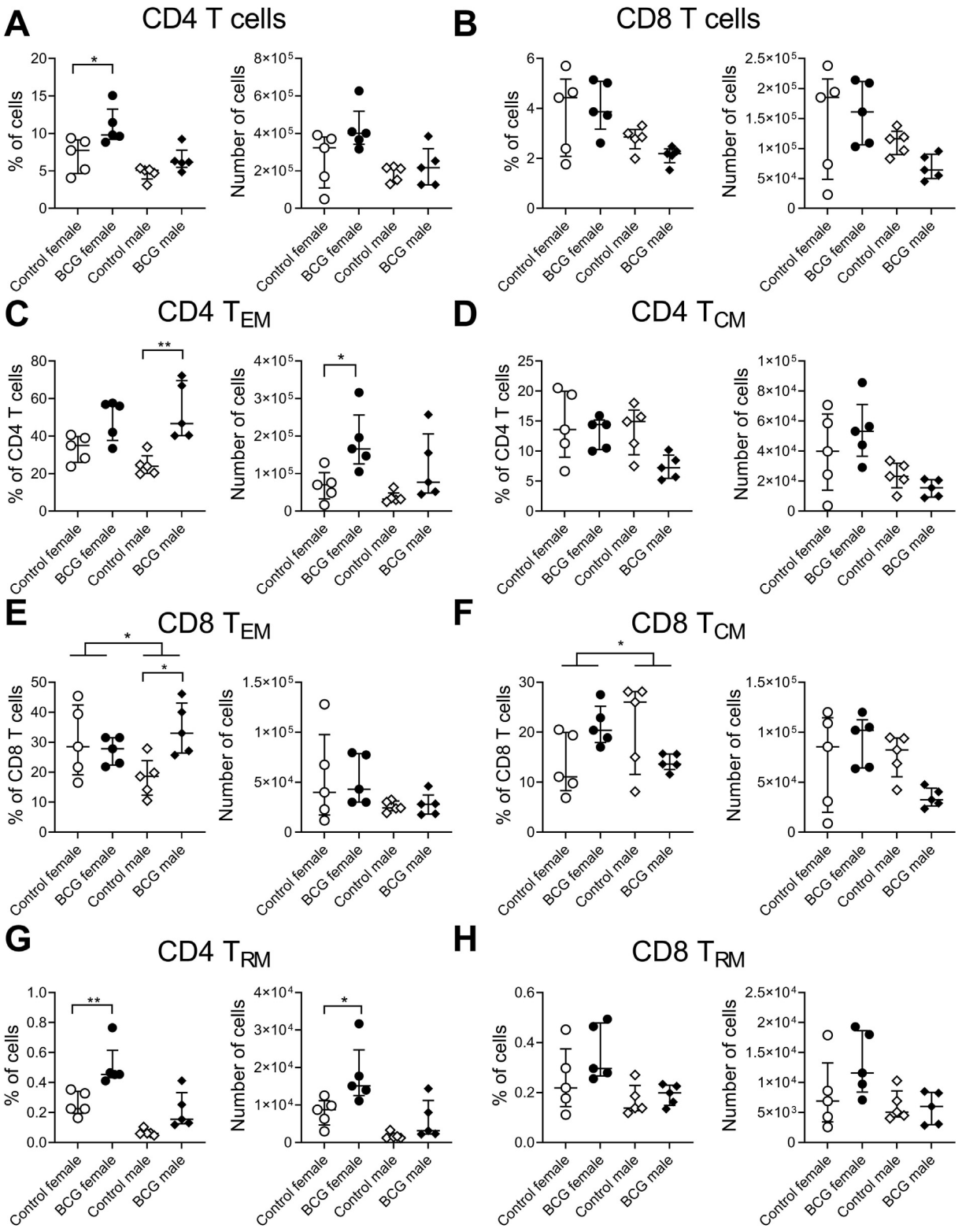

Fig. 5. Pulmonary T cell subsets of male and female BCG-vaccinated mice at day 18. Mice were vaccinated with 1 × 10E6 BCG and challenged by aerosol at day 60 post vaccination with 20–50 CFUs Mtb H37Rv. At day 18, lungs were harvested and cells were isolated by digestion with collagenase and DNAse. Lung cell populations were analysed by flow cytometry. A) CD4 T cells ($CD3^+CD4^+$). B) CD8 T cells ($CD3^+CD8^+$). C) CD4 T effector memory cells (T_{EM})($CD3^+CD4^+CD44^+CD62L^-$). D) CD4 T central memory cells (T_{CM}) ($CD3^+CD4^+CD44^+CD62L^+$). E) CD8 T_{EM} ($CD3^+CD8^+CD44^+CD62L^-$). F) CD8 T_{CM} ($CD3^+CD8^+CD44^+CD62L^+$). G) CD4 T resident memory cells (T_{RM}) ($CD3^+CD4^+CD103^+$-$CD69^+$). H) CD8 T_{RM} ($CD3^+CD8^+CD103^+CD69^+$). Statistical significance was calculated using linear modelling with contrast set within sex group and between groups and correction for multiple testing was performed. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. Representative of two experiments, n = 4–5 per group per experiment.

in response to changes in the local cytokine environment [31,38]. Interestingly, increased numbers of tissue resident leukocytes were previously reported in female mice during sepsis [39]. In this study, macrophages from female mice were found to be more effective in phagocytosis and bacterial killing, while the presence of proportionally more CD4 T cells in females than in males limited

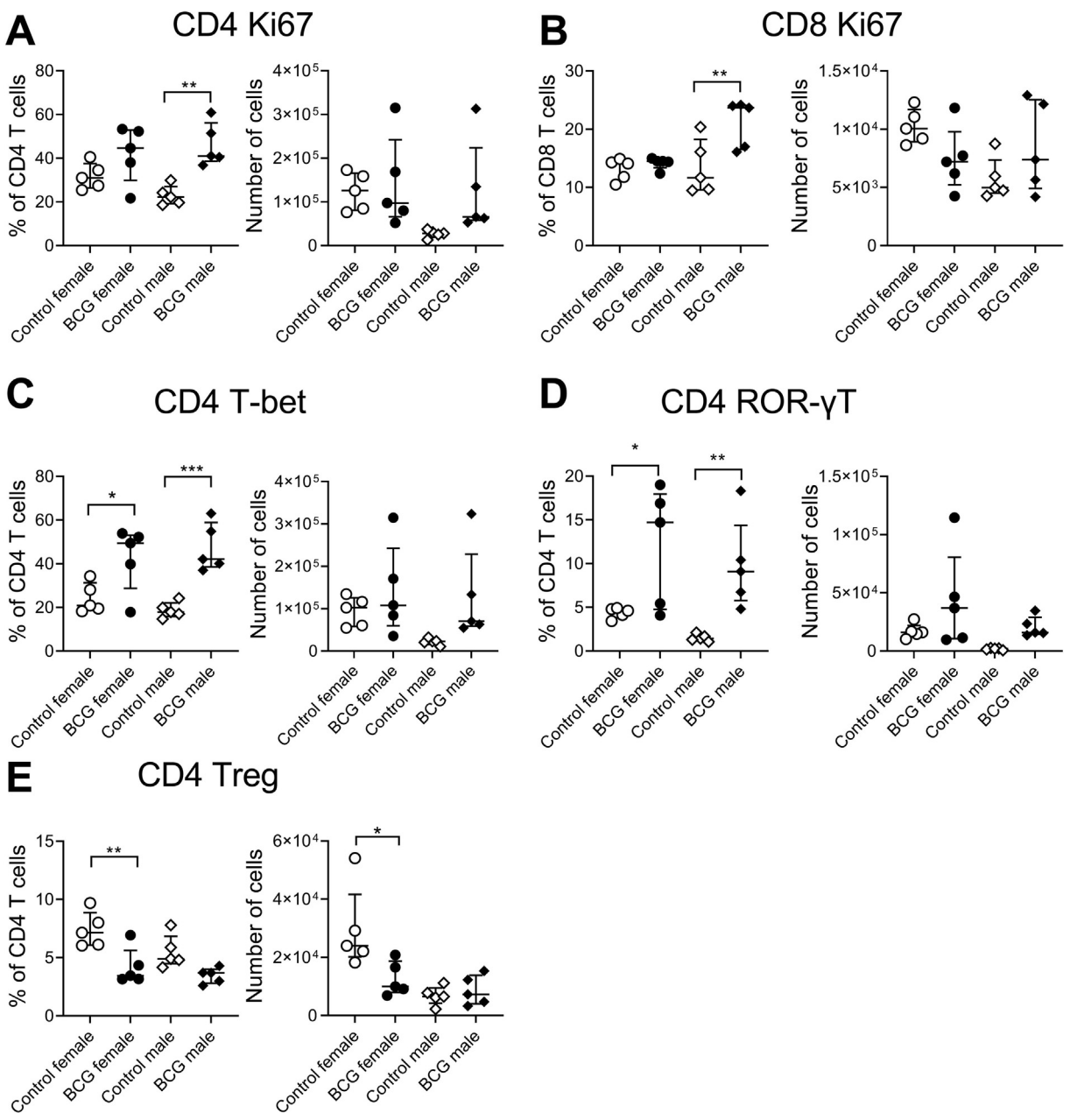

Fig. 6. Phenotype of T cells in the lungs of BCG-vaccinated and unvaccinated control mice at day 18. Mice were vaccinated with 1 × 10E6 BCG and challenged by aerosol at day 60 post vaccination with 20–50 CFUs Mtb H37Rv. At day 18, lungs were harvested and cells were isolated by digestion with collagenase and DNAse. Lung cell populations were analysed by flow cytometry. A) Ki67 expressing CD4 T cells. B) Ki67 expressing CD8 T cells. C) T-bet expressing CD4 T cells. D) ROR-γT expressing CD4 T cells. E) Regulatory T cells (Treg) ($CD3^+CD4^+CD25^+Foxp3^+$). Statistical significance was calculated using linear modelling with contrast set within sex group and between groups and correction for multiple testing was performed. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Representative of two experiments, n = 4–5 per group per experiment.

excessive cytokine production and recruitment of neutrophils, modulating tissue damage.

Overall, numbers of T cells were lower in males than in females, which is in line with the known effects of androgens on reducing T cell responses. Many studies have shown that healthy human women have higher $CD4^+$ T cell counts than age-matched men, and higher CD4/CD8 ratios [12]. In our study, we did not see differences in CD4/CD8 ratios between males and females. The role for Th1 cells, driven by T-bet, and Th17 cells, programmed by ROR-γT, in controlling *Mtb* infection is well-established. Both male and female BCG-vaccinated mice had increased percentages of T-bet^+ and ROR-γT^+ CD4 T cells and reduced percentages of T_{reg} cells, compared to unvaccinated mice. However, only males had increased percentages of CD4 and CD8 T cells bearing the proliferation marker Ki-67. T_{reg} cells can downregulate T cell responses, but also assist in controlling excessive inflammation. Human studies indicate higher numbers of T_{reg} cells in males than in females, but studies in mice show conflicting results, with difference patterns depending upon the disease and tissue studied [12]. Females also tend to have higher antibody levels than males in response to vaccination or infection [12,40]. However, in our model, both BCG-vaccinated and unvaccinated males and females had similar levels of mycobacteria-specific IgG after *Mtb* challenge.

To obtain an unbiased view of our data, we performed PCA analysis and hierarchical clustering. In the PCA analysis, the timepoint, vaccination status and mouse sex had the biggest effect on mouse grouping. The features that were most influential on the grouping of the mice tended to be the CD4 and CD8 T cell subsets, with CD4

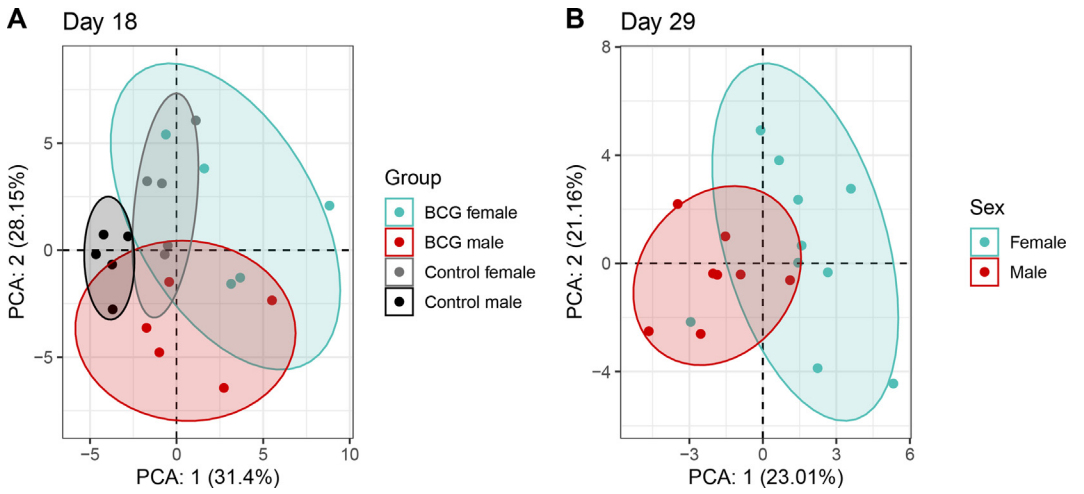

Fig. 7. Principal Component Analysis for all measurements collected at two different timepoints. A) Components projection at day 18, where colours represents different mouse sex and vaccination status. B) Components projection at day 29 only for BCG vaccinated mice, where colour represents mouse sex. In both panels, ellipses represent a 95% radius for multivariate t-distribution of each group, and the variance explanation of each component is presented in the axis description.

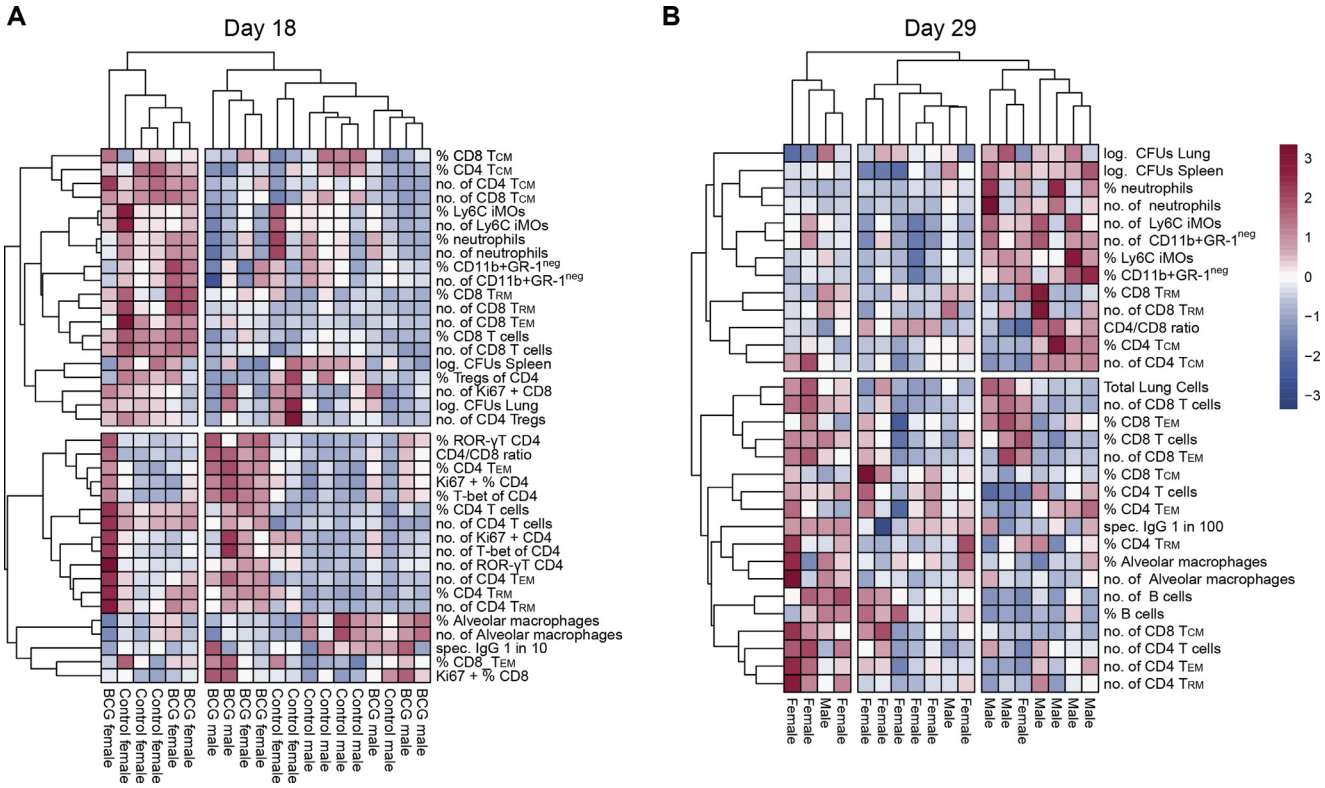

Fig. 8. Hierarchical clustering for all measurements collected at two different timepoints. A) HC day 18 on unvaccinated control and BCG-vaccinated mice with different sex. B) HC at day 29 performed only on BCG-vaccinated mice. In both panels, the x axis represents mice, while the y axis shows particular measured features. Red colour represents a high value for a particular feature in a group of investigated mice, while blue colour represents the opposite (normalized row-wise). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and CD8 T_{CM} and T_{RM} featuring prominently. This echoes the study from our group in which the T cell response was the major discriminator between resistant and susceptible mouse strains [28]. At day 29, myeloid cells also emerged as important features impacting the grouping of mice in the PCA. The hierarchical clustering provided additional insights. At day 18 mice tended to cluster predominantly according to sex and then according to vaccination status, suggesting sex specific responses to both *Mtb* and BCG. At day 29, myeloid cell responses had a strong influence on clustering.

Although bacterial loads, weight loss and symptom scores were similar in unvaccinated male and female mice, there were some trends to differences between males and females irrespective of BCG vaccination, for example, trends in males towards higher MTRs, higher numbers of alveolar macrophage but lower numbers of T_{RM} cells, proliferating ($Ki67^+$) T cells, and T-bet or ROR-γT^+ CD4 T cells. Thus, we cannot exclude some impact of sex on susceptibility to TB on the outcome of BCG vaccination. The overall outcome of vaccination will always depend on both susceptibility to infec-

tion and efficacy of the vaccine (and the impact of sex thereon). By using linear modelling, we could dissect differences in response to BCG between the sexes to some extent. There were significant differences in BCG response for symptom scores, % CD8 T_{EM} cells and % CD8 T_{CM} cells between male and female mice. Interestingly, the theme of CD8 T cells being important in sex-driven differences in vaccination outcome reoccurred in the PCA analysis and hierarchical analysis.

The preference for using female or male mice varies widely between research areas [41]. A survey of journal articles found that immunology studies favoured female mice [41]. Strikingly, the percentage of articles in which some portion of the results was analysed by sex was lowest in the field of immunology, and in the majority of immunology articles, mouse sex was not specified. Female mice may be favoured for long-term experiments as males are considered more likely to fight [42]. However, aggression in mice varies between strains and can be reduced by housing littermates together and optimising environmental conditions [42]. In some fields, male mice are favoured to avoid variability caused by the female hormonal cycle [41]. In the field of biology as a whole, females are underrepresented in both preclinical and clinical studies, which may negatively impact the medical care of women [41,43]. Underrepresenting male mice in immunology studies could have a similar effect on the medical care of men. As preclinical studies are the entry point for the clinical trial pipeline for novel drug and vaccine candidates, the NIH has recommended consideration of sex as a biological variable to improve translation of results into the clinical setting [14].

Although the difference in the prevalence of TB among men and women has been well documented, there is surprisingly little information available about sex-specific responses to BCG vaccination. The protective efficacy of BCG against TB was reported to be higher among females than males (78.6% vs. 65.8%) in a study from South Korea [44]. A study in North America showed similar results, with efficacy being slightly higher among women than men (79% vs 68%) [45]. Non-specific effects of BCG vaccination on overall neonatal mortality and the incidence of respiratory diseases were found to be sex-dependent [46–48]. In the first week after vaccination, a stronger protective effect on mortality was observed in boys [46], but in the long term the non-specific protective effect of BCG vaccination was stronger in girls [46–48].

Although not many clinical trials of BCG efficacy against *Mtb* have compared responses in males and females, sex differences have been examined using mycobacterial growth inhibition assays (MGIA), which were developed to assess vaccine immunogenicity *ex vivo* [49]. In these experiments, peripheral blood mononuclear cells (PBMCs) from BCG-vaccinated females co-cultured with mycobacteria controlled mycobacterial growth better than PBMCs from BCG-vaccinated males. BCG-vaccinated females also had a higher frequency of cytokine-producing NK cells and a higher CD4/CD8 ratio compared to males, while males had a higher frequency of monocytes and a trend towards a higher MLR. Another study investigated the effect of sex on modulation of inflammatory responses by the BCG vaccine [50]. Pre-vaccination levels of multiple inflammatory markers were significantly higher in males than in females, and BCG vaccination had a stronger effect on dampening this systemic inflammation in males than in females. In this study, no changes in the lymphocyte or monocyte percentages among PBMCs were observed.

In summary, female 129 S2 mice were better protected than males by BCG vaccination against TB. Our data strongly suggests that in pre-clinical trials, novel vaccine candidates should be tested in both males and females, to generate more representative results that encompass sex-specific differences in immune responses and vaccine efficacy. In clinical trials, stratified analysis of vaccine efficacy should be carried out in order to determine potential differences between men and women. It is possible that in some cases different vaccination strategies or even different vaccines may be optimal for each sex.

Data statement

Data is available upon request.

Funding

This study was funded by the Max Planck Society and the German Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung [BMBF]) "inVAC" (grant no. 03ZZ0806A) and DIAT ((grant no. 03ZZ0827B) to S.H.E.K. and N.E. N., and the Silesian University of Technology grant for Support and Development of Research Potential to J.Z.

CRediT authorship contribution statement

Natalie E. Nieuwenhuizen: Conceptualization, Formal analysis, Investigation, Writing - original draft. **Joanna Zyla:** Formal analysis, Writing - original draft, Writing – review and editing. **Ulrike Zedler:** Investigation. **Silke Bandermann:** Investigation. **Ulrike Abu Abed:** Formal analysis. **Volker Brinkmann:** Formal analysis. **Stefan H.E. Kaufmann:** Conceptualization, Funding acquisition, Writing - original draft.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Stefan Kaufmann is co-inventor and co-holder of a patent for the TB vaccine VPM1002 which has been licensed to Vakzine Projekt Management GmbH, Hannover and sublicensed to Serum Institute of India Ltd., Pune, India].

Acknowledgements

We thank Dr Gesa Rausch and the mouse facility staff for support with the animal caretaking, and Souraya Sibaei and Olaf Kostbahn for administrative support.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2021.09.039.

References

[1] Lange C. A. P., Behr M.A., Donald P., Kaufmann S.H.E., Netea M.G., Mandalakas A.M. 100 years of Mycobacterium bovis bacille Calmette Guérin: similia similibus curentur. Lancet Infect Dis. 2021 Sep 7;S1473–3099(21)00403–5.
[2] Calmette A. Preventive vaccination against tuberculosis with BCG. Proc R Soc Med 1931;24(11):1481–90.
[3] Mangtani P, Abubakar I, Ariti C, Beynon R, Pimpin L, Fine PE, et al. Protection by BCG vaccine against tuberculosis: a systematic review of randomized controlled trials. Clin Infect Dis 2014;58:470–80.
[4] Roy A, Eisenhut M, Harris RJ, Rodrigues LC, Sridhar S, Habermann S, et al. Effect of BCG vaccination against Mycobacterium tuberculosis infection in children: systematic review and meta-analysis. BMJ 2014;349.
[5] Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, et al. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA 1994;271:698–702.
[6] World Health Organisation. WHO global tuberculosis report 2020. Geneva, Switzerland; 2020.
[7] Smith CM, Proulx MK, Olive AJ, Laddy D, Mishra BB, Moss C, et al. Tuberculosis Susceptibility and Vaccine Protection Are Independently Controlled by Host Genotype. mBio 2016;7.

[8] Horton KC, MacPherson P, Houben RM, White RG, Corbett EL. Sex Differences in Tuberculosis Burden and Notifications in Low- and Middle-Income Countries: A Systematic Review and Meta-analysis. PLoS Med 2016;13.
[9] Neyrolles O, Quintana-Murci L. Sexual inequality in tuberculosis. PLoS Med 2009;6.
[10] Nhamoyebonde S, Leslie A. Biological differences between the sexes and susceptibility to tuberculosis. J Infect Dis 2014;209(suppl 3):S100–6.
[11] Fish EN. The X-files in immunity: sex-based differences predispose immune responses. Nat Rev Immunol 2008;8(9):737–44.
[12] Klein SL, Flanagan KL. Sex differences in immune responses. Nat Rev Immunol 2016;16(10):626–38.
[13] Holmes CB, Hausler H, Nunn P. A review of sex differences in the epidemiology of tuberculosis. Int J Tuberc Lung Dis 1998;2:96–104.
[14] Miller LR, Marks C, Becker JB, Hurn PD, Chen W-J, Woodruff T, et al. Considering sex as a biological variable in preclinical research. FASEB J 2017;31(1):29–34.
[15] Roden AC, Moser MT, Tri SD, Mercader M, Kuntz SM, Dong H, et al. Augmentation of T cell levels and responses induced by androgen deprivation. J Immunol 2004;173(10):6098–108.
[16] Arnold AP. Y chromosome's roles in sex differences in disease. Proc Natl Acad Sci U S A 2017;114(15):3787–9.
[17] Krementsov DN, Case LK, Dienz O, Raza A, Fang Q, Ather JL, et al. Genetic variation in chromosome Y regulates susceptibility to influenza A virus infection. Proc Natl Acad Sci U S A 2017;114(13):3491–6.
[18] Dibbern J, Eggers L, Schneider BE. Sex differences in the C57BL/6 model of Mycobacterium tuberculosis infection. Sci Rep 2017;7:10957.
[19] Bini EI, Mata Espinosa D, Marquina Castillo B, Barrios Payan J, Colucci D, Cruz AF, et al. The influence of sex steroid hormones in the immunopathology of experimental pulmonary tuberculosis. PLoS One 2014;9.
[20] Yamamoto Y, Saito H, Setogawa T, Tomioka H. Sex differences in host resistance to Mycobacterium marinum infection in mice. Infect Immun 1991;59(11):4089–96.
[21] Tsuyuguchi K, Suzuki K, Matsumoto H, Tanaka E, Amitani R, Kuze F. Effect of oestrogen on Mycobacterium avium complex pulmonary infection in mice. Clin Exp Immunol 2001;123:428–34.
[22] Brown IN, Glynn AA. The Ity/Lsh/Bcg gene significantly affects mouse resistance to Mycobacterium lepraemurium. Immunology 1987;62:587–91.
[23] Hertz D, Dibbern J, Eggers L, von Borstel L, Schneider BE. Increased male susceptibility to Mycobacterium tuberculosis infection is associated with smaller B cell follicles in the lungs. Sci Rep 2020;10:5142.
[24] Medina E, North RJ. Resistance ranking of some common inbred mouse strains to Mycobacterium tuberculosis and relationship to major histocompatibility complex haplotype and Nramp1 genotype. Immunology 1998;93:270–4.
[25] Dorhoi A, Yeremeev V, Nouailles G, Weiner J, Jörg S, Heinemann E, et al. Type I IFN signaling triggers immunopathology in tuberculosis-susceptible mice by modulating lung phagocyte dynamics. Eur J Immunol 2014;44(8):2380–93.
[26] Berry MPR, Graham CM, McNab FW, Xu Z, Bloch SAA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature 2010;466(7309):973–7.
[27] Maertzdorf J, Weiner J, Mollenkopf H-J, Network T, Bauer T, Prasse A, et al. Common patterns and disease-related signatures in tuberculosis and sarcoidosis. Proc Natl Acad Sci U S A 2012;109(20):7853–8.
[28] Domaszewska T, Scheuermann L, Hahnke K, Mollenkopf H, Dorhoi A, Kaufmann SHE, et al. Sci Rep 2017;7:12094.
[29] Gengenbacher M, Nieuwenhuizen NE, Kaufmann SHE. BCG - old workhorse, new skills. Curr Opin Immunol 2017;47:8–16.
[30] Osborn JF, Hobbs SJ, Mooster JL, Khan TN, Kilgore AM, Harbour JC, et al. Central memory CD8+ T cells become CD69+ tissue-residents during viral skin infection independent of CD62L-mediated lymph node surveillance. PLoS Pathog 2019;15.
[31] Takamura S, Kohlmeier JE. Establishment and Maintenance of Conventional and Circulation-Driven Lung-Resident Memory CD8(+) T Cells Following Respiratory Virus Infections. Front Immunol 2019;10:733.
[32] Ogongo P, Porterfield JZ, Leslie A. Lung Tissue Resident Memory T-Cells in the Immune Response to Mycobacterium tuberculosis. Front Immunol 2019;10:992.
[33] Zelmer A, Stockdale L, Prabowo SA, Cia F, Spink N, Gibb M, et al. High monocyte to lymphocyte ratio is associated with impaired protection after subcutaneous administration of BCG in a mouse model of tuberculosis. F1000Res 2018;7:296.
[34] Srivastava S, Ernst JD, Desvignes L. Beyond macrophages: the diversity of mononuclear cells in tuberculosis. Immunol Rev 2014;262(1):179–92.
[35] Naranbhai V, Hill AV, Abdool Karim SS, Naidoo K, Abdool Karim Q, et al. Ratio of monocytes to lymphocytes in peripheral blood identifies adults at risk of incident tuberculosis among HIV-infected adults initiating antiretroviral therapy. J Infect Dis 2014;209:500–9.
[36] Billahalli T, Ward RJB, Barnes KE, Marshall NJ, Spong VC, Bothamley GH. The monocyte-to-lymphocyte ratio: defining a normal range, sex-specific differences in the tuberculosis disease spectrum and diagnostic indices. In: medRxiv 2021 Plos One 2021 Aug 30;16(8):e0247745. https://doi.org/10.1371/journal.pone.0247745. eCollection.
[37] Anderson KG, Mayer-Barber K, Sung H, Beura L, James BR, Taylor JJ, et al. Intravascular staining for discrimination of vascular and tissue leukocytes. Nat Protoc 2014;9(1):209–22.
[38] Topham DJ, Reilly EC. Tissue-Resident Memory CD8(+) T Cells: From Phenotype to Function. Front Immunol 2018;9:515.
[39] Scotland RS, Stables MJ, Madalli S, Watson P, Gilroy DW. Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. Blood 2011;118:5918–27.
[40] Fischinger S, Boudreau CM, Butler AL, Streeck H, Alter G. Sex differences in vaccine-induced humoral immunity. Semin Immunopathol 2019;41(2):239–49.
[41] Beery AK, Zucker I. Sex bias in neuroscience and biomedical research. Neurosci Biobehav Rev 2011;35(3):565–72.
[42] Lidster K, Owen K, Browne WJ, Prescott MJ. Cage aggression in group-housed laboratory male mice: an international data crowdsourcing project. Sci Rep 2019;9:15211.
[43] Wald C, Wu C. Biomedical research. Of mice and women: the bias in animal models. Science 2010;327(5973):1571–2.
[44] Kang PS, Lee SK. A study on the effects of BCG Vaccination against Tuberculosis. Korean J Prev Med 1982;15:33–46.
[45] Stein SC, Aronson JD. The occurrence of pulmonary lesions in BCG-vaccinated and unvaccinated persons. Am Rev Tuberc 1953;68:695–712.
[46] Biering-Sorensen S, Jensen KJ, Monterio I, Ravn H, Aaby P, Benn CS. Rapid protective effects of early BCG on neonatal mortality among low birth weight boys: observations from randomized trials. J Infect Dis 2018;217:759–66.
[47] Roth A, Gustafson P, Nhaga A, Djana Q, Poulsen A, Garly ML, et al. BCG vaccination scar associated with better childhood survival in Guinea-Bissau. Int J Epidemiol 2005;34:540–7.
[48] Aaby P, Vessari H, Nielsen J, Maleta K, Benn CS, Jensen H, et al. Sex differential effects of routine immunizations and childhood survival in rural Malawi. Pediatr Infect Dis J 2006;25:721–7.
[49] Prabowo SA, Smith SG, Seifert K, Fletcher HA. Impact of individual-level factors on Ex vivo mycobacterial growth inhibition: Associations of immune cell phenotype, cytomegalovirus-specific response and sex with immunity following BCG vaccination in humans. Tuberculosis (Edinb) 2019;119.
[50] Koeken VA, de Bree LCJ, Mourits VP, Moorlag SJ, Walk J, Cirovic B, et al. BCG vaccination in humans inhibits systemic inflammation in a sex-dependent manner. J Clin Invest 2020;130:5591–602.