

Title: Mycobacterial exposure remodels alveolar macrophages and the early innate response to *Mycobacterium tuberculosis* infection

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Keywords:

innate immunity, alveolar macrophages, *Mycobacterium tuberculosis*, lung, vaccination, bacterial infection, host-pathogen interaction

1 **Abstract**

2 As innate sentinels in the lung, alveolar macrophages (AMs) play a critical role during
3 *Mycobacterium tuberculosis* (Mtb) infection as the first cells to encounter bacteria. We
4 previously showed that AMs initially respond to Mtb infection *in vivo* by mounting a cell-
5 protective, rather than pro-inflammatory response, yet whether the AM response could be
6 modified by environmental factors was unknown. Here, we characterize how previous exposure
7 to mycobacteria, either through subcutaneous vaccination with *Mycobacterium bovis* (scBCG)
8 or through a contained Mtb infection (coMtb), impacts the initial response by AMs and early
9 innate response in the lung. We find that both scBCG and coMtb accelerate early innate cell
10 activation and recruitment and generate a stronger pro-inflammatory AM response to Mtb *in*
11 *vivo*. AMs from scBCG vaccinated mice mount a robust interferon response, while AMs from
12 coMtb mice produce a broader and more diverse inflammatory response. Using single-cell RNA-
13 sequencing, we identify exposure-induced changes to airway-resident cells, with scBCG and
14 coMtb enriching for different AM subpopulations. *Ex vivo* stimulation assays reveal that AMs
15 from scBCG and coMtb mice switch on an interferon-dependent response, which is absent in
16 AMs from unexposed mice. Overall, our studies reveal significant, durable, and cell-intrinsic
17 modifications to AMs following exposure to *mycobacterium*, and comparison of scBCG and
18 coMtb models reveals that AMs can be reprogrammed into more than one state. These findings
19 highlight the plasticity of innate responses in the airway and underscore the unexplored
20 potential of targeting AMs through vaccination or host-directed therapy to augment host
21 responses.

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27 Introduction

28 *Mycobacterium tuberculosis* (Mtb), the causative agent of Tuberculosis (TB), leads to
29 more than 1.5 million deaths each year and, for the first time since 2005, the number of TB
30 deaths worldwide is increasing^{1,2}. These trends highlight the urgent need for new vaccination
31 strategies. Traditionally, vaccine design has focused on generating a rapid, robust, and effective
32 adaptive immune response. However, recent studies suggest that the innate immune system
33 can undergo long-term changes in the form of trained immunity³, which affect the outcome of
34 infection and may be important components of an effective TB vaccine^{4,5}. Initial studies focused
35 primarily on central trained immunity, long-term changes to hematopoietic stem cells that lead to
36 functional changes in short-lived innate cell compartments (i.e., monocytes, NK cells, dendritic
37 cells)³. In the context of TB, exposure to BCG and Mtb were found to have opposing effects on
38 central myelopoiesis: BCG vaccination augments myelopoiesis while Mtb infection limits it^{6,7}.
39 More recent studies have examined innate training in tissue-resident macrophages and
40 demonstrated that these cells can respond to remote injury and inflammation⁸, undergo long-
41 term changes³, and display altered responses to bacteria after pulmonary viral infection^{9,10,11}.

42 Lung resident alveolar macrophages (AMs) are the first cells to become infected with
43 inhaled Mtb and engage a cell-protective response, mediated by the transcription factor Nrf2,
44 that impedes their ability to control bacterial growth^{12,13}. In this study, we examined how prior
45 mycobacterial exposure reprograms AMs and alters the overall innate response in the lung to
46 aerosol challenge with Mtb. We chose to compare the effects of subcutaneous BCG vaccination
47 (scBCG) with those arising from a contained Mtb-infection model (coMtb) as these represent
48 two of the most common mycobacterial exposures in humans, and both exposures are
49 associated with changes to innate immunity. In mice with an established contained but
50 persistent lymph node Mtb infection, generated by intradermal inoculation, the AM response to
51 inhaled Mtb is more inflammatory and bacterial growth over the course of disease is restricted
52 compared to naïve mice^{14,15}. CoMtb also protects mice against heterologous challenges,

53 including infection with *Listeria monocytogenes* and expansion of B16 melanoma cells,
54 suggesting substantial remodeling of innate immune responses¹⁵. BCG, a live-attenuated TB
55 vaccine derived from *M. bovis*, provides protection against disseminated pediatric disease but
56 has lower efficiency against adult pulmonary disease^{16, 17}. In addition to enhancement of Mtb-
57 specific adaptive responses, based on shared antigens, BCG vaccination also leads to changes
58 in hematopoiesis and epigenetic reprogramming of myeloid cells in the bone marrow⁷, early
59 monocyte recruitment and Mtb dissemination¹⁸, and innate activation of dendritic cells critical for
60 T cell priming¹⁹. Intranasal BCG vaccination protects against *Streptococcus pneumoniae* and
61 induces long term activation of AMs²⁰. BCG vaccination is also associated with trained immunity
62 effects in humans^{21, 22, 23}, including well-described reductions in all-cause neonatal mortality and
63 protection against bladder cancer^{3, 24}.

64 Here, we show that while both coMtb and scBCG protect against low dose Mtb aerosol
65 challenge, they remodel the innate response in different ways. In AMs, scBCG enables a largely
66 interferon response to infection, while coMtb promotes a broader pro-inflammatory response.
67 Prior mycobacteria exposure also generates altered immune cellularity in the lung prior to
68 aerosol challenge and significant changes in the early dynamics of the overall innate response.
69 The observation that the innate response to Mtb, including intrinsic effects of AMs, can be
70 reprogrammed in multiple directions highlights the plasticity of tissue resident cells and suggests
71 opportunities to modulate their function through vaccination or host-directed therapies.

72

73 **Results**

74 **Prior mycobacterial exposure accelerates activation and innate cell recruitment that is** 75 **associated with protection from Mtb infection**

76 We first determined the earliest stage of infection when the immune response was
77 altered by prior exposure to mycobacteria. Mice were vaccinated with scBCG (1×10^6 *M. bovis*
78 BCG, Pasteur strain) or treated with coMtb (1×10^4 H37Rv, delivered intradermally in the ear),

79 rested for 8 weeks, and then challenged with low-dose (~100 CFU) H37Rv aerosol infection.
80 We measured both the cellularity and activation of innate immune cells in the lung at 10, 12 and
81 14 days following infection, the earliest timepoints when innate cells are known to be recruited¹²,
82 ^{13, 25}. We observed a significant increase in MHC II Median Fluorescence Intensity (MFI) as
83 early as day 10 for AMs from coMtb mice and day 12 for AMs from scBCG mice compared to
84 controls (**Fig 1A, S1**). There were no significant differences in MHC II expression prior to
85 challenge on day 0 (**Fig 1A**). There were significant increases in the numbers of monocyte-
86 derived macrophages (MDM), neutrophils (PMN), dendritic cells, Ly6C⁺ CD11b⁺ monocytes, and
87 Ly6C⁺ CD11b⁻ monocytes by day 10 in coMtb mice compared to controls, with further increases
88 by days 12 and 14 (**Fig 1B, Fig S1**). scBCG elicited similar increases in these populations
89 starting at day 10, but the increases were not as robust or rapid as those observed in the coMtb.
90 Significant differences between scBCG and coMtb groups were found at d10, d12, d14 in MDM,
91 d14 in PMN, d12, d14 in dendritic cells, and d14 in Ly6C⁺ CD11b⁺ monocytes (**Fig 1B**,
92 designated with +).

93 In addition to early changes in innate cell activation and recruitment, we observed earlier
94 recruitment of activated CD44⁺ CD4⁺ and CD8⁺ T cells in both coMtb and scBCG mice starting
95 at day 10 as well as TB antigen-specific T cells, ESAT6-tetramer⁺ CD4⁺ T cells and TB10.4-
96 tetramer⁺ CD8⁺ T cells in coMtb mice starting at day 10 compared to controls and to scBCG
97 mice (**Fig 1C, Fig S1**). The differences in the recruitment of ESAT6-tetramer⁺ CD4⁺ T cells
98 between scBCG and coMtb were expected, as the ESAT6 antigen is expressed by H37Rv but
99 not BCG.

100 We also evaluated whether these cell recruitment differences correlated with changes in
101 bacterial burden. We found that both modalities generated a significant reduction in bacterial
102 burden in the lung, spleen, and lung-draining lymph node (LN) at day 14 and at day 28, as has
103 been previously reported^{15, 18, 26} (**Fig S2A-D**). Interestingly, we also observed a significant
104 reduction in bacterial burden in the lung by day 12 in coMtb but not scBCG mice and significant

105 reduction in CFU in the LN in both models compared to controls, at a time when we first
106 detected innate cell recruitment to the lungs of coMtb mice (**Fig S2B**). The majority of control
107 mice had undetectable bacteria in spleen and LN at day 10 and there was no reduction in
108 bacterial burden in the lung at that time. Our results demonstrate that prior mycobacteria
109 exposure leads to accelerated innate activation and innate cell recruitment within the first two
110 weeks of infection, with coMtb generating a faster and more robust response compared to
111 scBCG. These early immune changes are associated with early reductions in bacterial load in
112 the lung, while reductions in bacterial burden in the LN and spleen suggest delays in bacterial
113 dissemination.

114

115 ***Mycobacterium* exposure alters the *in vivo* alveolar macrophage response to Mtb**

116 **infection**

117 To examine the earliest response to Mtb, we measured the gene expression profiles of
118 Mtb-infected AMs 24 hours following aerosol challenge with high dose mEmerald-H37Rv
119 (depositions: 4667, 4800) in scBCG-vaccinated mice and compared these measurements to
120 previously generated profiles of AMs from control (unexposed) mice¹² and coMtb mice¹⁵ (**Table**
121 **S1**). Mtb-infection induced distinct expression changes for coMtb, scBCG, and control AMs as
122 measured by Principal Component Analysis (**Fig 2A**) and the majority of up-regulated
123 Differentially Expressed Genes (DEG) (fold change > 2, FDR < 0.05) were unique to each
124 condition (control: 151 unique genes/257 total genes, scBCG: 222/289, coMtb: 156/229) (**Fig**
125 **2B**). The divergence in the responses of Mtb-infected AMs from each of the 3 conditions was
126 also reflected in the diversity in the Top 20 Canonical Pathways identified by Ingenuity Pathway
127 Analysis (**Fig S3**).

128 To identify trends between groups, we performed Gene Set Enrichment Analysis using a
129 set of 50 Hallmark Pathways. As we've shown previously, Mtb-infected AMs from control mice
130 at 24 hours had strong enrichment for "Xenobiotic Metabolism" and "Reactive Oxygen Species"

131 pathways, indicative of the Nrf2-associated cell-protective response (**Fig 2C**). While these two
132 pathways were not among most enriched pathways in the exposed groups, Mtb-infected AMs
133 from all groups upregulated genes associated with the cell-protective Nrf2-driven response¹²
134 (**Fig 2D**). Expression profiles for Mtb-infected AMs from scBCG mice showed the strongest
135 enrichment for “Interferon Alpha Response” and “Interferon Gamma Response” pathways,
136 which contain many shared genes (**Fig 2C**). The strength of the interferon response in these
137 AMs was further highlighted by examining gene expression changes in a set of Interferon
138 Stimulated Genes (ISGs) identified from macrophages responding to IFN α (fold change > 2, p-
139 value < 0.01)²⁷ (**Fig 2D**). Expression profiles for Mtb-infected AMs from coMtb mice showed a
140 weaker enrichment for interferon response pathways with fewer up-regulated ISGs compared to
141 scBCG, and instead showed enrichment across a number of inflammatory pathways including
142 “IL6 JAK STAT3 signaling” in comparison to the other groups (**Fig 2C, D**).

143 In summary, mycobacteria exposures alter the initial *in vivo* response of AMs to Mtb
144 infection 24 hours after challenge and remodel the AM response in different ways. AMs from
145 scBCG vaccinated animals mount a robust interferon associated response, while AMs from
146 coMtb mice generate a more expansive inflammatory program across multiple pathways in
147 response to infection.

148

149 ***Mycobacterium* exposure modifies the baseline phenotype of alveolar macrophages in** 150 **the airway**

151 Although scBCG and coMtb mice potentiate significantly altered AM responses to Mtb
152 infection *in vivo*, these effects are not widely evident in the transcriptomes prior to infection as
153 measured by population-level RNA-sequencing (**Fig S4**). However, we posited that remodeling
154 effects were likely not homogenous across the entire AM population and that small
155 heterogenous changes to baseline profiles might be detectable using a single cell approach. We
156 therefore analyzed pooled BAL samples taken from 10 age- and sex-matched mice from each

157 of the three conditions (control, scBCG, coMtb) eight weeks following mycobacteria exposure by
158 single cell RNA-sequencing (scRNAseq). Gross cellularity was unaffected by mycobacterial
159 exposure as measured by flow cytometry analysis of common lineage markers with AMs the
160 dominant hematopoietic cell type (57.4-85.8% of CD45⁺ live cells), followed by lymphocytes
161 (5.26-22.7% of CD45⁺ live cells) with small contributions from other innate cell populations (**Fig**
162 **S5**).

163 Six samples, with an average of 2,709 cells per sample (range: 2,117-4,232), were
164 analyzed together for a total of 17,788 genes detected. The most prominent expression cluster
165 mapped to an AM profile, with smaller clusters mapping to T and B lymphocytes, dendritic cells,
166 and neutrophils (**Fig 3A**). All cells that mapped to a macrophage profile were extracted and
167 reclustered into 11 macrophage subclusters (**Fig 3B, C**). All but one of the macrophage
168 subclusters expressed AM lineage markers (*Siglecf*, *Mertk*, *Fcgr1* (CD64), *Lyz2* (LysM), and
169 *Itgax* (CD11c) and had low expression of *Itgam* (CD11b) (**Fig 3D**). The one exception was
170 cluster 6 that showed high *Itgam* and *Lyz2* expression and lower *Siglecf* expression, likely
171 representing a small monocyte-derived macrophage population in the airway.

172 To interpret the various expression subclusters, we identified the genes that most
173 distinguished each cluster from the others (**Fig S6, Table S2**). As has been reported by other
174 groups^{28, 29}, a small proportion of the AMs formed two clusters (4, 10) with high expression of
175 cell cycle genes (i.e., *Top2a*, *Mki67*), indicative of cell proliferation (**Fig 3E, Table S2**). Cluster 0
176 formed the most abundant macrophage cluster with high expression of lipid metabolism genes
177 (i.e., *Abcg1*, *Fabp1*) and with a trend of slightly decreased relative frequency in scBCG and
178 coMtb samples compared to controls (**Fig 3F, Table S2**). Cluster 2 was significantly increased
179 in relative frequency for scBCG samples compared to coMtb ($p = 0.032$, *One-way ANOVA with*
180 *Tukey post-test*) and associated with oxidative stress response genes (*Hmox1*, *Gclm*) (**Fig 3G,**
181 **Table S2**). Cluster 7 was the only cluster with an increase in relative frequency trending for both
182 scBCG and coMtb ($p = 0.076$, *One-way ANOVA*). AMs in this cluster had high expression of

183 Interferon Stimulated Genes (**Fig 3H, Table S2**). Cluster 3 had significantly higher relative
184 frequency for coMtb samples compared to control and scBCG samples ($p = 0.021, 0.039$,
185 respectively, *One-way ANOVA with Tukey post-test*) and was distinguished by expression of the
186 macrophage-associated transcription factors (*Cebpb, Zeb2, Bhlhe40*)^{30, 31}, hemoglobin
187 metabolism (*Hba-a1, Hba-a2, Hbb-bs*), mitochondrial oxidative phosphorylation (*mt-Co1, mt-*
188 *Cytb, mt-Nd2*), chromatin remodeling (*Ankrd11, Baz1a*), and immune signaling including the
189 CARD9 complex (*Malt1, Spag9, Bcl10, Prkcd*) (**Fig 3I, S7, Table S2**). This expression profile
190 closely matches a subcluster of AMs previously described by Pisu et al, as an “interstitial
191 macrophage-like” AM population (labeled “AM_2”) that expanded in relative frequency in lung
192 samples 3 weeks following low-dose H37Rv infection²⁸. The changes in the baseline
193 phenotypes revealed by scRNAseq correspond to differences observed in the AM *in vivo*
194 response (**Fig 2**), with scBCG driving AMs towards an interferon response and coMtb shifting
195 AMs towards a pro-inflammatory response with more diverse qualities.

196 Interestingly, Cluster 2 (higher relative frequency in scBCG) and Cluster 3 (higher
197 relative frequency in coMtb) represent divergent endpoints of a pseudotime plot generated by
198 performing a trajectory inference analysis, regardless of whether the starting point is the most
199 abundant cluster in the control group (Cluster 0) (**Fig 3J, top**) or the cluster of proliferating cells
200 (Cluster 4) (**Fig 3J, bottom**). This result suggests that scBCG and coMtb drive AM phenotypes
201 in different directions and may indicate the possibility of driving different flavors of an innate
202 tissue-resident response, rather than flipping an “on/off” switch.

203

204 ***Mycobacterium* exposure modifies T cell populations in the airway**

205 While AMs are the dominant immune cell type in the airway, other cell populations make
206 up an average of 18.4% of the cells within the BAL in controls (range: 10.4-26.3%) and 31.3% in
207 exposed groups (range: 14.0-48.8%). To determine how mycobacteria exposure influenced
208 other cells in the airway, we focused on T cells and dendritic cells (DCs) which have the two

209 highest relative frequencies after AMs (**Fig 4A, B**). As expected, the overall frequency of T cells
210 in BAL was increased following exposure (**Fig 4B**). To examine the alterations in T cell
211 cellularity in detail, we combined and reclustered the two original T cell clusters into 7, which
212 were manually annotated using the closest Immgen profiles and the expression of key lineage
213 specific markers (**Fig 4A-C, Fig S8**). We focused specifically on the 5 most abundant T cell
214 subclusters (Clusters 0-4). While we observed subtle shifts in the relative frequency of each
215 group, none reached statistical significance. Cluster 0, the most abundant cluster, had an
216 expression profile most consistent with $\gamma\delta$ T cells, including expression of *Cd3e* with low to nil
217 *Cd4* and *Cd8a* and some expression of *Zbtb16* (PLZF) and *Tmem176a*, an ion channel
218 regulated by ROR γ t and reported to be expressed by lung $\gamma\delta$ T cells^{32, 33} (**Fig 4D-F, S8**). Cluster
219 1, consistent with a profile for effector CD4⁺ T cells, had slightly higher relative frequency in
220 scBCG samples (**Fig 4D-F, S8**). Cluster 2 had a profile associated with naïve CD8⁺ T cells, with
221 minimal differences in relative frequency between groups (**Fig 4D-F, S8**). Cluster 3, with high
222 relative frequency in just one of the two coMtb samples, had a profile consistent with effector
223 memory/resident memory CD8⁺ T cells (T_{EM/RM}). (**Fig 4D-F, Fig S7**). Lastly, Cluster 4, with
224 slightly higher relative frequency for both scBCG and coMtb samples compared to controls, had
225 a profile consistent with NK cells. Overall, *mycobacterium* exposure increases the total
226 frequency of T cells in the airway, but leads to only subtle shifts in relative frequencies of T cell
227 subclusters in the airway, mostly notably a slight increase in effector T cell or memory T cell
228 clusters and a decrease in naïve T cells, trends that have been documented previously^{18, 26}.

229

230 ***Mycobacterium* exposure modifies the dendritic cell airway landscape**

231 Similar re-clustering of DCs yielded 2 major clusters and 1 minor cluster (**Fig 4G**). BAL
232 from coMtb mice had a slightly higher relative frequency of Cluster 0, notable for expression of
233 *Clec9a*, *Itgae* (CD103), consistent with an expression profile of lung CD103⁺ cDCs³⁴. Cluster 0

234 also had high expression of antigen presenting molecules such as *H2-Ab1* and *H2-DMA* (**Fig**
235 **4I**). Cluster 1 had slightly higher relative frequency in control BAL and expressed *Batf3*, *Ccr7*,
236 and *Fscn1*. Cluster 2 had a mixed phenotype with expression of genes from both major clusters.
237 All of the clusters had high *Irf8* expression and low expression of *Xcr1*, *Irf4*, and *Itgam* (CD11b),
238 (**Fig 4I**). Antigen presentation genes were more highly expressed by Cluster 0, while *Ccr7*,
239 which is known to be important for migration of DC out of the lung into the draining lymph node
240 during Mtb infection³⁵, was more highly expressed by Cluster 1, hinting at a potential division of
241 labor for T cell priming. Overall, scRNAseq analysis shows that mycobacteria exposure alters
242 the airway landscape and may impact how airway resident T cells and DCs interact with AMs
243 and with each other following subsequent aerosol infection.

244

245 **Cell-intrinsic remodeling following *mycobacterium* exposure licenses an alveolar** 246 **macrophage interferon response *in vitro***

247 *In vivo* transcriptional analysis of the AM response within 24 hours of aerosol challenge
248 demonstrates that the very earliest immune response to Mtb is altered by previous
249 *mycobacterium* exposure. However, a trade-off to measuring responses *in vivo* is the inability to
250 discern whether observed changes are cell-intrinsic or dependent on the changed tissue
251 environment. Therefore, to determine whether mycobacteria exposure induces cell-intrinsic
252 changes to AM responses or whether AMs simply respond differently due to a changed
253 environment (i.e., the presence of greater number of antigen-specific T cells), we isolated AMs
254 from control, scBCG, or coMtb mice, stimulated them *ex vivo* with LPS (10 ng/ml), Pam3Cys (10
255 ng/ml), or H37Rv, and measured their transcriptional profile 6 hours later. AMs from both coMtb
256 and scBCG mice showed remarkably different responses than AMs from control mice to both
257 LPS and H37Rv stimulation, and minimal differences in response to Pam3Cys stimulation (**Fig**
258 **5A, S9, Table S3**). Performing Gene Set Enrichment Analysis, we found that the greatest
259 changes for LPS and H37Rv responses were associated with “Interferon Gamma Response”,

260 “Interferon Alpha Response”, “TNF α signaling via NF- κ B”, and “Inflammatory Response”
261 pathways (**Fig 5B**). To assess whether the cell-intrinsic changes observed were long-lasting, we
262 compared the responses of AMs at 8 or 23 weeks following scBCG vaccination by RT-qPCR.
263 Increases in gene expression were as robust or even enhanced 23 weeks following exposure
264 compared to 8 weeks, suggesting that exposure-induced changes to AMs are relatively long-
265 lived (**Fig S10**).

266 We observed that many of the genes whose response to H37Rv stimulation was altered
267 by mycobacteria exposure could be categorized as ISGs²⁷ (**Fig 5C, S9**). We previously defined
268 “Type I IFN dependent” (352 genes) or “Type I IFN independent” (339 genes) portions of the
269 response of bone-marrow-derived macrophages to H37Rv stimulation using cells from IFNAR^{-/-}
270 mice³⁶. Interestingly, expression of H37Rv-induced IFN dependent genes was minimally
271 induced by H37Rv in control AMs but strongly induced in AMs from *mycobacterium* exposed
272 mice (**Fig 5D, left**). In contrast, expression of H37Rv-induced IFN independent genes was
273 modestly upregulated in control AMs and only slightly altered by *mycobacterium* exposure (**Fig**
274 **5D, right**). These results demonstrate that previous *mycobacterium* exposure leads to cell-
275 intrinsic changes in AMs that are most evident following secondary stimulation and can be long-
276 lasting and that one of the most significant of these is the licensing of a more robust Type I
277 Interferon response.

278

279 **Discussion**

280 Here, we describe remodeling of AMs, long-lived airway-resident innate immune cells,
281 following two types of *mycobacterium* exposure, scBCG vaccination and coMtb, a model of
282 contained H37Rv infection. We find that prior *mycobacterium* exposure generates faster innate
283 cell activation and immune cell recruitment to the lung following Mtb aerosol infection, immune
284 modifications that are detectable within the first 10-14 days of infection. We observe that the AM
285 response within the first 24 hours of infection is substantially altered in mice previously exposed

286 to mycobacteria and that scBCG and coMtb lead to qualitatively different AM responses: AMs
287 from BCG vaccinated mice mount a robust and dominant interferon response, while AMs from
288 coMtb mice generate a broader inflammatory response that includes but is not dominated by
289 interferon pathways. Profiling the airway landscape by scRNAseq, we find sub-populations of
290 AMs that are differentially enriched following scBCG or coMtb exposures. These sub-
291 populations, defined by expression of ISGs, oxidative stress response genes, or interstitial
292 macrophage-like profiles, reveal how prior exposure may generate different flavors of innate
293 immune responses in the airway. Subtle shifts in the relative frequency and phenotype of T cells
294 and DCs within the airway following *mycobacterium* exposure highlight potential areas where
295 innate and adaptive interactions may be substantially altered during the early stages of
296 infection, complementing previous descriptions of accelerated immune responses following
297 vaccination^{18, 25, 26}. *Ex vivo* stimulations of AMs from either scBCG or coMtb mice demonstrate
298 cell-intrinsic effects, with the most robust expression changes occurring in genes that are IFN-
299 dependent, suggesting that prior *mycobacterium* exposure licenses AMs to mount an interferon
300 response that is otherwise lacking in control animals.

301 AMs are the first cells to be productively infected in the lung following aerosol Mtb
302 infection^{12, 13}, but their role during subsequent stages of infection and their contribution to
303 protection mediated by vaccination or concomitant immunity is not fully understood. We
304 previously showed that AMs initially respond to Mtb infection with a cell-protective, Nrf2-driven
305 program that is detrimental to early host control¹². Others have shown that depletion of AMs or
306 strategies that “skip” the AM stage including directly injecting antigen-primed DCs or activating
307 DCs accelerates the immune response and reduces bacterial burden^{19, 37, 38}. However,
308 strategies that target AMs to make them better early responders have not been well studied³⁹.
309 We demonstrate here that following mycobacteria exposure, whether from scBCG or coMtb, the
310 AM response to Mtb becomes more pro-inflammatory, including an up-regulation of ISGs. This
311 coincides with an accelerated innate response and early bacterial control. Typically, interferon

312 signatures are associated with active TB or TB disease progression in both humans and non-
313 human primates^{40, 41, 42}. In addition, type I IFN has known negative consequences for infection.
314 Host perturbations such as treatment with poly I:C or viral co-infection that induce type I IFN
315 lead to worsened disease^{43, 44}, type I IFN has been shown to block production of IL-1 β in
316 myeloid cells during Mtb infection⁴⁵, and type I IFN drives mitochondrial stress and metabolic
317 dysfunction in Mtb infected macrophages³⁶. However, there are also examples where AM
318 production of type I IFN is critical for host protection due to the ability to activate other innate
319 cells in the lung during acute viral infection^{46, 47}. Determining whether early type I IFN production
320 by AMs helps or hurts in the acceleration of the host response during Mtb will require further
321 study.

322 What signals are required to induce long-term remodeling in AMs? A number of recent
323 studies investigated how prior viral infection alters AM function, uncovering either enhanced AM
324 antimicrobial phenotypes^{9, 10, 11} or impaired responses^{48, 49} following exposure. Other studies
325 have discovered long-lasting changes to AMs following intranasal immunization of either
326 adenoviral-based or inactivated whole cell vaccines^{20, 50, 51}. Several reports have identified T
327 cell-derived IFN γ as critical for altering AM function, although the immunological outcome varies
328 substantially based on the context. In one study, T cell-derived IFN γ following adenoviral
329 infection leads to AM activation, innate training and protection from *S. pneumoniae*¹⁰, while in
330 another study influenza-induced T cell-derived IFN γ leads to AM dysfunction and impaired
331 clearance of *S. pneumoniae*⁴⁹. Importantly, a study of BAL samples from 88 SARS-CoV-2
332 patients identified AMs and T cell-derived IFN γ as part of a positive feedback loop in the airway
333 that generates more progressive disease and lung pathology⁵². IFN γ is a likely candidate driving
334 the effects of *mycobacterium* exposure on AMs described in this study. IFN γ was shown to be
335 important for the generation of trained immunity in bone marrow-derived myeloid cells following
336 BCG vaccination^{6, 7}. While pulmonary H37Rv infection is associated with induction of type I IFN

337 and a reduction in myeloid training⁶, we previously found that coMtb leads to low-level systemic
338 cytokinemia, including IFN γ production, and using WT:Ifngr1^{-/-} mixed bone marrow chimeras, we
339 showed that IFN γ signaling was responsible for monocyte and AM activation following
340 establishment of coMtb¹⁵.

341 By providing a side-by-side comparison of multiple remodeled states of AMs following
342 different initial exposures (scBCG vs coMtb), our study highlights the plasticity of AM
343 phenotypes and the impact of the local and/or systemic environments. Heterogeneity in myeloid
344 reprogramming is a feature that has also been demonstrated in human monocytes⁵³. While
345 scBCG and coMtb provide models of mycobacterial exposure that are highly relevant to human
346 biology and host protection derived from vaccination or concomitant immunity, they also have
347 drawbacks. One significant limitation is that perturbations of the likely mechanisms for scBCG
348 or coMtb-mediated AM reprogramming alter the initial exposures themselves. For example,
349 containment in the coMtb model is lost following T cell depletion or anti-IFN γ blockade¹⁴. For
350 this reason, we have not been able to dissect how different signals derived from scBCG versus
351 coMtb push AMs towards different phenotypic states. Instead, we envision future studies using
352 other models that can examine the specific effects of individual cytokines etc. on AM
353 remodeling.

354 There are many other remaining questions. We do not yet know whether the altered AM
355 phenotypes require constant exposure from the changed environment or whether the cell-
356 intrinsic nature is stable in the absence of environmental cues. These would require complex
357 cell transfer experiments that are beyond the scope of this study. Additionally, we do not yet
358 know the durability of these changes and whether they are mediated by epigenetic effects. Our
359 longest experiment showed retention of changes to AMs after 23 weeks. Whether the effects
360 derived from *mycobacterium* exposure require ongoing bacterial replication is also unclear. In
361 Nemeth et al, we showed that antibiotic treatment lessened the protection mediated by coMtb,

362 suggesting that ongoing replication is a key part of the effect¹⁵. However, we rarely find live
363 bacteria in the lungs of scBCG vaccinated mice after 8 weeks yet AMs from these mice show
364 robust alterations, suggesting that the effects can be mediated in the absence of ongoing
365 bacterial replication. We performed several of these studies with intravenous BCG vaccination
366 (ivBCG), which in the mouse model leads to much more bacterial dissemination and ongoing
367 replication (data not shown). While we saw similar changes to AMs in the ivBCG model, these
368 were not dramatically different than those of scBCG vaccination, despite major differences in
369 bacterial replication and far greater T cell recruitment to the airway.

370 There is still much unknown about the signals that drive reprogramming of tissue-
371 resident innate cells. Ideally, vaccines would be designed to leverage these signals and
372 generate the most effective interactions between innate and adaptive responses. Identifying the
373 ways that AMs are reprogrammed by inflammatory signals and the effects of their changed
374 phenotypes on the early stages of infection will help to improve future vaccines or host-directed
375 therapies.

376

377 **Materials and Methods**

378

379 **Mice**

380 C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed
381 and maintained in specific pathogen-free conditions at Seattle Children's Research Institute and
382 experiments were performed in compliance with the Institutional Animal Care and Use
383 Committee. 6-12 week old male and female mice were used for all experiments, except for
384 RNA-sequencing, which used only female mice for uniformity. Mice infected with Mtb were
385 housed in a Biosafety Level 3 facility in an Animal Biohazard Containment Suite.

386

387 **Mycobacteria exposure models: BCG immunization and establishment of coMtb**

388 BCG-Pasteur was cultured in Middlebrook 7H9 broth at 37°C to an OD of 0.1–0.3. Bacteria was
389 diluted in PBS and 1 x 10⁶ CFU in 200 ml was injected SC. Intradermal infections to establish
390 coMtb were performed as formerly described¹⁴, with some modifications as detailed
391 previously¹⁵. Briefly, 10,000 CFU of Mtb (H37Rv) in logarithmic phase growth were injected
392 intradermally into the ear in 10 µL PBS using a 10 µL Hamilton Syringe, following anesthesia
393 with ketamine/xylazine.

394

395 ***M. tuberculosis* Aerosol Infections and Lung Mononuclear Cell Isolation**

396 Aerosol infections were performed with wildtype H37Rv, including some transformed with an
397 mEmerald reporter pMV261 plasmid, generously provided by Dr. Chris Sasseti and Christina
398 Baer (University of Massachusetts Medical School, Worcester, MA). For both standard (~100
399 CFU) and high dose (~2,000-4,000 CFU) infections, mice were enclosed in an aerosol infection
400 chamber (Glas-Col) and frozen stocks of bacteria were thawed and placed inside the associated
401 nebulizer. To determine the infectious dose, three mice in each infection were sacrificed one
402 day later and lung homogenates were plated onto 7H10 plates for CFU enumeration. High dose
403 challenge and sorting of Mtb-infected AM was performed 4 weeks following scBCG vaccination
404 and 2 weeks following coMtb vaccination as previously described⁵⁴. All other analysis was
405 performed 8 weeks following mycobacterium exposures.

406

407 **Lung Single Cell Suspensions**

408 At each time point, lungs were removed, and single-cell suspensions of lung mononuclear cells
409 were prepared by Liberase Blendzyme 3 (70 ug/ml, Roche) digestion containing DNaseI (30
410 µg/ml; Sigma-Aldrich) for 30 mins at 37°C and mechanical disruption using a gentleMACS
411 dissociator (Miltenyi Biotec), followed by filtering through a 70 µM cell strainer. Cells were
412 resuspended in FACS buffer (PBS, 1% FBS, and 0.1% NaN₃) prior to staining for flow
413 cytometry. For bacterial enumeration, lungs were processed in 0.05% Tween-80 in PBS using a

414 gentleMACS dissociator (Miltenyi Biotec) and were plated onto 7H10 plates for CFU
415 enumeration.

416

417 **Alveolar Macrophage Isolation**

418 Bronchoalveolar lavage was performed by exposing the trachea of euthanized mice, puncturing
419 the trachea with Vannas Micro Scissors (VWR) and injecting 1 mL PBS using a 20G-1" IV
420 catheter (McKesson) connected to a 1 mL syringe. The PBS was flushed into the lung and then
421 aspirated three times and the recovered fluid was placed in a 15mL tube on ice. The wash was
422 repeated 3 additional times. Cells were filtered and spun down. For antibody staining, cells were
423 suspended in FACS buffer. For cell culture, cells were plated at a density of 5×10^4 cells/well
424 (96-well plate) in complete RPMI (RPMI plus FBS (10%, VWR), L-glutamine (2mM, Invitrogen),
425 and Penicillin-Streptomycin (100 U/ml; Invitrogen) and allowed to adhere overnight in a 37°C
426 humidified incubator (5% CO₂). Media with antibiotics were washed out prior to infection with *M.*
427 *tuberculosis*.

428

429 **Cell Sorting and Flow Cytometry**

430 Fc receptors were blocked with anti-CD16/32 (2.4G2, BD Pharmingen). Cell viability was
431 assessed using Zombie Violet dye (Biolegend). Cells were suspended in 1X PBS (pH 7.4)
432 containing 0.01% NaN₃ and 1% fetal bovine serum (i.e., FACS buffer). Surface staining,
433 performed at 4 degrees for 20 minutes, included antibodies specific for murine: Siglec F (E50-
434 2440, BD Pharmingen), CD11b (M1/70), CD64 (X54-5/7.1), CD45 (104), CD3 (17A2,
435 eBiosciences), CD19 (1D3, eBiosciences), CD11c (N418), I-A/I-E (M5/114.15.2), Ly6G (1A8),
436 and Ly6C (HK1.4) (reagents from Biolegend unless otherwise noted). MHC class II tetramers
437 ESAT-6 (I-A(b) 4-17, sequence: QQWNFAGIEAAASA) and MHC class I tetramers TB10.4 (H-
438 2K(b) 4-11, sequence: IMYNYPAM) were obtained from the National Institutes of Health
439 Tetramer Core Facility. Cell sorting was performed on a FACS Aria (BD Biosciences). Sorted

440 cells were collected in complete media, spun down, resuspended in TRIzol, and frozen at -80°C
441 overnight prior to RNA isolation. Samples for flow cytometry were fixed in 2% paraformaldehyde
442 solution in PBS and analyzed using a LSRII flow cytometer (BD Biosciences) and FlowJo
443 software (Tree Star, Inc.).

444

445 **Bulk RNA-sequencing and Analysis**

446 RNA isolation was performed using TRIzol (Invitrogen), two sequential chloroform extractions,
447 Glycoblue carrier (Thermo Fisher), isopropanol precipitation, and washes with 75% ethanol.
448 RNA was quantified with the Bioanalyzer RNA 6000 Pico Kit (Agilent). cDNA libraries were
449 constructed using the SMARTer Stranded Total RNA-Seq Kit (v2) - Pico Input Mammalian
450 (Clontech) following the manufacturer's instructions. Libraries were amplified and then
451 sequenced on an Illumina NextSeq (2 x 76, paired-end (sorted BAL cells) or 2 x 151, paired-end
452 (ex vivo stimulation samples)). Stranded paired-end reads were preprocessed: The first three
453 nucleotides of R2 were removed as described in the SMARTer Stranded Total RNA-Seq Kit -
454 Pico Input Mammalian User Manual (v2: 063017) and read ends consisting of more than 66% of
455 the same nucleotide were removed). The remaining read pairs were aligned to the mouse
456 genome (mm10) + Mtb H37Rv genome using the gsnap aligner⁵⁵ (v. 2018-07-04) allowing for
457 novel splicing. Concordantly mapping read pairs (~20 million / sample) that aligned uniquely
458 were assigned to exons using the subRead program and gene definitions from Ensembl
459 Mus_Musculus GRCm38.78 coding and non-coding genes. Genes with low expression were
460 filtered using the "filterByExpr" function in the edgeR package⁵⁶. Differential expression was
461 calculated using the "edgeR" package⁵⁶ from bioconductor.org. False discovery rate was
462 computed with the Benjamini-Hochberg algorithm. Hierarchical clusterings were performed in R
463 using 'TSclust' and 'hclust' libraries. Heat map and scatterplot visualizations were generated in
464 R using the 'heatmap.2' and 'ggplot2' libraries, respectively.

465

466 **Gene Set Enrichment Analysis (GSEA)**

467 Input data for GSEA consisted of lists, ranked by $-\log(p\text{-value})$, comparing RNAseq expression
468 measures of target samples and naïve controls including directionality of fold-change. Mouse
469 orthologs of human Hallmark genes were defined using a list provided by Molecular Signatures
470 Database (MSigDB)⁵⁷. GSEA software was used to calculate enrichment of ranked lists in each
471 of the respective hallmark gene lists, as described previously⁵⁸. A nominal p-value for each ES
472 is calculated based on the null distribution of 1,000 random permutations. To correct for multiple
473 hypothesis testing, a normalized enrichment score (NES) is calculated that corrects the ES
474 based on the null distribution. A false-discovery rate (FDR) is calculated for each NES. Leading
475 edge subsets are defined as the genes in a particular gene set that are part of the ranked list at
476 or before the running sum reaches its maximum value.

477

478 **Ingenuity Pathway Analysis (IPA)**

479 IPA (QIAGEN) was used to identify enriched pathways for differentially expressed genes
480 between naïve and Mtb-infected AMs (cut-off values: $FDR < 0.01$, $|FC| > 2$). The top 20
481 canonical pathways with enrichment score p-value < 0.05 with greater than 10 gene members
482 are reported.

483

484 **Single cell RNA-sequencing**

485 BAL from 10 mice per condition was pooled for each sample, with two independent replicates
486 per condition. Samples were prepared for methanol fixation following protocol “CG000136 Rev.
487 D” from 10X Genomics⁵⁹. Briefly, samples were filtered with 70 μm filters and red blood cells
488 were lysed with ACK lysis buffer. Samples were resuspended in 1 mL ice-cold DPBS using a
489 wide-bore tip and transferred to a 1.5 mL low-bind Eppendorf tube. Samples were centrifuged at
490 $700 \times g$ for 5 minutes at 4°C. Supernatant was carefully removed with a p1000 pipette, and the

491 cell pellet was washed two more times with DPBS, counted, and resuspended in 200 μ L ice-
492 cold DPBS/ 1×10^6 cells. 800 μ L of ice-cold methanol was added drop-wise for a final
493 concentration of 80% methanol. Samples were incubated at -20°C for 30 minutes and then
494 stored at -80°C for up to 6 weeks prior to rehydration. For rehydration, frozen samples were
495 equilibrated to 4°C , centrifuged at $1,000 \times g$ for 10 minutes at 4°C , and resuspended in 50 μ L of
496 Wash-Resuspension Buffer (0.04% BSA + 1mM DTT + 0.2U/ μ L Protector RNAase Inhibitor in
497 $3\times$ SSC buffer) to achieve $\sim 1,000$ cells/ μ L (assuming 75% sample loss).

498

499 **Single cell RNA-sequencing Analysis**

500 Libraries were prepared using the Next GEM Single Cell 3 \square Reagent Kits v3.1 (Dual Index)
501 (10X Genomics) following the manufacturer's instructions. Raw sequencing data were aligned to
502 the mouse genome (mm10) and UMI counts determined using the Cell Ranger pipeline (10X
503 Genomics). Data processing, integration, and analysis was performed with Seurat v.3⁶⁰.
504 Droplets containing less than 200 detected genes, more than 4000 detected genes (doublet
505 discrimination), or more than 5% mitochondrial were discarded. Genes expressed by less than 3
506 cells across all samples were removed. Unbiased annotation of clusters using the Immgen
507 database⁶¹ as a reference was performed with "SingleR" package⁶². Pseudotime analysis was
508 performed using the "SeuratWrappers" and "Monocle3" R packages⁶³. Data visualization was
509 performed with the "Seurat", "tidyverse", "cowplot", and "viridis" R packages.

510

511 **Alveolar Macrophage *Ex Vivo* Stimulation**

512 AMs were isolated by bronchoalveolar lavage and pooled from 5 mice per group. Cells were
513 plated at a density of 5×10^4 cells/well (96-well plate) in complete RPMI (RPMI plus FBS (10%,
514 VWR), L-glutamine (2mM, Invitrogen), and Penicillin-Streptomycin (100 U/ml; Invitrogen) and
515 allowed to adhere overnight in a 37°C humidified incubator (5% CO_2). Media with antibiotics and

516 non-adherent cells were washed out prior to stimulation. AM were stimulated with LPS (LPS
517 from Salmonella Minnesota, List Biologicals, #R595, 10 ng/ml), Pam3Cys (Pam3CSK4, EMC
518 Microcollections, GmbH, 10 ng/ml), or H37Rv (MOI 25:1). H37Rv was prepared by culturing
519 from frozen stock in 7H9 media at 37°C for 48 hours to O.D. of 0.1-0.3. The final concentration
520 was calculated based on strain titer and bacteria was added to macrophages for two hours.
521 Cultures were then washed three times to remove extracellular bacteria. Cell cultures were
522 washed once in PBS after 6 hours to remove dead cells and collected in TRIzol for RNA
523 isolation via chloroform/isopropanol extraction.

524

525 **Filtering for IFN dependent and independent gene sets**

526 “IFN dependent” and “IFN independent” gene sets were generated from data from Olson et al³⁶,
527 using the following filters starting from a total of 1,233 genes up-regulated in H37Rv-stimulated
528 WT BMDM with average CPM >1, log₂ fold change > 1 and FDR < 0.01:

529 “*IFN dependent*” = H37Rv-stimulated IFNAR^{-/-} BMDM: log₂ fold change < 1 AND H37Rv-
530 stimulated WT vs IFNAR^{-/-}: log₂ fold change > 2 = **352 genes**

531 “*IFN independent*” = H37Rv-stimulated IFNAR^{-/-} BMDM: log₂ fold change > 1, FDR < 0.01
532 AND H37Rv-stimulated WT vs IFNAR^{-/-}: log₂ fold change < 2 = **339 genes**

533

534 **qRT-PCR**

535 Quantitative PCR reactions were carried out using TaqMan primer probes (ABI) and TaqMan
536 Fast Universal PCR Master Mix (ThermoFisher) in a CFX384 Touch Real-Time PCR Detection
537 System (BioRad). Data were normalized by the level of EF1a expression in individual samples.

538

539 **Statistical Analyses**

540 RNA-sequencing was analyzed using the edgeR package from Bioconductor.org and the false
541 discovery rate was computed using the Benjamini-Hochberg algorithm. All other data are

542 presented as mean \pm SEM and analyzed by one-way ANOVA (95% confidence interval) with
543 Tukey post-test (for comparison of multiple conditions). Statistical analysis and graphical
544 representation of data was performed using either GraphPad Prism v6.0 software or R. PCA
545 plots generated using “Prcomp” and “Biplot” packages. Venn diagrams and gene set
546 intersection analysis was performed using Intervene⁶⁴. p-values, * $p < 0.05$, ** $p < 0.01$, *** $p <$
547 0.001.

548

549 **Acknowledgements:** We thank the staff at Seattle Children’s Research Institute vivarium for
550 animal care, Pamela Troisch and the Next Gen Sequencing core at the Institute for Systems
551 Biology, and members of the Aderem, Urdahl, and Rothchild labs for helpful discussions.

552 **Funding:** This work was supported by National Institute of Allergy and Infectious Disease of the
553 National Institute of Health under Awards U19AI135976 (A.A.), R01AI032972 (A.A.),
554 75N93019C00070-P00006-9999-1 (A.A., K.U., A.C.R.), and R21AI163809 (A.C.R.).

555 **Author contributions:** D.M., A.C.R., J.N., A.H.D., K.U., and A.A. designed the experiments.
556 A.C.R., D.M., A.J., T.M. conducted the experiments. A.H.D., A.C.R., M.M. performed
557 computational analyses. A.C.R., A.H.D. wrote the paper.

558 **Competing interests:** The authors declare no competing interests.

559 **Data and materials availability:** Raw and processed RNA-sequencing data can be accessed
560 from the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus
561 (GEO) database under accession number GSE212205
562 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE212205>]. (*Submission currently*
563 *private.*)

564

565 **Figure 1: Prior mycobacteria exposure leads to faster activation and innate cell**
566 **recruitment following aerosol Mtb challenge.** Control, scBCG, and coMtb mice, 8 weeks
567 following exposure, challenged with standard low-dose H37Rv. Lungs collected on day 10, 12,

568 and 14 post-infection. A) AM MHC II MFI. B) Total numbers of MDMs, PMN, DC, Ly6C⁺ CD11b⁺
569 and Ly6C⁺ CD11b⁺ monocytes. C) Total numbers of CD44⁺ CD4⁺ T cells, ESAT6-tetramer⁺
570 CD4⁺ T cells, CD44⁺ CD8⁺ T cells, and TB10.4-tetramer⁺ CD8⁺ T cells. Mean +/- SEM, 5 mice
571 per group, representative of 3 independent experiments. One-way ANOVA with Tukey post-test.
572 * p < 0.05, **p < 0.01, ***p < 0.001. B, C) *, **, and *** scBCG or coMtb vs control; +, ++ scBCG
573 vs coMtb.

574

575 **Figure 2: Mycobacterium exposure alters the alveolar macrophage transcriptional**
576 **response to Mtb infection in vivo.** Gene expression of naive and Mtb-infected AMs 24 hours
577 following high-dose mEmerald-H37Rv infection in mice previously exposed with scBCG or
578 coMtb or controls (controls- reported in Rothchild et al, 2019¹²; CMTB- reported in Nemeth et al,
579 2020¹⁵). A) Principal Component Analysis using DEG (|fold change| > 2, FDR < 0.05) in Mtb-
580 infected AMs. B) Venn Diagram and Intersection plot of overlap in up-regulated DEG between
581 the 3 conditions. C) Gene Set Enrichment Analysis of 50 Hallmark Pathways. D) Heatmap of
582 131 Nrf2-associated DEG at 24 hours in Mtb-infected AM (left), Interferon Stimulated Genes,
583 derived from macrophage response to IFN α (fold change >2, p-value < 0.01) Mostafavi et al,
584 2016²⁷ (middle), IL6 JAK STAT3 hallmark pathway (right). Compiled from 4 independent
585 experiments per condition for control, 2 independent experiments per condition for scBCG and
586 coMtb.

587

588 **Figure 3: Mycobacterium exposure modifies the alveolar macrophage phenotype in the**
589 **airway pre-challenge.** Single-cell RNA-sequencing of BAL samples from control, scBCG, and
590 coMtb mice. A) Compiled scRNAseq data for all BAL samples, highlighted by major clusters,
591 annotated based on closest Immgen sample match. B) Highlighting of the two clusters used for
592 macrophage recluster analysis. C) The 12 clusters generated by the macrophage recluster
593 analysis, separated by condition. D) Expression of major macrophage-specific markers: *Siglecf*,

594 *Mertk*, *Fcgr1*, *Lyz2*, *Itgam* (CD11b), and *Itgax* (CD11c). E-I) Relative frequency of each
595 macrophage sub-cluster by condition. Expression level of representative genes that are
596 distinguished by that particular cluster compared to other clusters. J) Pseudotime analysis using
597 Monocle3 with starting node at the largest cluster in control, Cluster 0 (*top*) and at the cluster of
598 proliferating cells, Cluster 4,9 (*bottom*). Data is compiled from two independent experiments with
599 3 conditions each for a total of 6 scRNA-seq BAL samples. One-way ANOVA with Tukey post-
600 test, * $p < 0.05$.

601

602 **Figure 4: Mycobacterium exposure modifies airway T cell and dendritic cell profiles.**

603 Single-cell RNA-sequencing of BAL samples from control, scBCG, and coMtb mice. A)
604 Compiled scRNAseq data for all BAL samples, with major T cell and dendritic cell clusters
605 highlighted. B) Relative frequency of T cells, DC #1, and DC #2 clusters for each condition. C-F)
606 T cell subcluster analysis. C) T cell subclusters compiled (left) and split by condition (right).
607 Annotations below were made following Immgen profile matches and manual marker inspection.
608 D) Relative frequency of Clusters 0-4 for each condition. E) UMAP gene expression plot for
609 general T cell markers. F) UMAP gene expression plot cluster-specific markers split by
610 condition. G-I) Dendritic cell subcluster analysis. G) Dendritic cell subcluster, colored by each of
611 3 different clusters, compiled (top) and split by conditions (bottom). H) Relative frequency of
612 Clusters 0-2 for each condition. I) Violin plots for cluster-specific markers and genes of interest.
613 Data is compiled from two independent experiments with 3 conditions each for a total of 6
614 scRNA-seq BAL samples.

615

616 **Figure 5: Alveolar macrophage remodeling following *mycobacterium* exposure licenses**

617 **an interferon response upon re-stimulation *ex vivo*.** AMs were stimulated for 6 hours with
618 Pam3Cys (10 ng/ml), LPS (10 ng/ml), and H37Rv (MOI 10:1). A) Scatterplots for \log_2 fold
619 change for stimulated versus unstimulated AMs for each background (control, scBCG, coMtb).

620 Differentially expressed genes (DEG) are highlighted for one or both conditions ($|\text{Fold change}| >$
621 2 , $\text{FDR} < 0.05$ for Pam3Cys and LPS; $|\text{Fold change}| > 2$, $\text{FDR} < 0.2$ for H37Rv). B) Gene Set
622 Enrichment Analysis results for 50 HALLMARK pathways. Pathways shown have $\text{NES} > 1.5$
623 and $\text{FDR} < 0.05$ for at least one of the conditions. C) Scatterplots for \log_2 fold change for
624 stimulated versus unstimulated AMs for each background. Genes highlighted are Interferon
625 Stimulated Genes. D) Heatmaps depicting \log_2 fold change for AM (control, scBCG, coMtb)
626 following 6 hour H37Rv stimulation for IFN-dependent genes (352 total) and IFN-independent
627 genes (339 total) based on WT vs IFNAR^{-/-} BMDM bulk RNA-seq dataset (Olson et al, 2021).

628
629 **Figure S1 (related to Figure 2): Flow cytometry gating schemes.** Gating strategies for
630 myeloid (A) and T cell (B) analysis.

631
632 **Figure S2 (related to Figure 1): Mycobacterium exposure provides protection against**
633 **standard dose H37Rv challenge.** A) Lung, spleen, and lung-draining lymph node (LN) CFU in
634 control mice at deposition, day 10, 12, 14, and 28. B-E) Summary plots of CFU change (log) in
635 lung, spleen, and LN following low-dose infection with H37Rv at day 10 (B), day 12 (C), day 14
636 (D), and day 28 (E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. One-way ANOVA with Tukey post-test.
637 Data compiled from 2-3 independent experiments per condition, with 5 mice per group for each
638 experiment.

639
640 **Figure S3 (related to Figure 2): Top 20 Canonical Pathways by Ingenuity Pathway**
641 **Analysis for up-regulated genes by Mtb-infected alveolar macrophages.** IPA analysis for
642 Mtb-infected AMs from control, scBCG, and coMtb mice 24 hours following high dose
643 mEmerald-H37Rv infection. Data representative of 3 independent experiments per condition.

644

645 **Figure S4 (related to Figure 3): Transcriptional changes to naive alveolar macrophages**
646 **following mycobacterium exposure by bulk RNA-sequencing.** Volcano plots depicting
647 changes in baseline gene expression of naive AMs from scBCG (A) and coMtb (B) mice
648 compared to naive AMs from control mice. Significantly changed genes ($FDR < 0.05$, $|FC| > 2$)
649 highlighted and labeled. Compiled from 2 independent experiments for each condition.

650
651 **Figure S5 (related to Figure 3): Flow analysis of BAL samples prepared for 10X single-cell**
652 **RNA-sequencing.** Percentage of each population (AM, lymphocytes, eosinophils, MDM, other
653 $CD45^+$) out of $CD45^+ ZV^-$. AM = Siglec $F^+ CD64^+$, Eosinophils = Siglec $F^+ CD64^-$, lymphocytes =
654 $CD3/CD19^+$, MDM = Siglec $F^- CD64^+$, other $CD45^+ = CD3^- CD19^- Siglec F^- CD64^-$. Note: One of
655 the two coMtb samples analyzed by flow cytometry did not have an accompanying 10X sample.
656 The second coMtb 10X sample was processed separately without flow analysis.

657
658 **Figure S6 (related to Figure 3): Top 10 genes differentially expressed for each of 11**
659 **macrophage sub-clusters** Heatmap of genes that are most differentially expressed for each of
660 11 clusters with all other clusters. Genes filtered with log fold change threshold of > 0.25 and
661 minimum percentage expression of 25% of cells. All genes but one (*Gsto1*) had an adjusted p-
662 value of $< 1.0 \times 10^{-5}$. *Five genes (*Fabp4*, *Fabp5*, *Stmn1*, *Mki67*, *Cbr2*) met this criterion for
663 more than one cluster, grouped with the more abundant cluster. Data is compiled from two
664 independent experiments, 3 conditions each, for a total of 6 scRNA-seq BAL samples.

665
666 **Figure S7 (related to Figure 4): UMAP gene expression plots for genes associated with**
667 **macrophage sub-cluster 3 and found in AM_2 (Pisu et al).** Genes associated with
668 mitochondrial oxidative phosphorylation (*mt-Co1*, *mt-Cytb*, *mt-Nd2*), chromatin remodeling
669 (*Ankrd11*, *Baz1*), macrophage-associated transcription factors (*Cebpb*, *Zeb2*, *Bhlhe40*, *Hif1a*),
670 CARD9 signaling (*Malt1*, *Bcl10*, *Prkcd*), hemaglobin metabolism (*Hba-a1*, *Hba-a2*, *Hbb-bs*).

671 Data is compiled from two independent experiments with 4 conditions each for a total of 8
672 scRNA-seq BAL samples.

673

674 **Figure S8 (related to Figure 4): UMAP gene expression plots of cluster and lineage**
675 **marker genes of interest for T cell subclusters.** Data is compiled from two independent
676 experiments with 4 conditions each for a total of 8 scRNA-seq BAL samples.

677

678 **Figure S9 (related to Figure 5): Volcano plots of alveolar macrophage *ex vivo***
679 **stimulations.** AMs were stimulated for 6 hours with Pam3Cys (10 ng/ml), LPS (10 ng/ml), or
680 H37Rv (MOI 10:1). Volcano plots depict fold change (log2) and P-value (-log10) for each
681 stimulation condition for each of the three groups (control scBCG, coMtb) compared to the
682 respective unstimulated control. DEG (p-value < 0.001; |fold change| > 2) highlighted and
683 labeled, space permitting. Compiled from 3 independent experiments.

684

685 **Figure S10 (related to Figure 5): Cell-intrinsic changes in alveolar macrophage response**
686 **is retained 23 weeks following vaccination.** Gene expression of *Mx1*, *Cxcl10*, *Il1b*, *Cxcl2*,
687 *Irf7*, and *Il6* as measured by qPCR in AMs isolated by BAL from mice 8 and 23 weeks following
688 scBCG vaccination and from age-matched controls, with and without LPS (10 ng/ml)
689 stimulation. Data is representative of technical AM duplicates from a single experiment

690

691 **Table S1: RNA-Sequencing data for alveolar macrophages 24 hours following high dose**
692 **H37Rv-mEmerald challenge from scBCG mice**

693

694 **Table S2: Top differentially expressed genes for individual clusters for macrophage, T**
695 **cell, and dendritic cell sub-cluster analysis**

696

697 **Table S3: RNA-Sequencing data for *ex vivo* stimulated alveolar macrophages**

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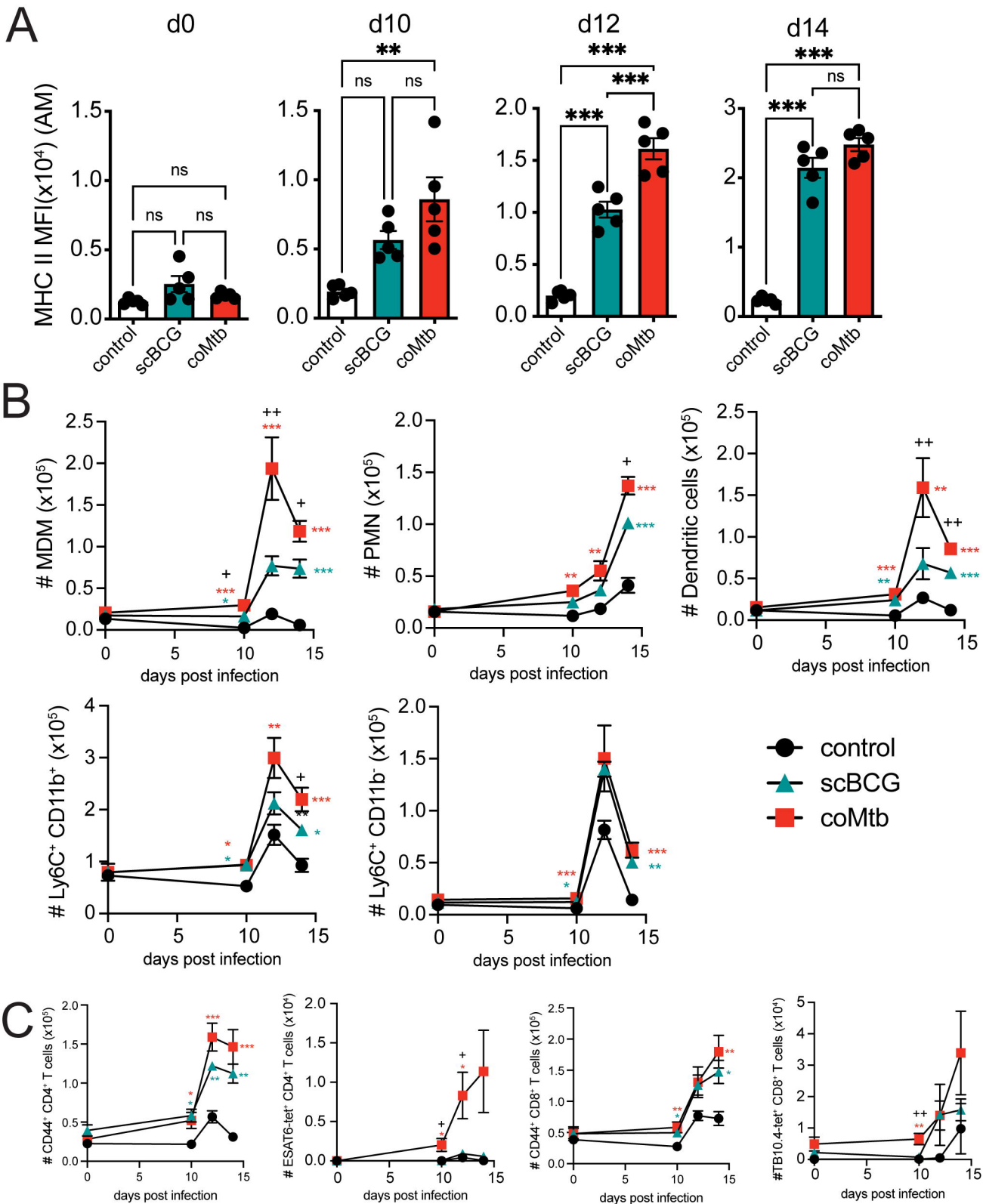


Figure 1: Prior mycobacteria exposure leads to faster activation and innate cell recruitment following aerosol Mtb challenge. Control, scBCG, and coMtb mice, 8 weeks following exposure, challenged with standard low-dose H37Rv. Lungs collected on day 10, 12, and 14 post-infection. A) AM MHC II MFI. B) Total numbers of MDMs, PMN, DC, Ly6C⁺ CD11b⁺ and Ly6C⁺ CD11b⁻ monocytes. C) Total numbers of CD44⁺ CD4⁺ T cells, ESAT6-tetramer⁺ CD4⁺ T cells, CD44⁺ CD8⁺ T cells, and TB10.4-tetramer⁺ CD8⁺ T cells. Mean \pm SEM, 5 mice per group, representative of 3 independent experiments. One-way ANOVA with Tukey post-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. B, C) *, **, and *** scBCG or coMtb vs control; +, ++ scBCG vs coMtb.

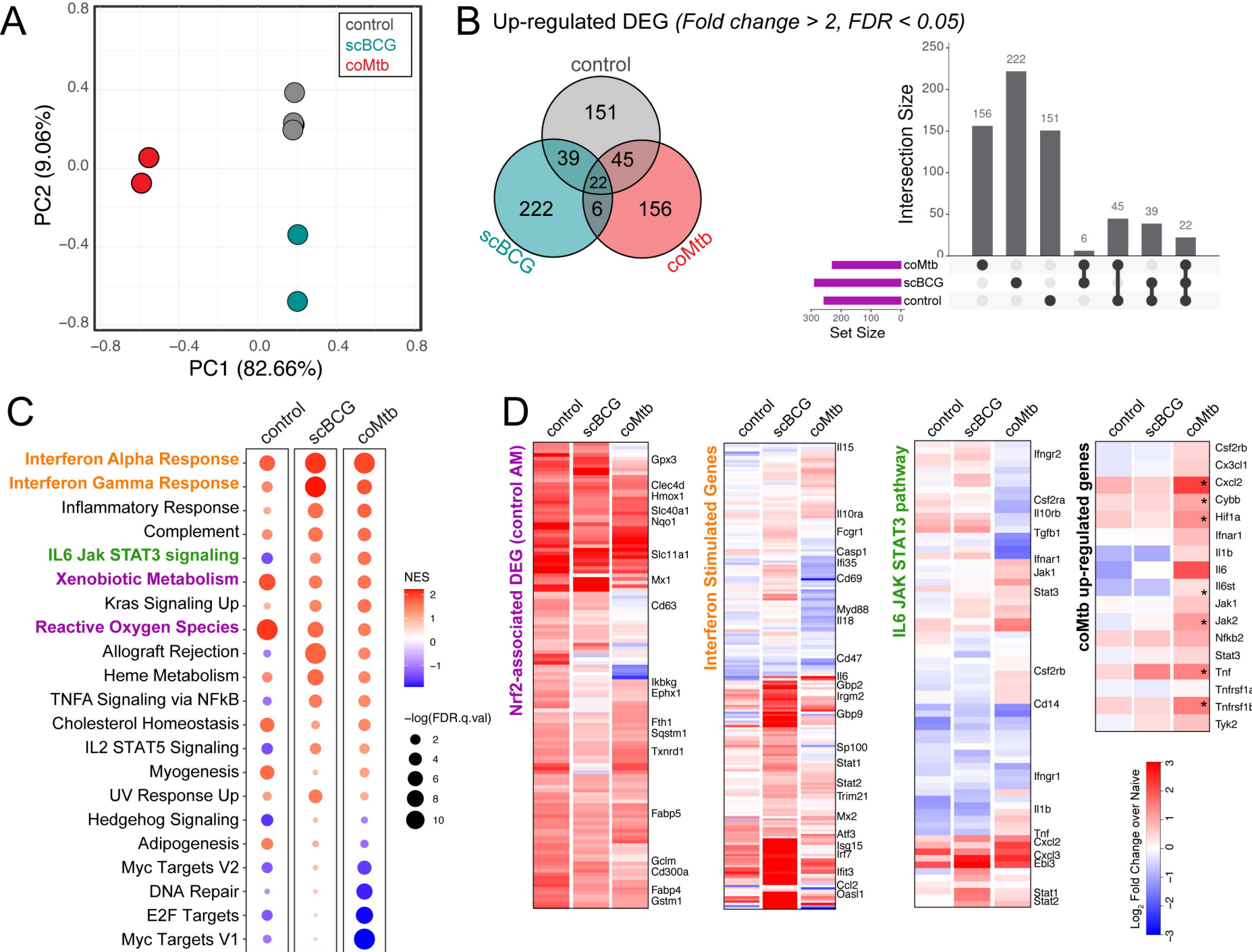


Figure 2: *Mycobacterium* exposure alters the alveolar macrophage transcriptional response to *Mtb* infection *in vivo*. Gene expression of naive and *Mtb*-infected AMs 24 hours following high-dose mEmerald-H37Rv infection in mice previously exposed with scBCG or coMtb or controls (*controls*- reported in Rothchild et al, 2019; *CMTB*- reported in Nemeth et al, 2020). A) Principal Component Analysis using DEG ($|\text{fold change}| > 2$, $\text{FDR} < 0.05$) in *Mtb*-infected AMs. B) Venn Diagram and Intersection plot of overlap in up-regulated DEG between the 3 conditions. C) Gene Set Enrichment Analysis of 50 Hallmark Pathways. D) Heatmap of 131 *Nrf2*-associated DEG at 24 hours in *Mtb*-infected AM (*left*), Interferon Stimulated Genes, derived from macrophage response to IFN α ($\text{fold change} > 2$, $p\text{-value} < 0.01$) (Mostafavi et al, 2016) (*middle-left*), IL6 JAK STAT3 hallmark pathway (*middle-right*) and selected coMtb signature genes (*right*, * $\text{FDR} < 0.05$, $\text{FC} > 2$). Compiled from 4 independent experiments per condition for control, 2 independent experiments per condition for scBCG and coMtb.

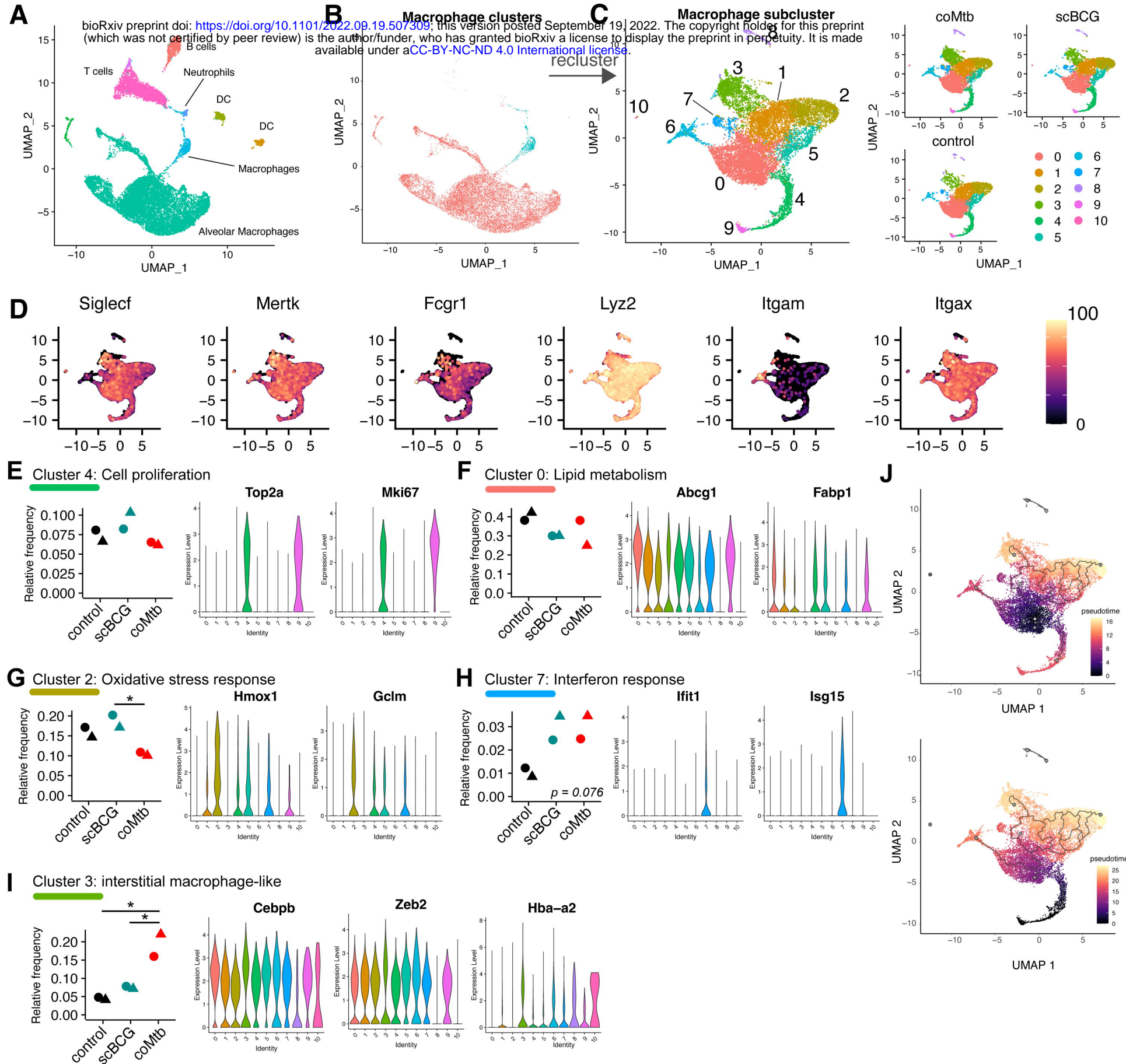


Figure 3: *Mycobacterium* exposure modifies the alveolar macrophage phenotype in the airway pre-challenge. Single-cell RNA-sequencing of BAL samples from control, scBCG, and coMtb mice. A) Compiled scRNAseq data for all BAL samples, highlighted by major clusters, annotated based on closest Immgen sample match. B) Highlighting of the two clusters used for macrophage reclustering analysis. C) The 12 clusters generated by the macrophage reclustering analysis, separated by condition. D) Expression of major macrophage-specific markers: Siglec f, Mertk, Fcgr1, Lyz2, Itgam (CD11b), and Itgax (CD11c). E-I) Relative frequency of each macrophage sub-cluster by condition. Expression level of representative genes that are distinguished by that particular cluster compared to other clusters. J) Pseudotime analysis using Monocle3 with starting node at the largest cluster in control, Cluster 0 (top) and at the cluster of proliferating cells, Cluster 4,9 (bottom). Data is compiled from two independent experiments with 3 conditions each for a total of 6 scRNA-seq BAL samples. One-way ANOVA with Tukey post-test, * $p < 0.05$.

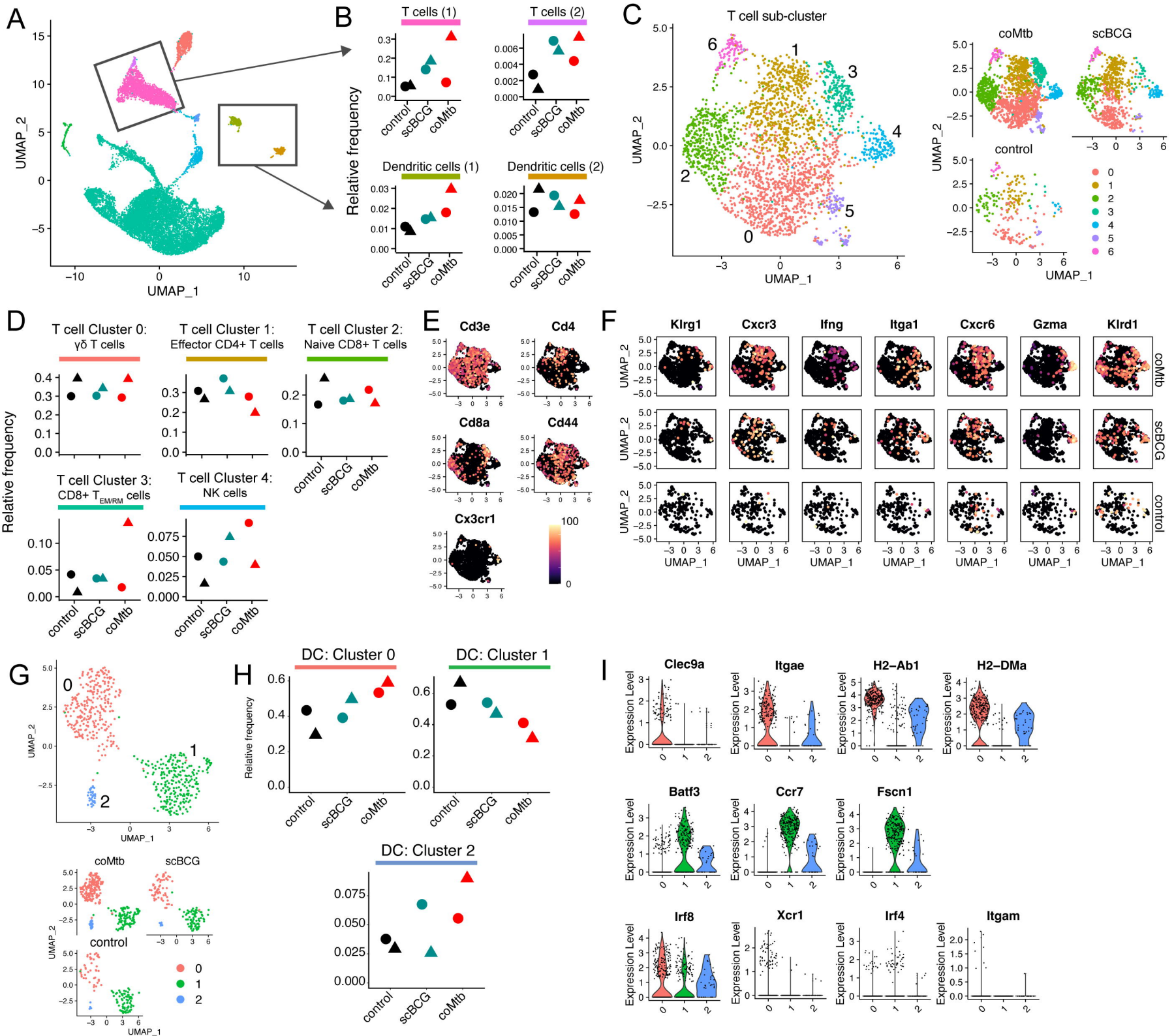


Figure 4: *Mycobacterium* exposure modifies airway T cell and dendritic cell profiles. Single-cell RNA-sequencing of BAL samples from control, scBCG, and coMtb mice. A) Compiled scRNAseq data for all BAL samples, with major T cell and dendritic cell clusters highlighted. B) Relative frequency of T cells, DC #1, and DC #2 clusters for each condition. C-F) T cell subcluster analysis. C) T cell subclusters compiled (*left*) and split by condition (*right*). Annotations below were made following Immgen profile matches and manual marker inspection. D) Relative frequency of Clusters 0-4 for each condition. E) UMAP gene expression plot for general T cell markers. F) UMAP gene expression plot cluster-specific markers split by condition. G-I) Dendritic cell subcluster analysis. G) Dendritic cell subcluster, colored by each of 3 different clusters, compiled (*top*) and split by conditions (*bottom*). H) Relative frequency of Clusters 0-2 for each condition. I) Violin plots for cluster-specific markers and genes of interest. Data is compiled from two independent experiments with 3 conditions each for a total of 6 scRNA-seq BAL samples.

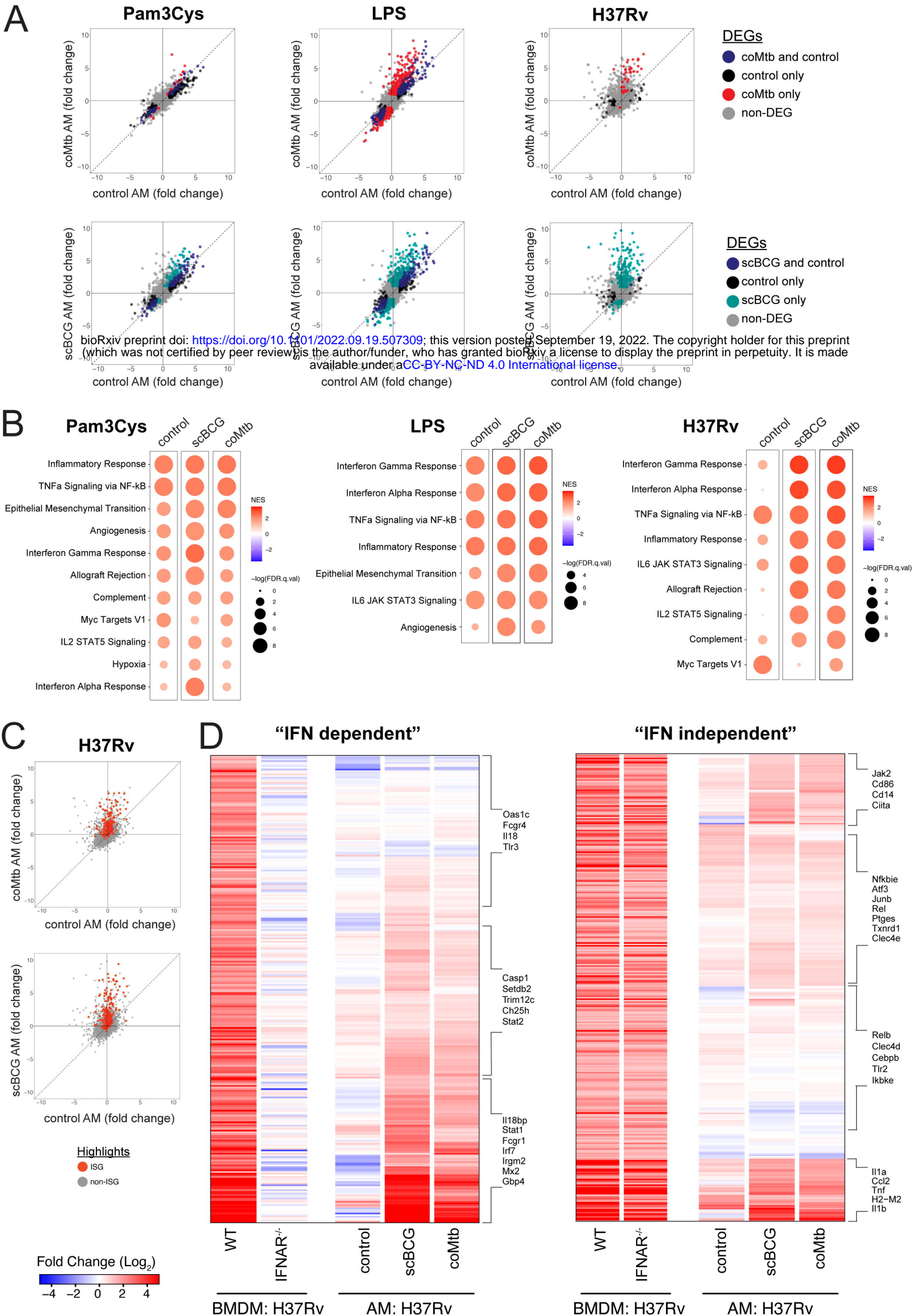


Figure 5: Alveolar macrophage remodeling following *mycobacterium* exposure licenses an interferon response upon re-stimulation *ex vivo*. AMs were stimulated for 6 hours with Pam3Cys (10 ng/ml), LPS (10 ng/ml), and H37Rv (MOI 10:1). A) Scatterplots for log_2 fold change for stimulated versus unstimulated AMs for each background (control, scBCG, coMtb). Differentially expressed genes (DEG) are highlighted for one or both conditions ($|\text{Fold change}| > 2$, $\text{FDR} < 0.05$ for Pam3Cys and LPS; $|\text{Fold change}| > 2$, $\text{FDR} < 0.2$ for H37Rv). B) Gene Set Enrichment Analysis results for 50 HALLMARK pathways. Pathways shown have NES > 1.5 and $\text{FDR} < 0.05$ for at least one of the conditions. C) Scatterplots for log_2 fold change for stimulated versus unstimulated AMs for each background. Genes highlighted are Interferon Stimulated Genes. D) Heatmaps depicting log_2 fold change for AM (control, scBCG, coMtb) following 6 hour H37Rv stimulation for IFN-dependent genes (352 total) and IFN-independent genes (339 total) based on WT vs IFNAR^{-/-} BMDM bulk RNA-seq dataset (Olson et al, 2021).

