1 **_Title:_**

2 Generation and utilization of a HEK-293T murine GM-CSF expressing cell line

3 **_Authors:_**

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9 **_Abstract:_**

10 Macrophages and dendritic cells (DCs) are innate immune cells that play a key role in defense against pathogens.

11 _In vitro_ cultures of bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs) are well-established

12 and valuable methods for immunological studies. Typically, commercially available recombinant GMCSF is

13 utilized to generate BMDCs and is also used to culture alveolar macrophages. We have generated a new HEK-293T

14 cell line expressing murine GM-CSF that secretes high levels of GM-CSF (~180ng/ml) into complete media as an

15 alternative to commercial GM-CSF. Differentiation of dendritic cells and expression of various markers were

16 kinetically assessed using the GM-CSF HEK293T cell line, termed supGM-CSF and compared directly to purified

17 commercial GMCSF. After 7-9 days of cell culture the supGM-CSF yielded twice as many viable cells compared

18 to the commercial purified GM-CSF. In addition to differentiating BMDCs, the supGM-CSF can be utilized to

19 culture alveolar macrophages without an altering inflammatory activation cascade. Collectively, our results show

20 that supernatant from our GM-CSF HEK293T cell line supports the differentiation of mouse BMDCs or alveolar

21 macrophage culturing, providing an economical alternative to purified GM-CSF.

22 **_Introduction:_**

23 Colony-stimulating factors (CSF) including macrophage colony-stimulating factor (M-CSF), granulocyte

24 colony-stimulating factor (G-CSF), and granulocyte-macrophage colony stimulating factor (GM-CSF) are crucial

25 for survival, proliferation, differentiation and functional activation of hematopoietic cells, including macrophages

26 and dendritic cells (DCs) (_1_). Macrophages and DCs are innate immune cells found in tissues and lymphoid organs

27   that play a key role in defense against pathogens (*2*). While there are a multitude of macrophage and dendritic cell

28   subsets, GM-CSF is critical for the development of conventional dendritic cells (cDCs) and alveolar macrophages

29   (AMs) (*2*). Due to cell number limitations from harvesting cDCs and AMs directly from mice, well-established *in*

30   *vitro* culturing of bone marrow and bronchoalveolar lavage fluid for dendritic cells and alveolar macrophages,

31   respectively, using GM-CSF have become invaluable for immunological and molecular biology studies (*2*). This

32   has led to the use of CSF proteins in the purified form, as well as to the generation of recombinant cell lines that

33   secrete the desired protein in the supernatant for cost efficiency (*3–6*). One of the most widely adapted cell lines,

34   utilized for differentiating and culturing murine bone-marrow derived macrophages (BMDMs) is the NCTC clone

35   929 strain L line, also known as L929 (*7*). Supernatant from cultured L929 can be utilized, in lieu of purified M-

36   CSF, to culture and differentiated BMDMs because it secretes murine M-CSF (*7*).

37   The Human embryonic kidney 293T (HEK293T) cell line is the ideal choice for expressing a CSF protein

38   for differentiating primary immune cells from mouse bone marrow as it has been shown not to express innate

39   immune pattern recognition receptors or naturally secrete immune-related cytokines (*8*, *9*). This ensures that it is

40   predominantly only your protein of choice being expressed and that there is no inadvertent activation of the

41   inflammatory cascade. Previously, murine GM-CSF has been cloned into J558L, a mouse B myeloma cell line (*4*),

42   which is known to express cytokines, including IL-10 (*10*). Using J558L can therefore alter the results of *in vitro*

43   experiments, by activating anti-inflammatory pathways when cultured with BMDCs or alveolar macrophages.

44   In this study we constructed HEK293T cell lines stably expressing and secreting murine GM-CSF. We

45   utilized GM-CSF to generate and culture BMDCs and alveolar macrophages (AMs). HEK293T cells have no

46   expression of human GM-CSF and the constructed cell lines thus express only the stably transfected gene of murine

47   GM-CSF. We have found that our line is very stable, producing GM-CSF at a concentration of ~200ng/ml even

48   following freeze thaw cycles of the line. We differentiated BMDCs and cultured AMs using our supGM-CSF and

49   compared them to commercially available purified GM-CSF (pGM-CSF) and found that our supGM-CSF yields a

50   higher number of cells, purity of DCs is not altered, and have an intact immune signaling cascades.

51   ***Materials and Methods***

52   *Cloning Strategy of mGM-CSF*

53 The mGM-CSF gene, including PspXI and NotI restriction sites, was amplified from the pCR3.1-mGM-CSF vector

54 (Addgene, 74465). The PCR was set up with:

55 25 μl 2X Phusion High-Fidelity PCR Master Mix (Thermo Scientific), 1 μl PspXI_hMCSF_fwd (20mM):

56 TCCGCTCGAGCCACCATGTGGCTGCAGAATTTACTTTTCC, 1 μl NotI_hMCSF_rev (20mM):

57 GACGCGGCCGCTCATTTTTGGCCTGGTTTTTTGC, 1 μl pCR3.1-mGM-CSF (20ng) and 22 μl DEP-C

58 nuclease-free water. PCR program: 95℃ 3 minutes, 35 cycles of 95℃ 30 sec, 60℃ 30 sec, and 72℃ 1 min, and

59 end the PCR with 72℃ and 12℃ hold. The PCR product was purified using the PCR Clean-up Kit (Macherey-

60 Nagel) and was subsequently digested with PspXI (New England BioLabs) and NotI-HF (New England BioLabs)

61 using recommended digestion conditions (https://nebcloner.neb.com/#!/redigest) and was cloned into our custom

62 681 bidirectional lentiviral vector (sequence in supplemental). Sequence was confirmed by Sequetech (Sanger)

63 sequencing.

64 *Lentivirus Generation*

65 HEK-293T cells (4e5 cells/well) were plated onto a 6-well plate (353046, Corning) with complete DMEM [10%

66 heat-inactivated FCS (Gibco, 26140-079), 100μg/ml penicillin (Thermo, 15140122), and 100μg/ml streptomycin

67 (Thermo, 15140122)]. 24hr later, 500 ng of 681-mGM-CSF or empty vector control, 250ng psPAX2 (Addgene,

68 12260), and 250ng pMD2.G (Addgene, 12259) were mixed in 200μl of serum-free Opti-MEM (Gibco) and 5μl

69 Lipofectamine 2000 (Thermo Fisher) was added to mix and incubated for 20 minutes at room temperature.

70 Transfection reaction was added to HEK-293T cells and allowed to transfect for 72 hours, and supernatant was

71 harvested, passed through 0.45μm filters (Millipore, Stericup), and aliquots were stored at -80°C.

72 *Construction of GM-CSF-producing HEK-293T Cells*

73 HEK-293T cells were transduced 2 days with 200ul of lentivirus per 1e5 cells. 48hrs after infection, the HEK-293T

74 cells were selected with puromycin (2ug/ml) for >3 days, monitoring viability and increase of mCherry expression

75 using FACS (Attune NxT Flow Cytometer). Once mCherry expression exceeded 85%, puromycin was removed,

76 and cell-line was expanded and used to produce GM-CSF. A detailed method for GM-CSF supernatant production

77 is described in extended methods.

78 *Enrichment of DCs and Macrophages from BM and in-vitro stimulation*

79   Bone marrow (BM) cells were harvested from the femurs and tibia of wild-type C57BL/6 between 6- and 18-weeks

80   old mice and depleted of erythrocytes. 1e6-3e6 BM cells were plated per well of a 6-well tissue culture plate

81   (353046, Corning) in complete DMEM supplemented with either 10% M-CSF from L929 cells, 5-10% supernatant,

82   or 10-25ng/ml of recombinant mGM-CSF [PMC2015, Thermo Fisher]. Media was replaced on day 3 and every 2

83   days henceforward. Cells were scraped and moved onto a larger plate as they proliferated. After 7 to 14 days of

84   enrichment, cells were stimulated by adding 200ug/ml LPS (Sigma, L2630-10MG) to the media and harvested for

85   analysis after 0, 6, or 24 hours of stimulation. Total cell count was determined by staining cells with trypan blue

86   and using a Light Microscope and hemocytometer.

87   *Surface Staining for Flow Cytometry*

88   Stimulated BMDCs and BMDMs were harvested and centrifuged at 300 g for 5 minutes. The pellets were

89   resuspended in 100μl sorting media (2% FCS, 5 mM EDTA, 1X PBS). Each sample was blocked with 100μl of Fc

90   block CD16/CD32 (BD Biosciences) diluted 1:250 in sorting media and incubated at room temperature for 15

91   minutes. Antibodies and viability dye were then used to stain the samples for 30 minutes on ice in the dark.

92   Antibodies and dye: APC-eFluor 780 anti-CD45 (Invitrogen, 47-0451-82), Alexa-Fluor 700 anti-CD11c (BD

93   Pharmingen, 560583), FITC anti-CD11b (Thermo Fisher, MA1-10081), PE-eFluor 610 anti-F4/80 (eBioScience,

94   61-4801-80), PE anti-MHC Class II (BD Pharmingen, 562010), and Fixable Aqua Dead Cell Stain (Thermo Fisher,

95   L34957). Cells were washed twice in 1 ml sorting media, spinning at 300 g for 5 minutes. The resulting pellet was

96   resuspended in 200μl of sorting media and analyzed by FACS (Attune NxT Flow Cytometer).

97   *Inflammasome Activation Assay and ELISA*

98   supGM-CSF (25ng/ml) or recGM-CSF (25ng/ml) differentiated BMDCs were plated at 2e5cells/well in a 96 well

99   plate with complete DMEM media. Cells were first primed with 200ng/ml LPS for 3h prior to treatment with

100   different agents. Poly(dA:dT) DNA (dsDNA mimic) was transfected using Lipofectamine 2000 at a concentration

101   of 1.5μg/ml, 6hrs prior to harvesting supernatant. The supernatant was harvested using a p300 multichannel pipette

102   and stored at -80°C. Commercial ELISAs were used to measure the following analytes in triplicate mice following

103   the manufacturer's instructions: IL-6 (mouse IL-6 DuoSet, R&D Systems DY406) and IL-1β (Mouse IL-1β/IL-1F2

104   R&D Systems DY401-05).

105 *BALF Harvest and Alveolar Macrophage Culturing*

106 Bronchoalveolar Lavage Fluid (BALF) was harvested as previously stated by Cloonan *et al*. (PMID:26752519). 40

107 mice were euthanized by CO_2 narcosis, the tracheas cannulated, and the lungs lavaged with 0.5-ml increments of

108 ice-cold PBS eight times (4 ml total), samples were combined in 50ml conical tubes. BALF was centrifuged at 500*g*

109 for 5 min. 1 ml red blood cell lysis buffer (Sigma-Aldrich) was added to the cell pellet and left on ice for 5 min

110 followed by centrifugation at 500*g* for 5 min. The cell pellet was resuspended in 500μl PBS, and leukocytes were

111 counted using a hemocytometer. Specifically, 10μl was removed for cell counting (performed in triplicate) using a

112 hemocytometer. Cells were plated in sterile 12 well plates at 5e5/well (total of 8 wells) and use complete DMEM

113 with 25ng/ml supGM-CSF.

114 *Culturing of Alveolar Macrophages*

115 24hrs post-BALF isolation, media was removed and fresh complete DMEM with 25ng/ml supGM-CSF is added.

116 All cells that adhere to the surface of the plate are considered alveolar macrophages (AM) as previously determined

117 by Chen *et al.* (PMID:3288696). After new media is added, AMs are stimulated with 200ng/ml LPS (Sigma, L2630-

118 10MG). Harvest supernatant 6hrs post-stimulation. Harvested supernatant was sent to Eve technologies for cytokine

119 analysis. Statistics were performed using GraphPad prism.

120 *Cytotoxicity Assay*

121 Cytotoxicity was assessed by measuring the release of LDH into the media (LDH-Cytotoxicity Colorimetric Assay

122 Kit II; BioVision) according to the manufacturer's protocol.

123 **Results:**

124 *Production of a murine GM-CSF-secreting HEK-293T cell line*

125 A murine GM-CSF (mGM-CSF) containing plasmid (Addgene, 74465) was used as a template to amplify mGM-

126 CSF, which we inserted into a plasmid with a pSico backbone (SFig1, Supplemental File). The vector contains a

127 bidirectional EF1a promoter driving puromycin resistance gene and mCherry on one side and mGM-CSF on the

128 other (SFig1B, Fig1A). Lentivirus containing mGM-CSF, or a control construct was generated and transduced into

129 HEK293Ts and monitored using flow cytometry (SFig2). Post puromycin selection for 7 days, our control and

130 mGM-CSF constructs were incorporated into the genomes of HEK293T cells (at a rate of >85%) (Fig1B). In order

131 to determine how much GM-CSF was being produced by the HEK293T line we plated and grew $2x10^6$ cells for 3

132 days, isolated the supernatant and performed an ELISA to measure the concentration which was found to be

133 181.7ng/ml (Extended Methods). The cell line was subsequently frozen down into aliquots and thawed to assess

134 stability. Protein secretion was assessed again, by ELISA, and the stock secreted mGM-CSF at 181.9±ng/ml

135 (Fig1C). There was no significant difference between the concentration of mGM-CSF in the supernatant of the

136 initial or the freeze thawed HEK293T cell line indicating that the line is stable.

137 *Bone-Marrow Cells differentiated with supGM-CSF yield a higher number of Dendritic Cells compared to pGM-*

138 *CSF*

139 To assess the efficacy of the mGM-CSF-rich supernatant (supGM-CSF), bone marrow (BM) cells were treated with

140 10ng/ml or 25 ng/ml of supGM-CSF and compared to the same concentrations of a commercially obtained purified

141 GM-CSF (pGM-CSF) (*11–13*). As a control for differentiation, we also generated bone-marrow derived

142 macrophages (BMDMs) using supM-CSF obtained from L929 cells (*14*) (Fig2A).

143 Cells incubated with M-CSF, pGM-CSF and supGM-CSF were fully differentiated by day 7 and could be cultured

144 until day 14 (Fig2A). Morphologically, BM differentiated with supGM-CSF and pGM-CSF, at either 10ng/ml or

145 25ng/ml, have a more stellate morphology as is expected in dendritic cells compared to M-CSF differentiated

146 macrophage cells. There is no morphological difference between pGM-CSF or supGM-CSF differentiated cells, at

147 either 10ng/ml or 25ng/ml (Fig 2B). At day 9 of differentiation, cells were harvested and counted. When comparing

148 the pGM-CSF to the supGM-CSF, supGM-CSF yields significantly more viable cells in comparison to pGM-CSF

149 (Fig 2C). After culture with M-CSF, pGM-CSF or supGM-CSF, cells were assessed for purity by flow cytometry

150 based on previously published panels (*15, 16*). After gating on Live+/CD45+/CD11b+ cells purity was determined

151 based on the proportion of the population expressing CD11c and F4/80, shown as a quadrant (SFig3, Fig2D-H).

152 Percent of cells in quadrant 2 (F4/80+ and CD11c+) was significantly higher for all GM-CSF treated cells, in

153 comparison to M-CSF treated cells, as is expected since CD11c is a dendritic cell marker. However, pGM-CSF and

154 supGM-CSF were not significantly different (Fig2 I). As expected, the mean fluorescence intensity for dendritic

155 cell marker CD11c+ cells was significantly higher for all GM-CSF treated cells in comparison to M-CSF (Fig2 J),

156 while F4/80+ expression was equal for every treatment (Fig2 K). While supGM-CSF is able to generate more viable

157   cells than pGM-CSF, there is no significant difference in cellular purity between supGM-CSF and pGM-CSF (both

158   at ~85% purity).

159   *BMDCs generated using supGM-CSF function as efficiently as BMDCs generated using pGM-CSF*

160   Dendritic cells are the professional antigen presenting cells of the immune system. In order to test the ability of our

161   supGM-CSF to generate functional DCs we measured their levels of MHC-Class II expression using flow cytometry

162   (*17*). M-CSF differentiated cells express little MHC class II, while pGM-CSF and supGM-CSF express robust levels

163   of MHC class II, when differentiated with 10ng/ml or 25ng/ml concentration of GM-CSF (Fig3A-B, SupFig3A-B).

164   Dendritic cells differentiated with 10ng/ml GM-CSF show statistically higher mean fluorescence intensity for MHC

165   class II for supGM-CSF in comparison to pGM-CSF (SFig.4 C). Interestingly, dendritic cells differentiated with

166   25ng/ml of either pGM-CSF or supGM-CSF exhibit no significant difference in MHC class II protein expression

167   when evaluated by mean fluorescence intensity (Fig.3 C). In addition to assessing MHC Class II levels, we also

168   assessed the ability of the pGM-CSF and supGM-CSF BMDCs to respond to inflammatory stimulation. Both pGM-

169   CSF and supGM-CSF BMDCs were stimulated with LPS (200ng/ml) for the indicated time course and IL6 mRNA

170   expression was measured by quantitative PCR (qPCR) (Fig.3 C). Thus, determining that the supGM-CSF derived

171   cells maintain their inflammatory activity. Finally, AIM2 inflammasome activity was measured through IL6 and

172   IL1b protein secretion of both purified and supernatant GM-CSF. Here we observe that supGM-CSF produces

173   significantly higher expression of IL6 and IL1b (Fig.3 D-E) suggesting that these dendritic cells are more sensitive

174   to inflammatory activation compared to DCs generated using pGM-CSF.

175   *supGM-CSF can maintain viable and inflammatory inducible Alveolar Macrophages*

176   mGM-CSF is a critical protein factor that is not only necessary for driving primary dendritic cell differentiation,

177   but also for maintaining primary alveolar macrophages (AM) in culture (*18*, *19*). To test the ability of supGM-CSF

178   to maintain AM in culture, we harvested bronchiolar lavage fluid (BALF) from 10 WT wild-type mice. We pooled

179   these cells, counted and then plated them in 25ng/ml supGM-CSF supplemented media. 24 h post-harvest, using a

180   light microscope, we tested that AMs attached (Fig. 4). Viability of AMs were assessed by measuring the amount

181   of lactate dehydrogenase (LDH) in the media. The amount of measured LDH correlates directly with the cell number

182   lysed (*20*). Our data indicate that supGM-CSF does not negatively affect the cell culture, while the controls indicate

183 there are healthy cultured cells not resistant to apoptosis (Fig.4 B). AMs were stimulated with LPS for 24 hr and

184 cytokines were measured by ELISA. Inflammatory inducible proteins are significantly upregulated in AMs cultured

185 in supGM-CSF media, including proteins IL6, TNFa, MDC, MCP-1, IP10, KC and Rantes (Fig.4 C-I). supGM-

186 CSF cultured primary alveolar macrophages maintain their pro-inflammatory activation programming.

187 ***Discussion:***

188 Dendritic cells and macrophages have been cultured and studied for the last 40 years, leading to many

189 advances in culturing protocols. Flt3L is often utilized as a factor for BMDC differentiation, it is now appreciated

190 that Flt3L DCs are representative of steady-state resident DCs, while GM-CSF BMDCs mirror the transcriptional

191 programing of pro-inflammatory recruited cells (*21*, *22*). Granulocyte-macrophage colony-stimulating factor (GM-

192 CSF), differentiated bone marrow cells are widely used as a model system for conventional DC development (*23*,

193 *24*), as well as sustaining primary alveolar macrophages in culture (*25*). Current strategies for the generation of

194 murine BMDCs or culturing of primary alveolar macrophages utilize store bought purified recombinant GM-CSF

195 or a stable cell-line expressing recGM-CSF called J558L (*10*). J558L, a murine GM-CSF, secreting cell-line is

196 utilized for both BMDC and alveolar macrophage culturing, however it is an immune cell that also secretes IL-10

197 which can alter the transcriptional programing of the cells when culturing and only secretes 80ng/ml (*10*, *18*, *19*).

198 Using supernatant from GM-CSF secreting HEK293T cells, can serve as an alternative to purified GM-

199 CSF for murine BMDCs or maintenance of primary alveolar macrophages. We have successfully cloned and stably

200 integrated murine GM-CSF into HEK293T cells, which consistently secrete 180ng/ml of GM-CSF (Fig. 1). Bone

201 marrow differentiated with our supGM-CSF produces more cells by day 9 compared to pGM-CSF, but both GM-

202 CSF sources generate an equal percentage of pure dendritic cells based on previously published gating strategies

203 (*15*, *16*) (Fig1C and I). Additionally, using more GM-CSF also will produce more viable and proliferating cells,

204 which can be further enhanced with higher concentrations of supGM-CSF (Fig2C). Commercial GM-CSF can be

205 used instead of our HEK293T supGM-CSF, but it is expensive, and GM-CSF has to be added every 2 days to cells

206 during the differentiation process. Our supGM-CSF is much more cost effective compared to purified GM-CSF

207 from a number of companies (Supplemental Table 1). From one harvest of supGM-CSF you can generate ~10 ug,

208 which will be the cost of 50ml of complete media (Extended Methods), while 10 ug of purified GM-CSF from

209 Thermo Fisher will cost $276. The purity of BMDCs generated from pGM-CSF and supGM-CSF does not differ

210 when assessed by flow cytometry based on previously published panels (*15*, *16*) (SFig3, Fig2D-H).

211   DCs are professional antigen presenting cells and therefore express high levels of MHC Class II (*21*, *26*).

212 BMDCs differentiated with either Flt3L or GM-CSF have comparable T cell activation, and therefore MHC II

213 expression (*22*). BMDCs differentiated with either pGM-CSF or supGM-CSF express MHC-II at a comparable

214 level, when measured by mean fluorescence through flow cytometry (Fig3A-C).

215   As previously stated, picking a cell line to express a recombinant protein is very important. If an immune

216 cell is chosen, one risks the chance of having immune factors being secreted in the supernatant, leading to priming

217 and activation of either pro- or anti- inflammatory transcriptional programs (*10*, *18*, *19*). By performing a time-

218 course stimulation or overnight LPS stimulation, our study indicates supGM-CSF does not inhibit the inflammatory

219 program transcriptionally when measuring the transcript or protein level of IL6 (Fig 3D-E). supGM-CSF and pGM-

220 CSF differentiated BMDCs both retain the ability to activate the inflammasome pathway, leading to the secretion

221 of IL1b (Fig3D-F), while this pathway is now contested in DCs our results for GM-CSF is true (*27*).

222   Not only does our study provide data to support the use of supGM-CSF compared to pGM-CSF for BMDCs,

223 but we also show that supGM-CSF can be utilized to sustain the culturing of primary alveolar macrophages from

224 murine bronchoalveolar lavage fluid (BALF) (*10*, *18*). After 48 h of supGM-CSF cultured BALF, the adhered

225 alveolar macrophages (AMs) were >95% viable (Fig4B). More importantly, when stimulating the cells with LPS

226 overnight cytokines such as IL6, TNFa, KC and Rantes were all inducible (Fig. 4C-I). Thus, supGM-CSF maintains

227 AMs in culture and does not inhibit the pro-inflammatory programming of the immune cells.

228   Taken together our results show that a pure BMDC population and inflammatory inducible DCs or AMs

229 can be established by culturing BM cells or BALF with a crude supernatant from our GM-CSF HEK293T cell line.

230 This line will provide the research community with a more cost-effective alternative to commercially available GM-

231 CSF.

232 ***Funding***

234 ***Author Contributions:***

235   SCo and SCa has designed the study and cell line. EKR has analyzed data for all figures. SZ has performed all flow

236   cytometry for figures 1, 2 and 3. SZ has characterized the cell line and generated extended methods. EKR has

237   personally performed experiments for data used in Figure 3 and 4 and supplemental figure 3 and table. EKR has

238   written and edited manuscript. SCa, SCo, SZ have edited manuscript. SCa has provided necessary funding for

239   project.

240   ***Competing Interests:***

241   There are no competing interests.

242   ***Data and Materials Availability:***

243   Our vector maps are available in supplemental materials.

244

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313

314

315

316 *<u>Figure Legends:</u>*

317 **Figure 1: Generation of mouse GM-CSF secreting HEK293T cell line. (A)** Schematic of lentiviral plasmid

318 construct used to generate the stable HEK293T mGM-CSF expressing cell line. **(B)** Uninfected, Control plasmid

319 and mGM-CSF plasmid lentivirally infected HEK239T cells were puromycin selected for mCherry expression and

320 assessed using flow cytometry. **(C)** Protein secretion and cell-line stability of mGM-CSF HEK293T cells was

321 confirmed ELISA, using initial and stock cell-line. Error bars represent the standard deviation of biological

322 triplicates. Student's t-tests were performed using GraphPad Prism. (SN) indicates not significantly different.

323 **Figure 2: Generation of primary Dendritic Cells using purified GM-CSF or supernatant GM-CSF. (A)**

324 Schematic of differentiation of primary bone marrow-derived macrophages (BMMs) or primary bone marrow-

325 derived dendritic cells (BMDCs). **(B)** Images of primary BMMs and BMDCs under a light microscope after 7 days

326 of differentiation. **(C)** Total number of live cells generated from using one of 5 conditions: M-CSF, purified GM-

327 CSF (pGM-CSF) at 10ng/ml, pGM-CSF at 25ng/ml, supernatant GM-CSF (supGM-CSF) at 10ng/ml or supGM-

328 CSF at 25ng/ml. **(D)** Gating of CD45+ and CD11b+ M-CSF differentiated cells are represented in a quadrant

329 contour plot to determine the expression of F4/80 and CD11c. Same gating strategy is used for **(E)** pGM-CSF at

330 10ng/ml, **(F)** pGM-CSF at 25ng/ml, **(G)** supGM-CSF at 10ng/ml and **(H)** supGM-CSF at 25ng/ml. **(I)** Graphical

331 representation for the percentage of M-CSF or GM-CSF differentiated cells that are F4/80+ and CD11c+ (Q2). (J)

332 Graphical representation for the mean fluorescence of CD11c+. (K) Graphical representation for the mean

333 fluorescence of F4/80+. Error bars represent the standard deviation of n=5. Student's t-tests were performed using

334 GraphPad Prism. Asterisks indicate statistically significant differences between mouse lines ($*p \geq 0.05$, $**p \geq 0.01$,

335 $***p \geq 0.005$).

336 **Figure 3: Dendritic Cells differentiated with supGM-CSF retain Dendritic Cell activity and are more**

337 **inflammatory in comparison to pGM-CSF. (A)** Histogram overlay of CD45+ and CD11b+ M-CSF (grey) and

338 pGM-CSF (pink) or **(B)** supGM-CSF (orange) differentiated cells expressing MHC II. **(C)** Graphical representation

339 of MHC II mean fluorescence intensity (MFI) of M-CSF, pGM-CSF, or supGM-CSF differentiated cells. **(D)** Il6

340 transcript measure by RTq-PCR from pGM-CSF (pink) or supGM-CSF (orange) DCs stimulated with LPS for 0, 6,

341 and 24hrs. (E) Dendritic cells differentiated with pGM-CSF (pink) or sup-GM-CSF (orange) were treated with

342   lipofectamine, LPS (24hrs) or LPS (2hrs) and polydA:dT (6hrs) secreted IL-6 or IL-1β **(E)** was measured by ELISA.

343   Student's t-tests were performed using GraphPad Prism. Asterisks indicate statistically significant differences

344   between mouse lines (∗p ≥ 0.05, ∗∗p ≥ 0.01, ∗∗∗p ≥ 0.005).

345   **Figure 4: supGM-CSF can differentiate functional Alveolar Macrophages. (A)** Schematic of alveolar

346   macrophage supGM-CSF harvesting and differentiating experiment. **(B)** The cellular viability of differentiated

347   alveolar macrophages was measured by LDH, where media, cells, and lysis of cells were measured. **(C)** Secreted

348   IL6 protein was measured from supGM-CSF differentiated alveolar macrophages treated with and without LPS for

349   6hrs, as well as **(D)** TNFa, **(E)** MDC, **(F)** MCP-1, **(G)** IP10, **(H)** KC and **(I)** Rantes. Student's t-tests were performed

350   using GraphPad Prism. Asterisks indicate statistically significant differences between mouse lines (∗p ≥ 0.05, ∗∗p

351   ≥ 0.01, ∗∗∗p ≥ 0.005).

352   **Supplemental Figure 1: Validation of cloned murine GM-CSF. (A)** Image of PspXI and NotI digested PCR

353   product of murine GM-CSF. **(B)** Detailed 266 vector map of mGM-CSF, restriction sites, promoter, antibiotic

354   selection and mCherry sequence. **(C)** Alignment of Sanger sequencing results of mGM-CSF and 266 Vector map

355   strategy. **(D)** Quality of sanger sequencing results of murine GM-CSF.

356   **Supplemental Figure 2: mCherry expression in HEK293T cell line.** HEK-293T cell line lentiviral integration of

357   GM-CSF was selected using puromycin (2ug/ml) over a week. Flow cytometry was used to assess fluorescence

358   intensity.

359   **Supplemental Figure 3: Gating strategy for identifying dendritic cell and macrophage populations.** M-CSF

360   and GM-CSF differentiated cells were both put through the same gating strategy. The 5 gating plots are for the **(A)**

361   M-CSF, **(B)** pGM-CSF (10ng/ml), **(C)** pGM-CSF (25ng/ml), **(D)** supGM-CSF (10ng/ml), **(E)** supGM-CSF

362   (25ng/ml) differentiated cells. Non-debris cells were gated for in gate 1, then singlets in gate 2, followed by CD45+

363   and live cells were gated for in gate 3, then CD11b+ cells were gated in gate 4 finally this population of cells were

364   visualized using F4/80+ and CD11c+ markers and gate were put into quadrants.

365   **Supplemental Figure 4: Expression of MHC II on dendritic cell populations differentiated with 10ng/ml**

366   **pGM-CSF or supGM-CSF. (A)** Histogram overlay of CD45+ and CD11b+ M-CSF (grey) and pGM-CSF (pink)

367   or **(B)** supGM-CSF (orange) differentiated cells expressing MHC II. **(C)** Graphical representation of MHC II mean

368   fluorescence of M-CSF, pGM-CSF, or supGM-CSF differentiated cells. Student's t-tests were performed using

369   GraphPad Prism. Asterisks indicate statistically significant differences between mouse lines ($*p \geq 0.05$, $**p \geq 0.01$,

370    $***p \geq 0.005$).

371

372   *__Extended Methods__*

373   Comprehensive Methods in the Creation of an mGM-CSF-Producing HEK293T Cell Line, Collection of mGM-

374   CSF-rich Supernatant, and Using Cell Line Supernatant to Generate Dendritic Cells From Myeloid Progenitors

375   *__Creating Cell Line__*

376   1. Acquire mGM-CSF gene (Addgene: Plasmid #74465)

377   a. Sequence to check for correct composition

378   2. PCR mGM-CSF gene

379   a. Create and acquire primers with required restriction sites (NotI-HF and PspXI)

380   b. PCR

381   3. Purify/isolate mGM-CSF gene (band size?)

382   4. Ligation of mGM-CSF gene with 681 bidirectional vector plasmid

383   a. Restriction digest 681 bidirectional vector with Not1-HF and PspXI and purify

384   b. Restriction digest modified mGM-CSF gene with Not1-HF and PspXI and purify

385   c. Ligate vector with gene

386   5. Transform E. coli. with 681 + GM-CSF plasmid construct

387   6. Extract and isolate 681 + GM-CSF plasmid construct

388   a. Incubate E. coli in liquid LB

389   b. Miniprep

390   c. Colony PCR and run through the gel to determine sample with GM-CSF gene inserted in 681

391   vector

392   7. Create lentiviral constructs containing plasmid

393   8. Infect HEK293 cells using lentiviral constructs

394   9. Select for cells with 681 + GM-CSF plasmid construct

395   a. Allow cells to recuperate from infection and grow to confluence

396   b. Puro-select

397   c. FACS to determine concentration of mCherry+ cells (>90% required)

398 *Collecting mGM-CSF-rich supernatant*

399 1. Allow for HEK293 cells with plasmid construct to grow to confluency

400 2. (To be redone in T-175) Plate 2 million cells per 10 cm plate

401 3. Incubate for 3 days

402 4. Collect supernatant

403 5. If needed to confirm the concentration of mGM-CSF in the supernatant, perform ELISA (~200 ng/ml)

404 **In a T-175 flask*

405 1. Allow for HEK293 cells with plasmid construct to grow to confluency

406 2. Plate 9 million cells in 50 ml DMEM per T-175 flask

407 3. Incubate for 3 days

408 4. Collect supernatant

409 5. If needed to confirm the concentration of mGM-CSF in the supernatant, perform ELISA (~200 ng/ml)

410 *Generation of Bone-Marrow-Derived DCs*

411 *Day 0*

412 1. Sacrifice one or two mice and reserve femur and tibia of mice

413 2. Extract bone marrow cells from femur and tibia

414 3. In 6 well plate, plate equal amounts of cells into each well

415   a. Total media should be equal to 2 ml per well (cells + DMEM + cytokine)

416 4. Incubate for three days
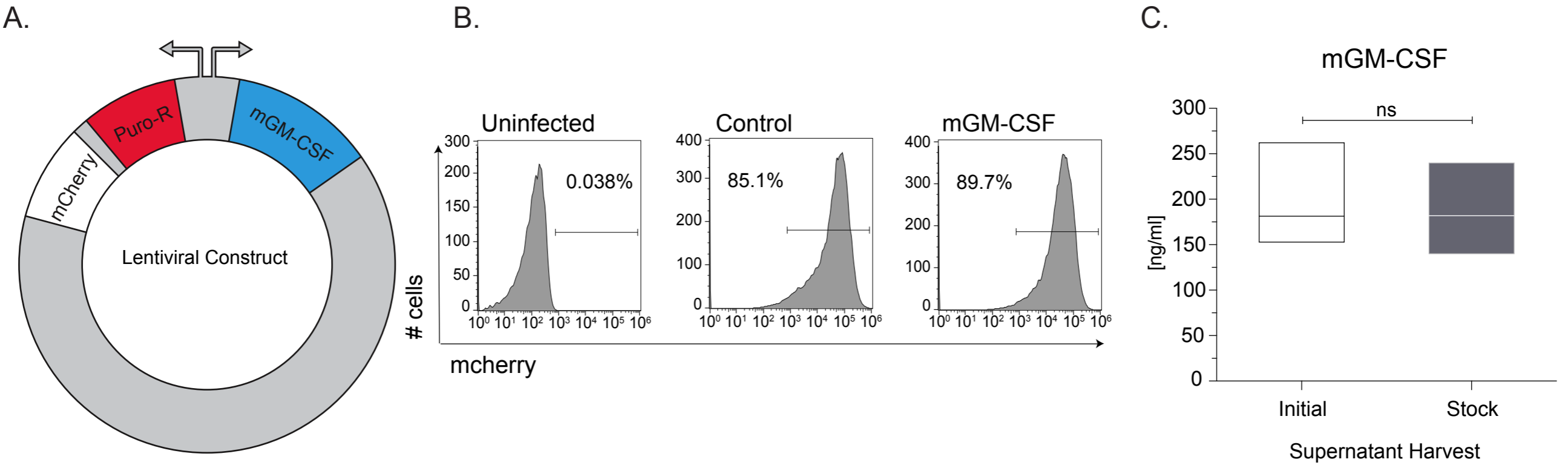
417 *Day 3*

418 5. On third day of differentiation, wash off dead cells

419   a. Aspirate media

420   b. Perform a rough PBS wash

421   c. Aspirate PBS

422   d. Replace media
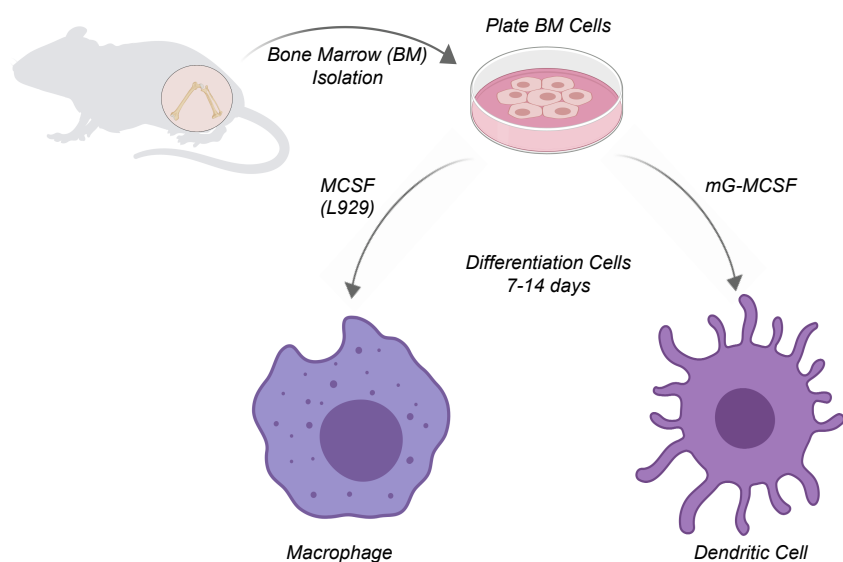
423 *Day 4 (if cells reach confluency)*

424   6. On fourth day of differentiation, move cells onto 10 cm plate

425   a. Save conditioned media

426   b. Perform normal PBS wash?

427   c. Aspirate PBS

428   d. Using cell scraper, gently scrape cells off plate

429   e. Transfer cells onto 10 cm plate

430   f. Replace media

431   i. Total media should be 10 ml (cells + DMEM + cytokine)

432   7. Incubate for 2-3 days

433 *Day 5 or 6*

434   8. Replace media

435   a. Save conditioned media

436   b. Perform normal PBS wash?

437   c. Aspirate PBS

438   d. Replace media (10 ml total; 6.750 ml DMEM + 2 ml conditioned media + ~1.250 ml supernatant)

439   9. Incubate for 1-2 days

440 *Day 7 or until usage before day 14*

441   10. Replace media every 2-3 days

442   11. Cells finished differentiation. Ready for use.

Figure 1.

A.

B.

C.

Figure 2

Figure 3.

Figure 4

A.

B.

LDH Release: Viability Assay

% Cytotoxicity vs (Media Only, Cells + Media, Lysis)

C.

Il6 (Protein)

[pg/mL] vs Treatment (Control, LPS)

D.

TNFα (Protein)

[pg/mL] vs Treatment (Control, LPS)

E.

MDC (protein)

[pg/mL] vs Treatment (Control, LPS)

F.

MCP-1 (protein)

[pg/mL] vs Treatment (Control, LPS)

G.

IP10 (protein)

[pg/mL] vs Treatment (Control, LPS)

H.

KC (protein)

[pg/mL] vs Treatment (Control, LPS)

I.

Rantes (protein)

[pg/mL] vs Treatment (Control, LPS)

Supplemental Figure 1.

A.

PspXI/NotI
mGM-CSF
(450bp)

B.

Vector266_mGM-CSF_puro-cherryNLS
8456 bp

8000

1000

2000

3000

4000

5000

6000

mCherry

Puromycin RG

short EIF-1a promoter
promoter

mGM-CSF (CSF2) ORF

NotI_hMCSF_rev (3004 .. 3037)

NotI (3008)

PspXI (3446)
PspXI_hMCSF_fwd (3416 .. 3455)

C.

```
0>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~>0
1000<GNAAANNNGNNCAGTACNNGACATCACTTNNCCAGTNNNCCGCGCCNNTTCTCTAGGCACCGGTTCAATTGCCGACCCCTCCCCCCAACTNTCGGGGACT<901

1>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~acggtgggaggcctatataagca>23
900<GTGGGCGATGTGCGCTCTGCCCACTGACGGGCACNCTAGAGCTCTAGAGATATCAAGCTTCTCGAGGGTAGGCGTGTACGGTGGGAGGCCTATATAAGCA<801

24>gagctcgtttagtgaaccgtcagatcgcctggagacgccatccacgctgttttgacctccatagaagacaccgggaccgatccagcctccgctcgagcca>123
800<GAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCATAGAAGACACCGGGACCGATCCAGCCTCCGCTCGAGCCA<701

124>ccATGTGGCTGCagaatttacttttcctgggcattgtggtctacagcctctcagcacccacccgctcacccatcactgtcacccggccttggaagcatgt>223
700<CCATGTGGCTGCAGAATTTACTTTTCCTGGGCATTGTGGTCTACAGCCTCTCAGCACCCACCCGCTCACCCATCACTGTCACCCGGCCTTGGAAGCATGT<601

224>agaggccatcaaagaagccctgaacctcctggatgacatgcctgtcacgttgaatgaagaggtagaagtcgtctctaacgagttctccttcaagaagcta>323
600<AGAGGCCATCAAAGAAGCCCTGAACCTCCTGGATGACATGCCTGTCACGTTGAATGAAGAGGTAGAAGTCGTCTCTAACGAGTTCTCCTTCAAGAAGCTA<501

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500<ACATGTGTGCAGACCCGCCTGAAGATATTCGAGCAGGGTCTACGGGGCAATTTCACCAAACTCAAGGGCGCCTTGAACATGACAGCCAGCTACTACCAGA<401

424>catactgccccccaactccggaaacggactgtgaaacacaagttaccacctatgcggatttcatagacagcgcttaaaaccttTctgactgatatcccctt>523
400<CATACTGCCCCCCAACTCCGGAAACGGACTGTGAAACACAAGTTACCACCTATGCGGATTTCATAGACAGCGCTTAAAACCTTTCTGACTGATATCCCCTT<301

524>tgaatgcaaaaaaccaggccaaaaatgagcggccgcgtcgagagatggggggaggctaactgagctagcatgcatcctaaccggtactagcttagtcatgc>623
300<TGAATGCAAAAAAACCAGGCCAAAAATGAGCGGCCGCGTCGAGAGATGGGGGGAGGCTAACTGAGCTAGCATGCATCCTAACCGGTACTAGCTTAGTCATGC<201

624>accgggctgcaggaattcgatgacaggcgcagcttccgagggatttgagatccagacatgataagatacattgatgagtttggacaaaccaaaactagaa>723
200<ACCGGGCTGCAGGAATTCGATGACAGGCGCAGCTTCCGAGGGATTTGAGATCCAGACATGATAAGATACATTGATGAGTTTGGACAAACCAAAACTAGAA<101

724>tgcagtgaaaaaaatgccttatttgtgaaatttgtgatgctattgccttatttgtaaccattataagctgcaataaaca~~~~~~~~~~~~~~~~~~~~>802
100<TGCAGTGAAAAAAATGCCTTATTTGTGAAATTTGTGATGCTATTGCCTTATTTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATGNNNN<1
```

Legend:
purple = kozak
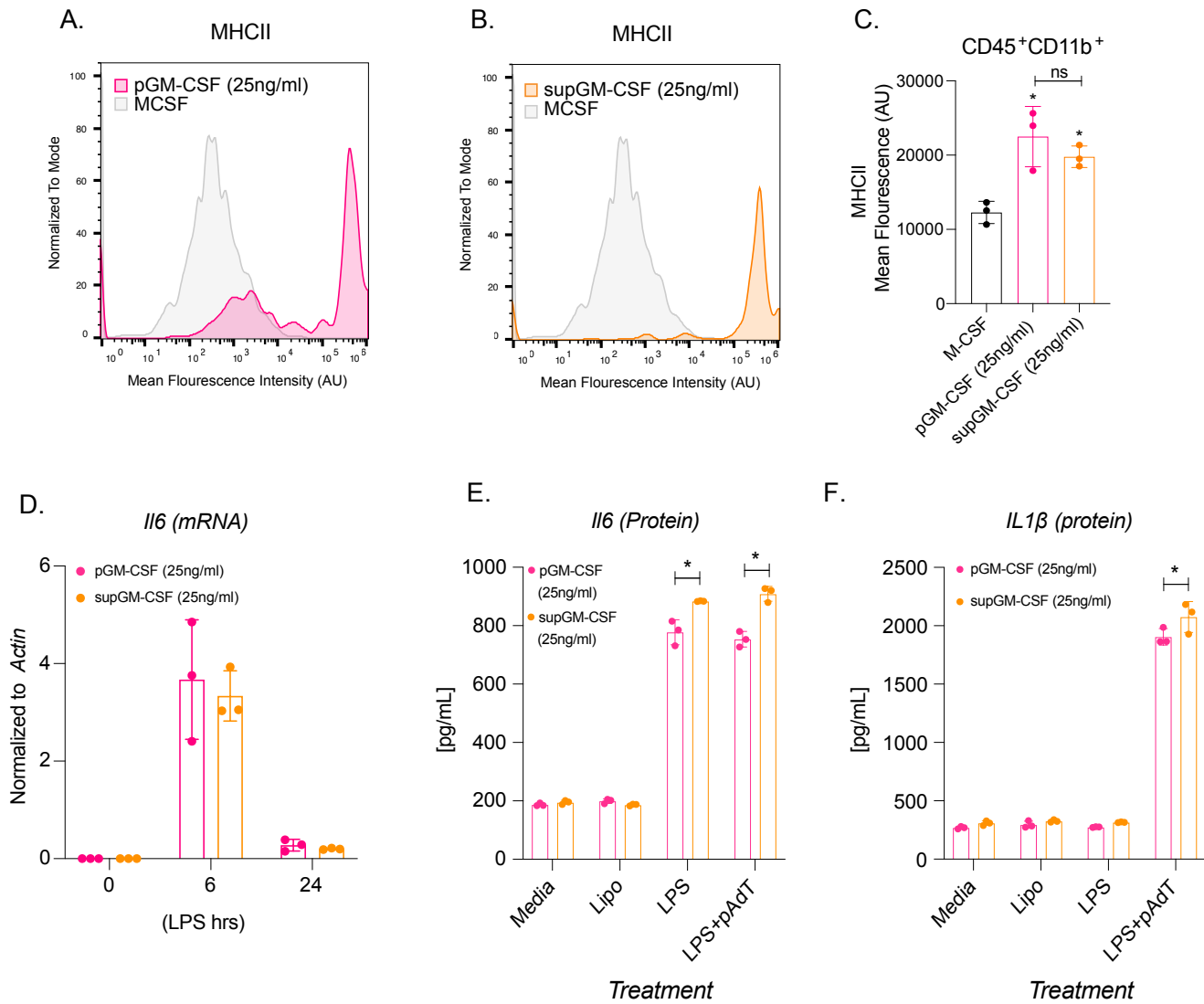green = ATG (start codon)
red = TGA (stop codon)
gray = GMCSF

D.

Supplemental Figure 2

Supplemental Figure 3

A. M-CSF

SSC / FSC — Gate 1

FSC-H / FSC — Gate 2
77.8

CD45 / Live/Dead — Gate 3
90.2

SSC / CD11b — Gate 4
98.8

F4/80 / CD11c
Macrophage	Dendritic Cell
Q1 97.0	Q2 0.76
Q4 1.97	Q3 0.24

B. pGM-CSF (10ng/ml)

SSC / FSC — Gate 1

FSC-H / FSC — Gate 2
54.2

CD45 / Live/Dead — Gate 3
73.3

SSC / CD11b — Gate 4
85.1

F4/80 / CD11c
Macrophage	Dendritic Cell
Q1 32.6	Q2 53.1
Q4 11.0	Q3 3.36

C. pGM-CSF (25ng/ml)

SSC / FSC — Gate 1

FSC-H / FSC — Gate 2
70.0

CD45 / Live/Dead — Gate 3
86.3

SSC / CD11b — Gate 4
92.2

F4/80 / CD11c
Macrophage	Dendritic Cell
Q1 40.4	Q2 44.0
Q4 13.6	Q3 1.93

D. supGM-CSF (10ng/ml)

SSC / FSC — Gate 1

FSC-H / FSC — Gate 2
58.6

CD45 / Live/Dead — Gate 3
78.8

SSC / CD11b — Gate 4
88.2

F4/80 / CD11c
Macrophage	Dendritic Cell
Q1 19.6	Q2 60.5
Q4 9.81	Q3 10.0

E. supGM-CSF (25ng/ml)

SSC / FSC — Gate 1

FSC-H / FSC — Gate 2
62.2

CD45 / Live/Dead — Gate 3
80.5

SSC / CD11b — Gate 4
89.5

F4/80 / CD11c
Macrophage	Dendritic Cell
Q1 32.3	Q2 56.2
Q4 8.63	Q3 2.87

Supplemental Figure 4

Supplemental Table 1: Cost of purified murine GM-CSF

	Biolegend			Abcam	Millipore Sigma			Thermo Fischer					Gold Biotechnology					
ug	10	100	500	20	5	20	50	2	10	25	100	1000	5	20	100	250	500	1000
ng	10000	100000	500000	2000	500	2000	5000	200	1000	2500	10000	100000	500	2000	10000	25000	50000	100000
$	125	650	1700	295	248	343	521	124	276	594	1202	3885	78	115	355	640	982	1584
$/ug	12.5	6.5	3.4	14.75	49.6	17.15	10.42	62	27.6	23.76	12.02	3.885	15.6	5.75	3.55	2.56	1.964	1.584
Catalog					G0282	SRP3201	G0282	PMC2014	PMC2015	PMC2016	PMC2011	PMC2013	1320-03-05	1320-03-20	1320-03-100	1320-03-250	1320-03-500	1320-03-1000
25ng/ml	400	4000	20000	80	20	80	200	8	40	100	400	4000	20	80	400	1000	2000	4000
	10ml per exp			**10ml per exp**	**10ml per exp**			**10ml per exp**					**10ml per exp**					
No./Exp	40	400	2000	8	2	8	20	0.8	4	10	40	400	2	8	40	100	200	400
Cost/Exp	3.125	1.625	0.85	36.875	124	42.875	26.05	155	69	59.4	30.05	9.7125	39	14.375	8.875	6.4	4.91	3.96
	25ml per exp			**25ml per exp**	**25ml per exp**			**25ml per exp**					**25ml per exp**					
No./Exp	16	160	800	3.2	0.8	3.2	8	0.32	1.6	4	16	160	0.8	3.2	16	40	80	160
Cost/Exp	7.8125	4.0625	2.125	92.1875	310	107.1875	65.125	387.5	172.5	148.5	75.125	24.28125	97.5	35.9375	22.1875	16	12.275	9.9
	50ml per exp			**50ml per exp**	**50ml per exp**			**50ml per exp**					**50ml per exp**					
No./Exp	8	80	400	1.6	0.4	1.6	4	0.16	0.8	2	8	80	0.4	1.6	8	20	40	80
Cost/Exp	15.625	8.125	4.25	184.375	620	214.375	130.25	775	345	297	150.25	48.5625	195	71.875	44.375	32	24.55	19.8