Supplemental figures and legends

Differentiation of Human Macrophages In Vitro

With Enhanced Antimycobacterial Activity

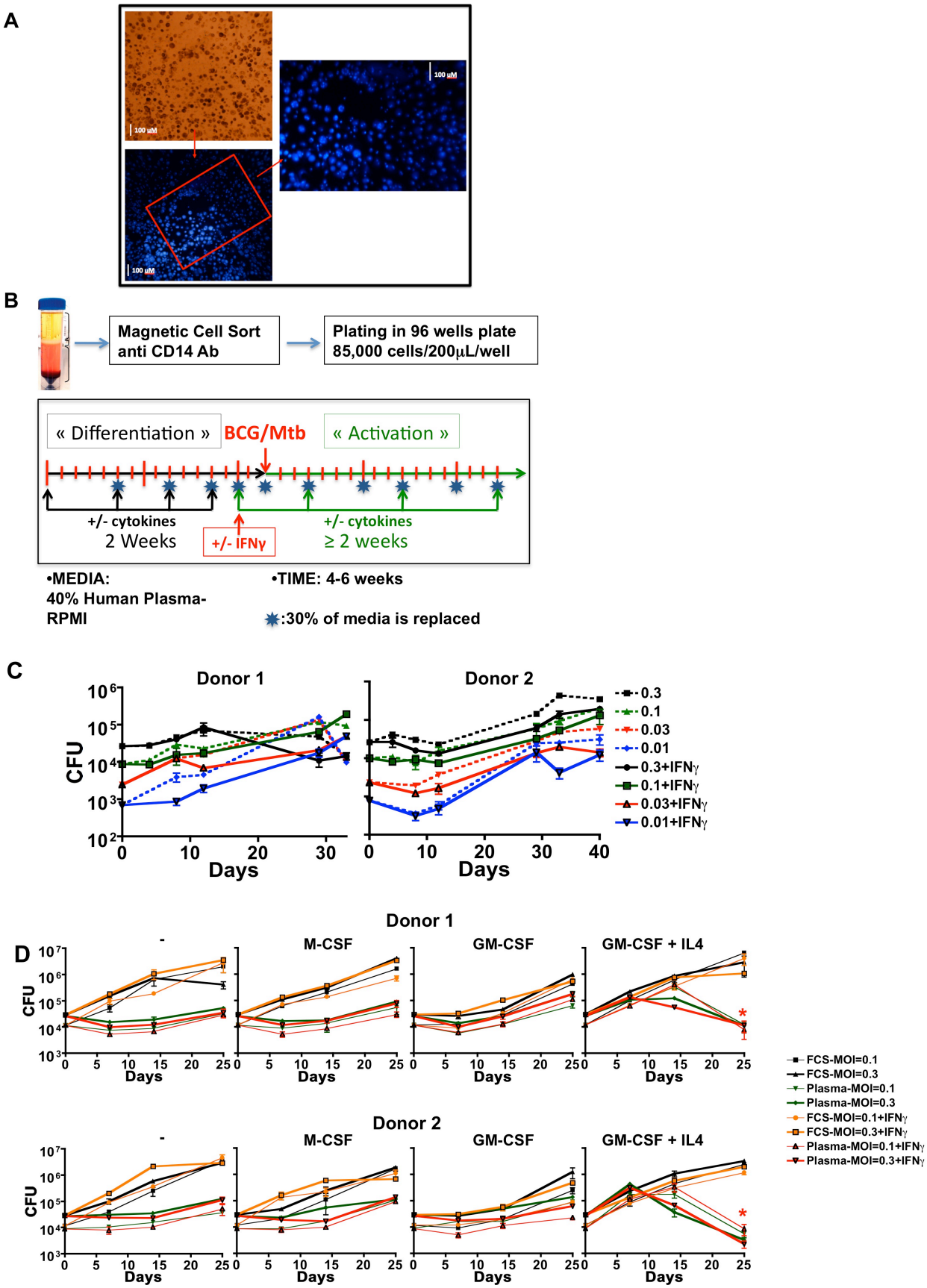
Guillaume Vogt[1] & Carl Nathan[1]

Department of Microbiology and Immunology, Weill Cornell Medical College,

1300 York Avenue, New York, NY 10065, USA

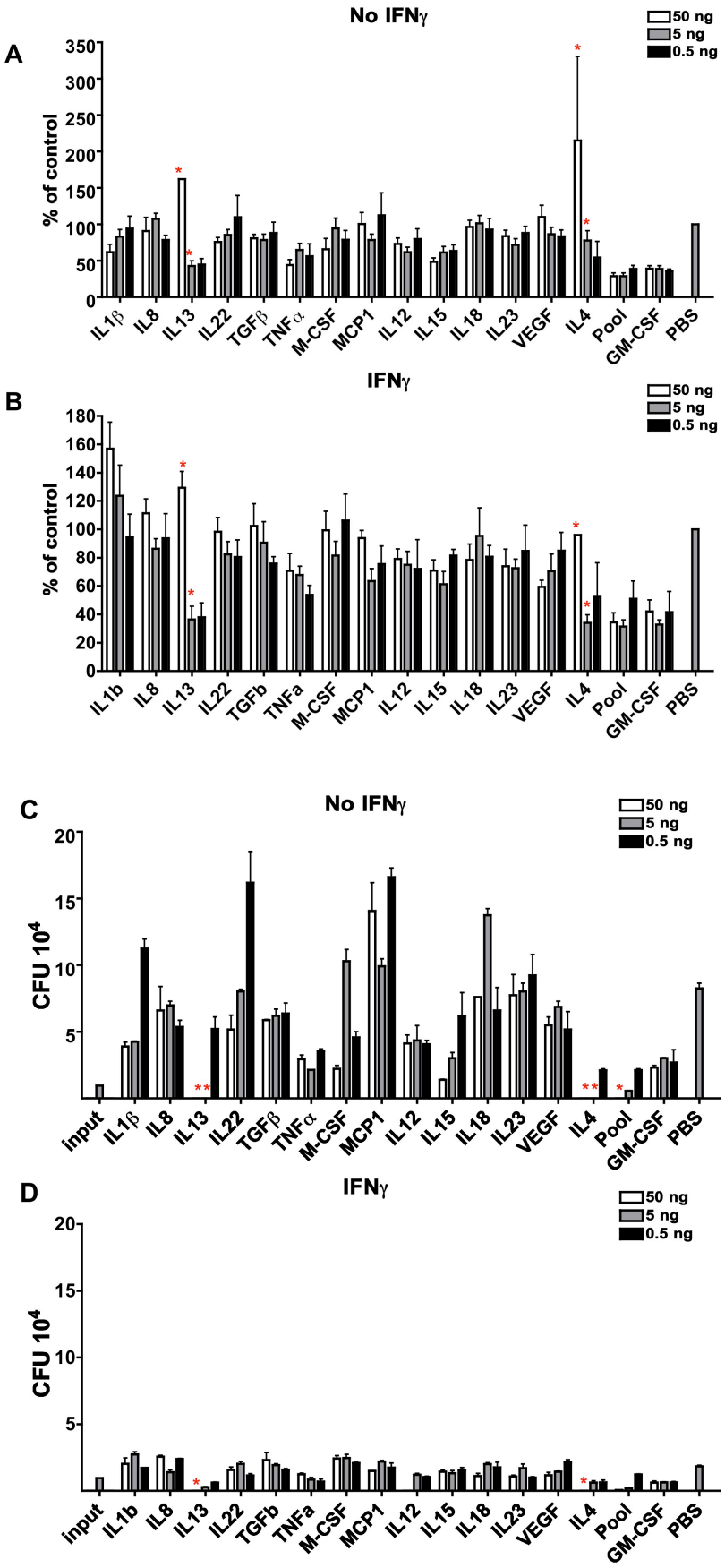
[1]To whom correspondence should be addressed at Guillaume Vogt, St. Giles Laboratory of Human Genetics of Infectious Diseases, Box 163, The Rockefeller University, 1230 York Avenue, New York, NY 10065. Tel: 212 327-7336 Fax: 212 327 7330. email guillaume.vogt@inserm.fr; Carl Nathan, Department of Microbiology and Immunology, Weill Cornell Medical College, 1300 York Avenue, New York, NY 10065, USA. Tel: 212 746 6505. Fax: 212746 8587. e-mail cnathan@med.cornell.edu

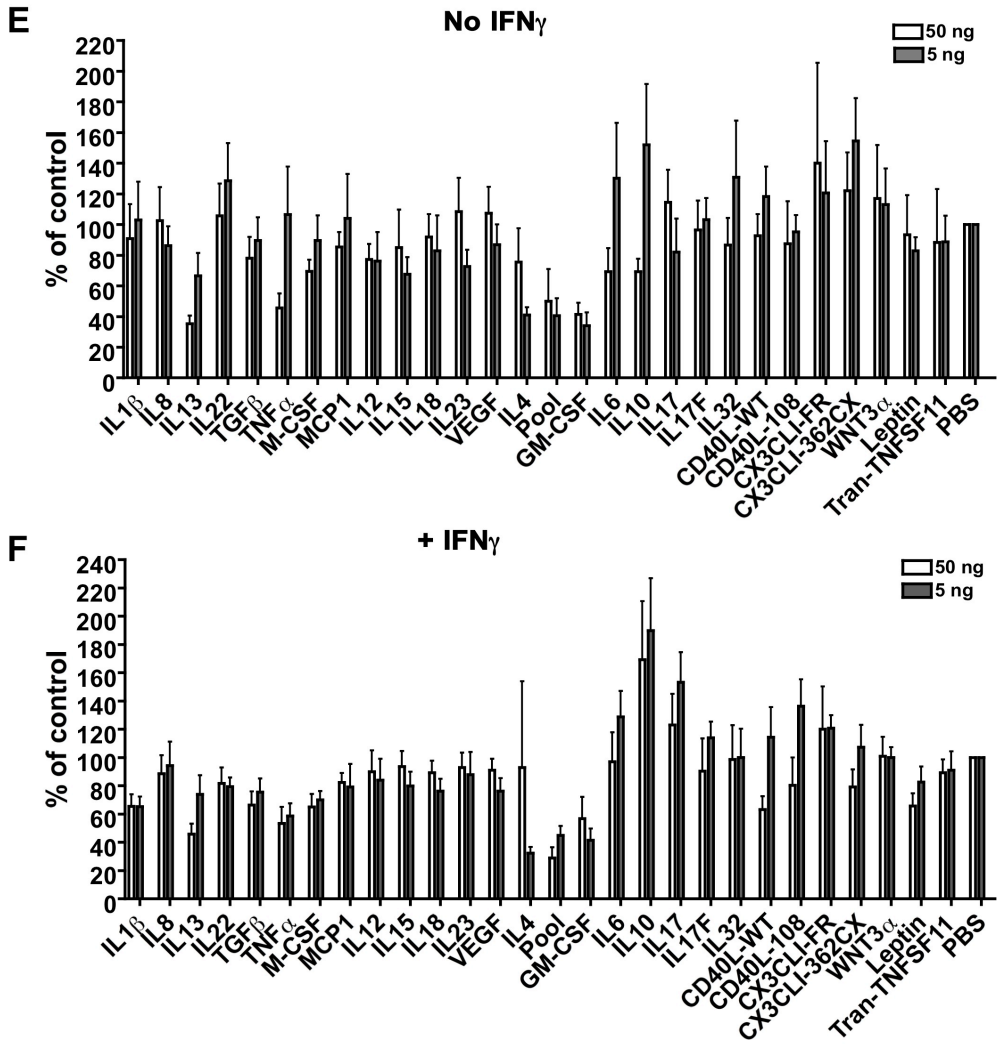
Supplemental Figure 1



Supplemental Figure 1. Impact of the MOI, and GM-CSF, M-CSF and IL-4 during the differentiation period on MDM control of BCG. (**A**) *Morphology of multinucleated giant cells.* MDM were differentiated with RPMI-40% human plasma (no added cytokines) for 14 days and micrographs were taken. Top panel, differential interference contrast. Other panels, nuclear staining with DAPI. Scale bar indicates 100 μm. (**B**) *Protocol of differentiation.* Peripheral monocytes purified by CD14 positive beads were were differentiated for 14 days with or without exogenous cytokines under various tensions of O_2. Cells were activated with the indicated cytokines on day 14 (and others indicated days) and infected with BCG or *M. tuberculosis* on day 16. Cells were lysed 2-8 weeks later to determine CFU. Blue stars represent changes of 30% of the medium. (**C**) *Effect of MOI on CFU.* MDM differentiated with RPMI-40% human plasma (no added cytokines)for 14 days were treated or not on day 14 with IFNg and infected with BCG at the indicated MOIs on day 16. Cells were lysed for CFU determination at the days following infection indicated on the X axis. Results from 2 donors are presented. (**D**) *Effect of cytokines during the differentiation period*. MDM were differentiated for 14 days as in (**C**) except that where indicated they were exposed to M-CSF, GM-CSF or the combination of GM-CSF + IL4. Each cytokine was at 50 ng/mL. On day 14, MDM were treated or not with IFNg infected with BCG at the indicated MOIs on day 16. Cells were lysed for CFU determination at the days following infection indicated on the X axis. The red "*" refers to visual cell destruction. Results from 2 donors are presented.

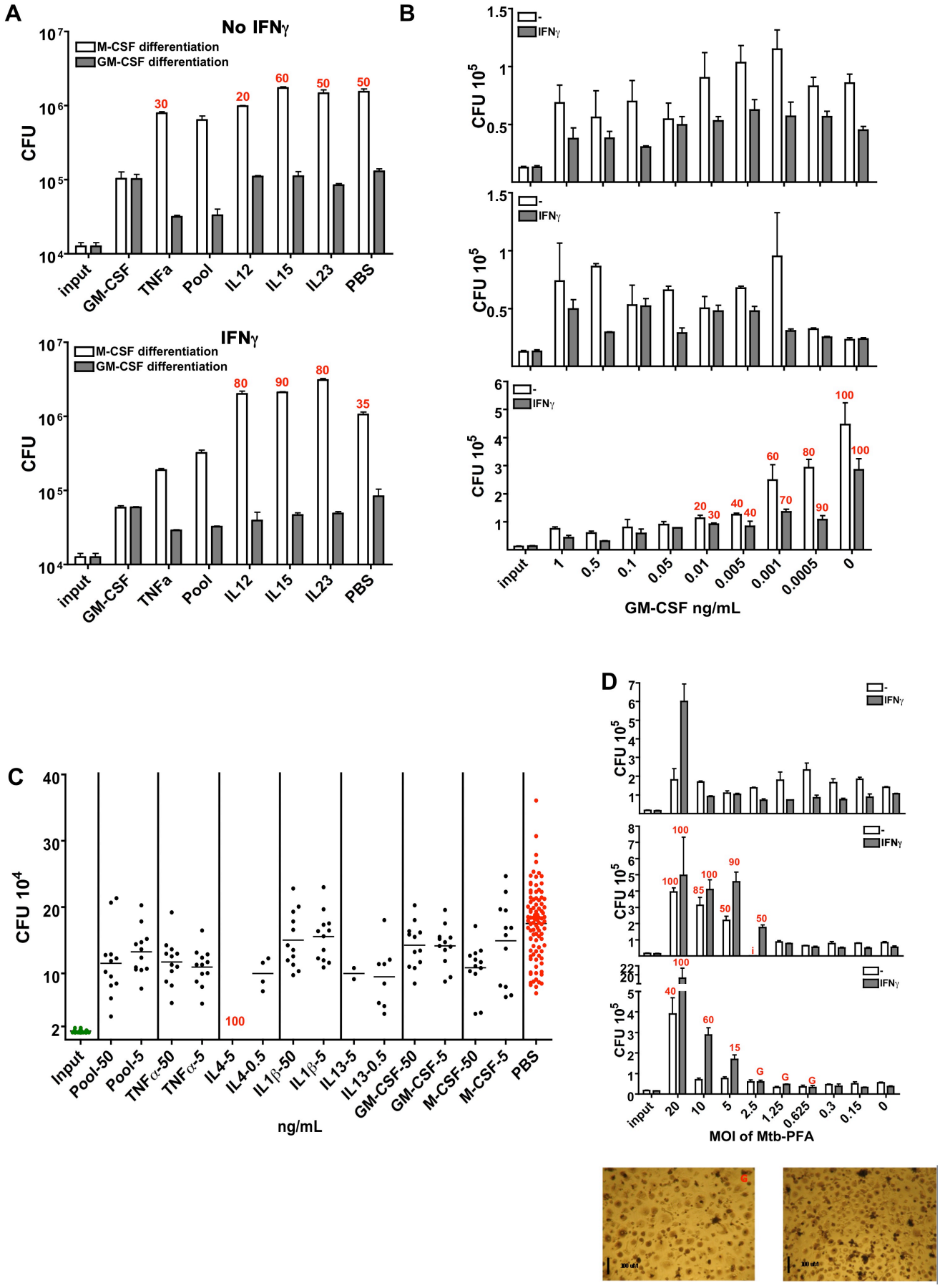
Supplemental Figure 2



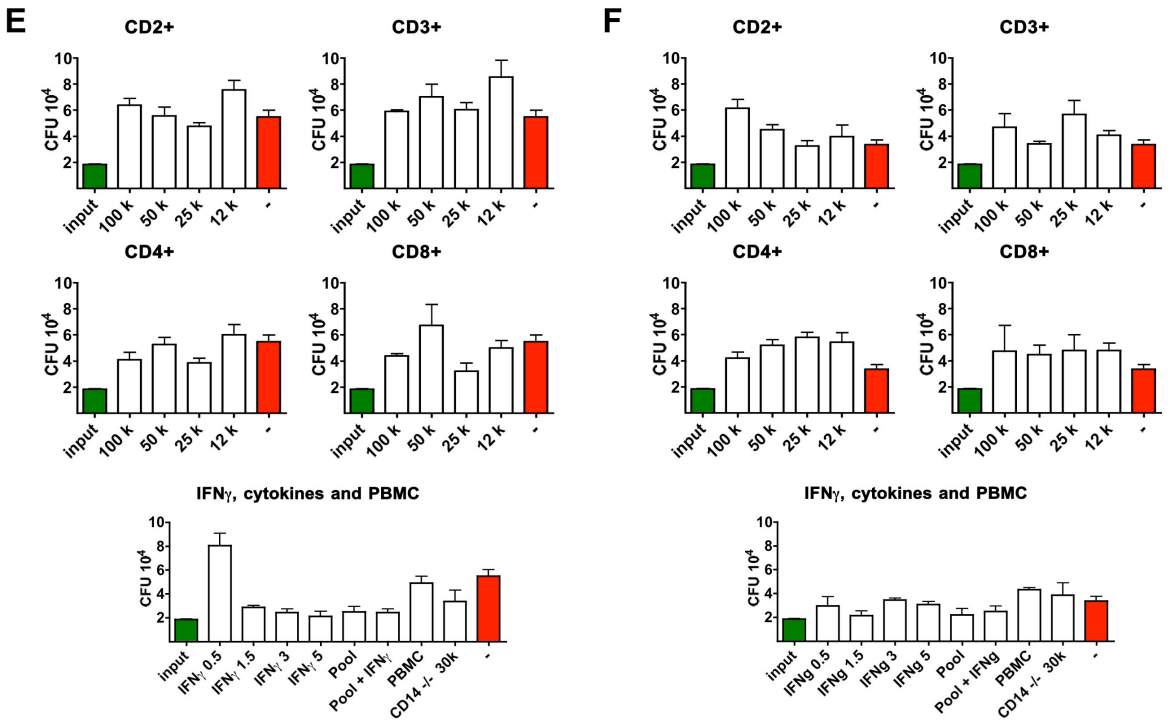


Supplemental Figure 2. Role of IFNγ and cytokines in the activation phase for the control and killing of BCG by MDM (A) and **(B)** *Role of cytokines in the activation phase* MDM were differentiated for 14 days with human plasma-RPMI. On day 14, MDM were treated **(B)** or not **(A)** with IFNγ (2.5 ng/mL) alone or with the other cytokines at the indicated concentrations. On day 16, MDM were infected *M. tuberculosis* (MOI = 0.1). Cells were lysed 3 weeks later to determine CFU. **(C)** and **(D)** MDM differentiated with human plasma-RPMI (no added cytokines) for 14 days were treated or not on day 14 without **(A)** and **(E)** or with **(B)** and **(F)** IFNg. Additionally, cells were activated with various concentrations of the indicated cytokines on day 14 and infected with BCG (MOI = 0.1) on day 16. Cells were lysed 3 weeks later to determine CFU. Percentage of control of BCG growth compared to MDM receiving PBS instead of cytokines. The red "*" refers to visual cell destruction. These experiments are representative of five independent experiments from two donors.

Supplemental Figure 3



Supplemental Figure 3

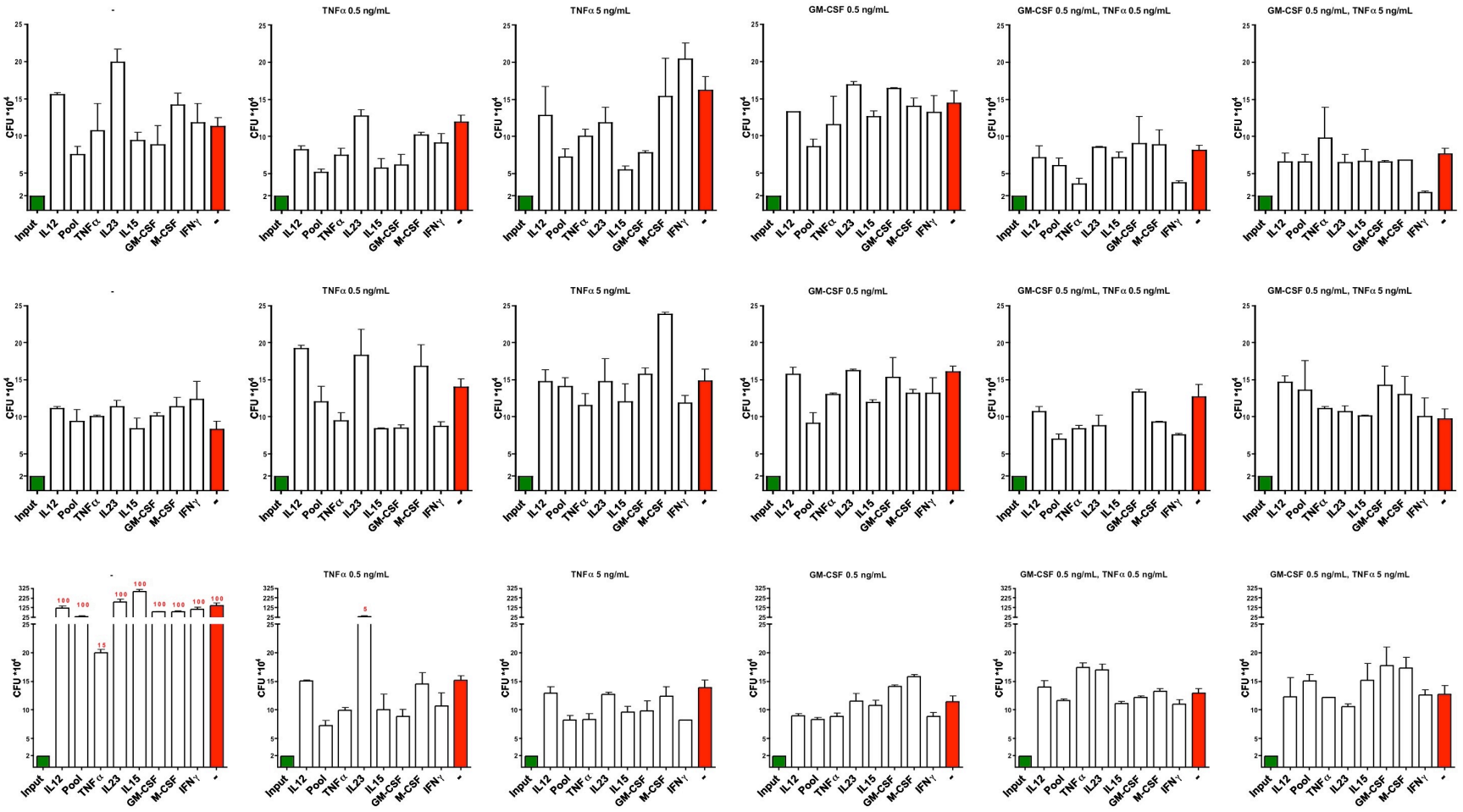


Supplemental Figure 3. MDM control of *M. tuberculosis*: Role of cytokines and PFA-fixed (killed) *M. tuberculosis* in the activation period and GM-CSF versus M-CSF during the differentiation period. (A) *Negative effect of M-CSF versus GM-CSF during the differentiation period.* MDM were differentiated for 14 days with human plasma-RPMI and exposed to M-CSF or GM-CSF (each cytokine was at 50 ng/mL) in 10% O_2. On day 14, MDM were treated without (upper panel) or with (lower panel) IFNγ (2.5 ng/mL) alone or with the other cytokines indicated. On day 16, MDM were infected with *M. tuberculosis* (MOI = 0.1). Cells were lysed for CFU determination at two weeks. Results were similar with cells from 4 donors **(B)** *Optimal concentration of GM-CSF during the differentiation period.* MDM were differentiated for 14 days as in **(A)** except that where indicated, on the X axis, they were exposed different amount of GM-CSF (from 1 to 0 ng/mL). On day 14, MDM were treated with or without IFNγ (2.5 ng/mL). On day 16, MDM were infected with *M. tuberculosis* (MOI = 0.1). Cells were lysed for

CFU determination at two weeks. Results are represented for 3 donors (upper, middle and lower panels). **(C)** *Role of cytokines in the activation phase.* MDM from three donors (each tested twice in independent experiments) were differentiated for 14 days with GM-CSF (0.5 ng/mL) with human plasma-RPMI in 10% O_2. On day 14, MDM were treated with IFNγ (2.5 ng/mL) alone or with the other cytokines indicated. On day 16, MDM were infected with *M. tuberculosis* (MOI = 0.1). Cells were lysed 2 weeks later to determine CFU. **(D)** *Effect of PFA-fixed M. tuberculosis during the activation period*. MDM from 3 donors were differentiated for 14 days as in **(c)**. On day 14, MDM were treated with or without IFNγ (2.5 ng/mL) alone or with various MOI of *M. tuberculosis* fixed by paraformaldehyde as indicated on the X axis. On day 16, MDM were infected *M. tuberculosis* (MOI = 0.1). Cells were lysed 2 weeks later to determine CFU. The red "G" refers to giant cells observed as illustrated in the micrographs in Fig. S3 taken 14 days after infection. In **A-D**, red numbers and "i" refer to cell destruction expressed in percent and to infected well, respectively, estimated visually. (**E**) and (**F**) *Effect of coculture with other cell types during the activation period on MDM for the control of M. tuberculosis.* MDM from two donors (donor 1 (**E**) and donor 2 (**F**)) were differentiated for 14 days with GM-CSF plus TNF-α (0.5 ng/mL each) under 7.5% O_2. Cells were activated with freshly purified autologous CD2+, CD3+, CD4+, CD8+, CD14- cells or PBMC at different cell densities ("k" refers to factor 1000) or by IFNγ at the indicated concentration on day 14 and infected with *M. tuberculosis* (MOI = 0.1) on day 16. Cells were lysed 2 weeks later to determine CFU.

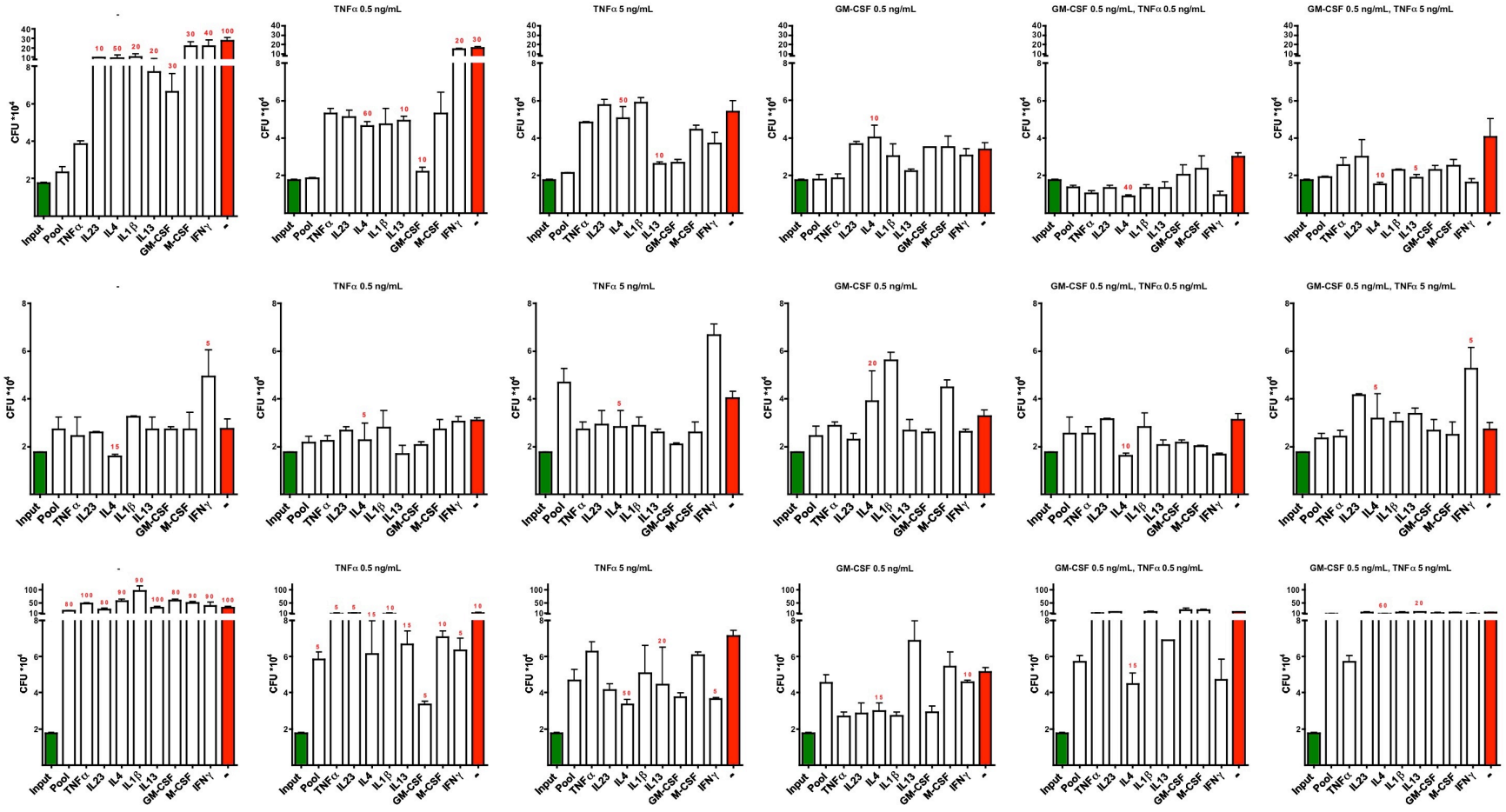
Supplemental Figure 4

A

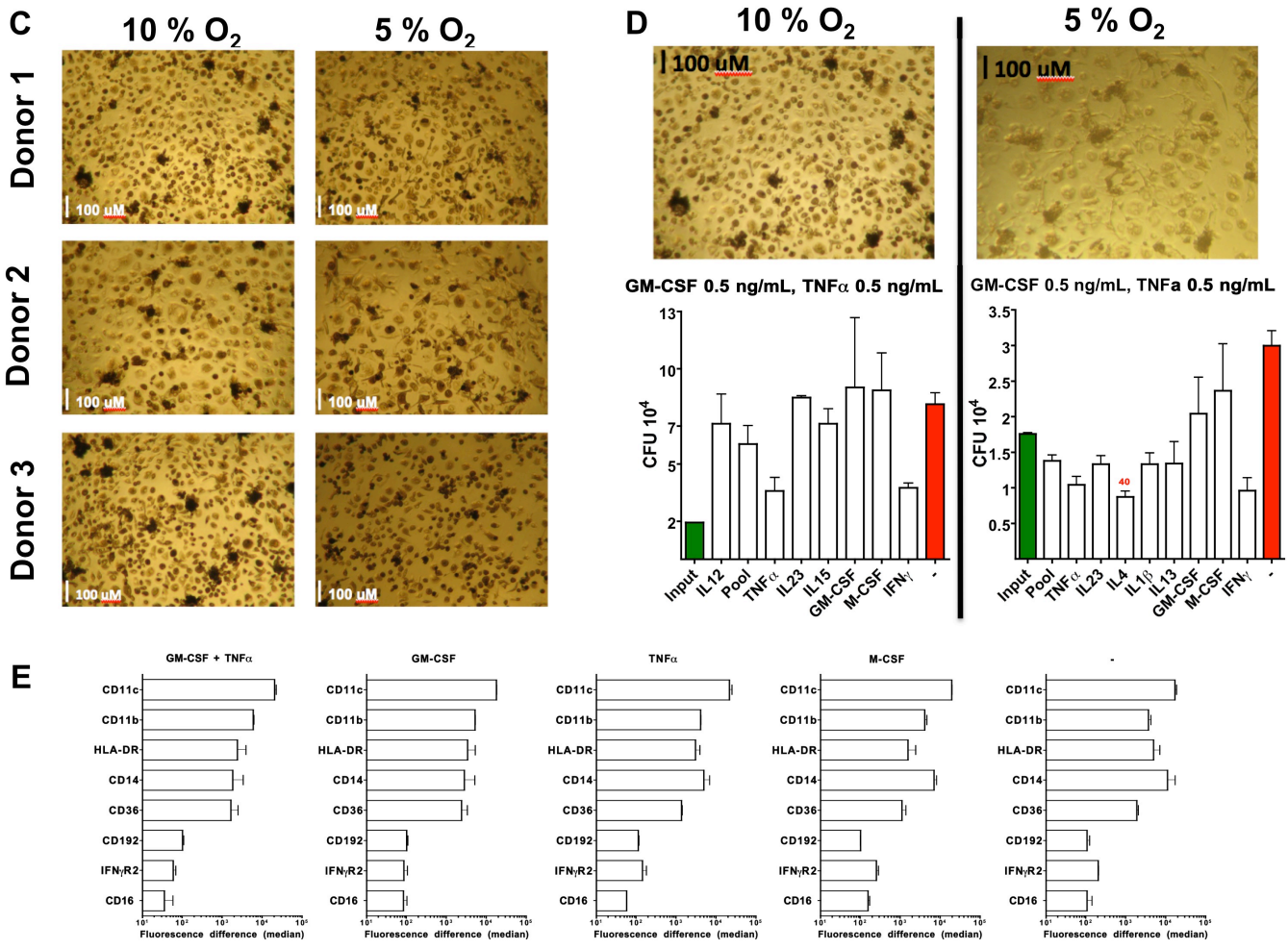


Supplemental Figure 4

B

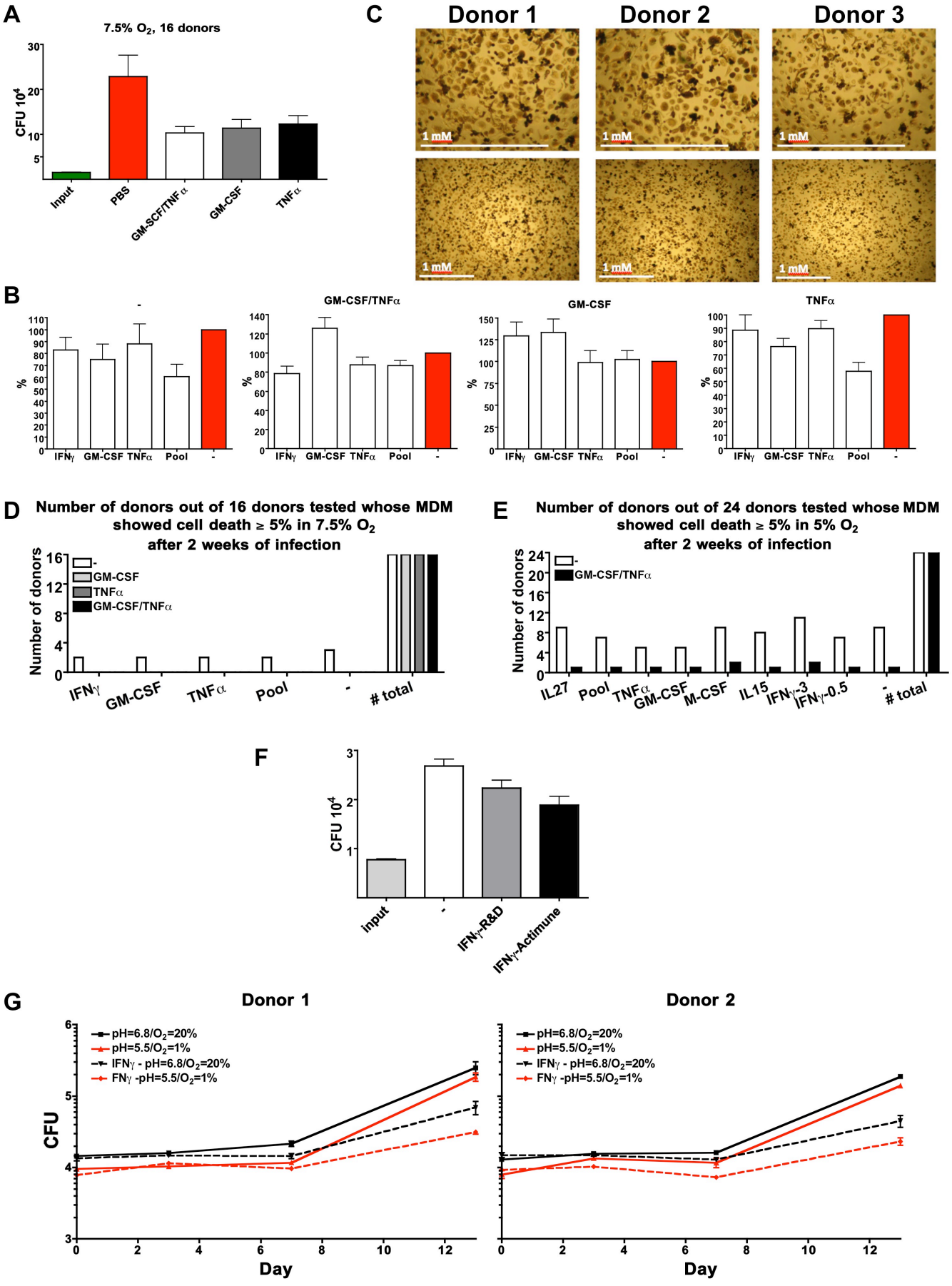


Supplemental Figure 4

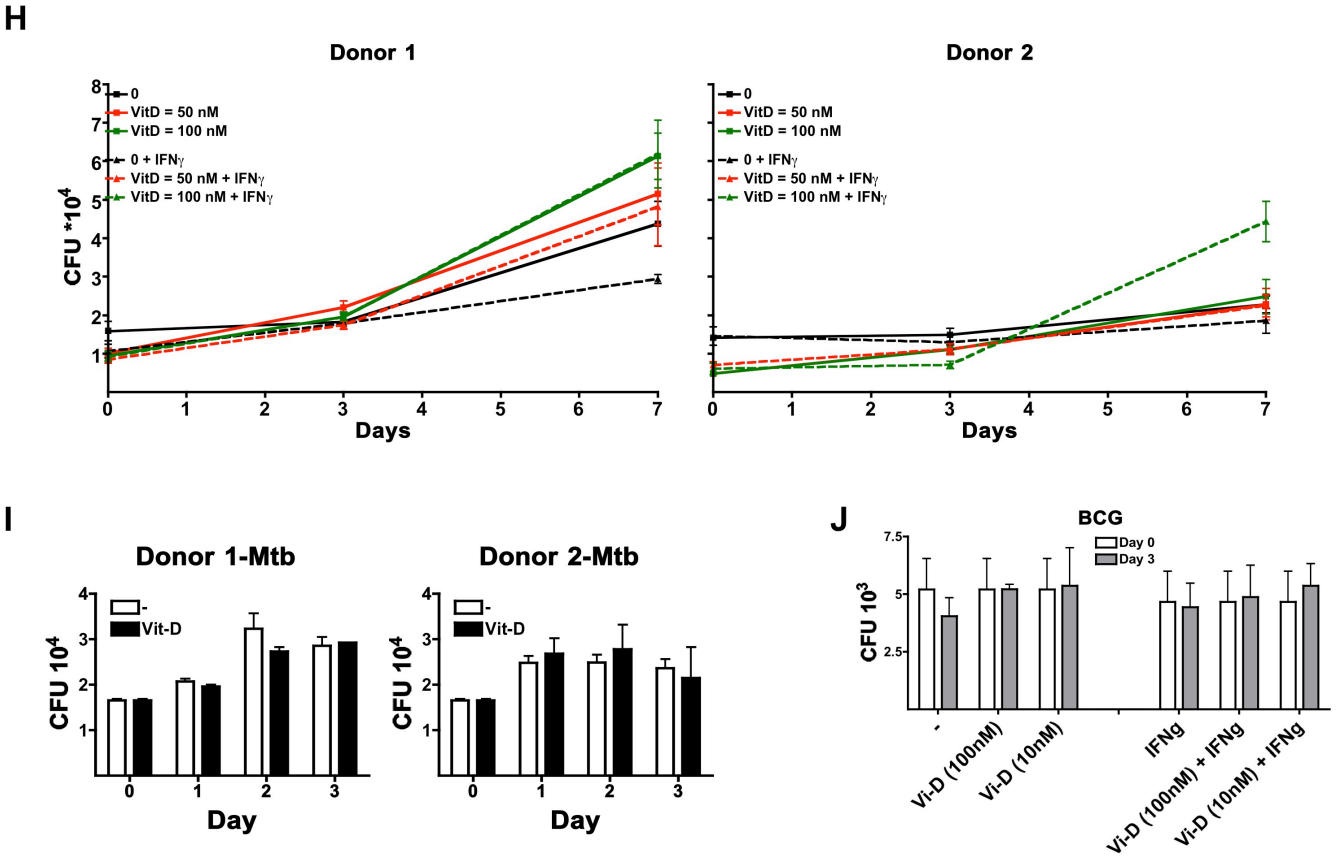


Supplemental Figure 4. Comparison of 10% and 5% O_2 and different combination of GM-CSF and TNFα during differentiation for control and killing of *M. tuberculosis* by MDM activated with various cytokines. (A) *CFU for cultures incubated in 10% O_2.* MDM were differentiated for 14 days with no exogenous cytokines, GM-CSF, TNF-α or with GM-CSF plus TNF-α (0.5 ng/mL for GM-CSF ; 0.5 and 5 for TNF-α) under 10% O_2. Cells were activated with the indicated cytokines (50 ng/mL each) on day 14 and infected with *M. tuberculosis* (MOI = 0.1) on day 16. Cells were lysed 2 weeks later to determine CFU. 3 donors are represented (upper, middle and lower panels). (**B**) *CFU for cultures incubated in 5% O_2.* Experiments were performed as in (**A**) except that the O_2 concentration was further reduced. (**C**) Morphology *of MDM under physiologic O_2 tension.* Cells were prepared as in **(A)** 10% O_2 and (**B**) 5% O_2 except that MDM were differentiated with GM-CSF (0.5 ng/mL) plus TNF-α (0.5 ng/mL) for the first 14 days and then activated with TNF-α (50 ng/mL). Micrographs were taken 14 days after infection in experiments with cells from three donors. Scale bar indicates 100 μm. (**D**) *Comparison of the control of M. tuberculosis under 5% and 10% O_2.* Cells were prepared as in **(A)** and (**B**) except that MDM were differentiated with GM-CSF (0.5 ng/mL) plus TNF-α (0.5 ng/mL) for the first 14 days and then activated with various cytokines (50 ng/mL) or with IFNγ. Micrographs from TNF-α activation were taken 14 days after infection to illustrate the cells' integrity. Scale bar indicates 100 μm. **(E)** ***Markers expressed by MDM.*** MDM were differentiated in 40% human plasma for 14 days with GM-CSF (0.5 ng/mL) plus TNF-α (0.5 ng/mL), GM-CSF (0.5 ng/mL), TNF-α (0.5 ng/mL), M-CSF (50 ng/mL) or without cytokines under 5% O_2. Cells were analyzed by flow cytometry. Results are means for cells from two different donors.

Supplemental Figure 5



Supplemental Figure 5



Supplemental Figure 5. Reproducibility of control of *M. tuberculosis* replication by MDM differentiated with different combinations of GM-CSF and TNFα and activated with various cytokines in physiologic levels of O_2. (A) *Reproducible control of M. tuberculosis with appropriately differentiated MDM that were activated with IFNγ in 7.5% O_2.* MDM from 16 donors were differentiated in 7.5% O_2 with no cytokines, GM-CSF, TNF-α or GM-CSF plus TNF-α (0.5 ng/mL each) for 14 days and infected with *M. tuberculosis* on day 16 (MOI = 0.19). Cells were lysed 2 weeks later to determine CFU. (**B**) *Comparison of IFNγ and other cytokines during the activation period at 7.5% O_2.* In the same experiments illustrated in (A), MDM were also activated with the indicated cytokines, each at 50 ng/mL except for IFNγ (3 ng/mL). CFU counts from cultures lysed 2 weeks after infection are presented as a percent of those for MDM given PBS instead of exogenous cytokines during the activation period. (**c**) Morphology *of MDM under physiologic O_2 tension.* Cells were prepared as in

(**A**) except that MDM were differentiated under 5% O_2. MDM were activated with TNF-α (50 ng/mL) on day 14 and infected with *M. tuberculosis* (MOI = 0.1) on day 16. Micrographs were taken 14 days after infection in experiments with cells from three donors. Scale bar indicates 1 mm (*sic*). (**D**) Number of donors (out of 16 tested) whose MDM underwent at least 5% cell death after two weeks of infection with *M. tuberculosis*, depending on the cytokines used for differentiation as shown in the inset and the cytokines used for activation as shown on the X-axis, both in 7.5% O_2. Conditions were otherwise as in (**A**) and (**B**). (**E**) As in (**D**) but testing MDM from 24 donors in 5% O_2. (**F**) Impact *of different sources of IFNγ*. MDM differentiated with RPMI-40% human plasma (no added cytokines) for 14 days were treated or not on day 14 with IFNγ from R&D or with the approved biologic "Actimune" and infected with BCG on day 16. Cells were lysed 3 weeks later to determine CFU. Means ± SD from 24 replicate wells for each condition in one experiment representative of 2 experiments each with a different donor. "*P*" refers to P-values from Student's t test, as compared with corresponding control. (**G**) *CFU for cultures infected in 5% O_2 with M. tuberculosis prepared by growth either at pH 6.8 under 20 % O_2 or at pH 5.5 under 1 % O_2*. MDM were differentiated for 14 days with GM-CSF plus TNF-α (0.5 ng/mL each) under 5% O_2. Cells were activated or not with IFNγ on day 14 and infected with *M. tuberculosis* (MOI = 0.2) on day 16 using *M. tuberculosis* prepared by two weeks of growth under two different conditions. MDM were lysed 0, 3 and 7 days later to determine CFU. Results are from 3 donors (upper, middle and lower panels). (**H**) *CFU for MDM differentiated with or without 1,25 dihydroxyvitamin D in 5% O_2*. MDM were differentiated in 10% FBS for 14 days with GM-CSF plus TNF-α (0.5 ng/mL each) with or without vitamin D (50 nM or 100 nM) under 5% O_2. Cells were activated with IFNγ on day 14 and infected with *M. tuberculosis* (MOI = 0.2) on day 16. Cells were lysed 0, 3 and 7 days later. Cellular destruction was observed after 7 days due to the culture in FBS. Results from 2 donors are represented. (**I**) *CFU for MDM activated with or without Vitamin D in 10% O_2*. MDM were differentiated in RPMI and 40% human plasma for 14 days with GM-CSF plus TNF-α (0.5 ng/mL each) under 5% O_2. Cells were activated with or without vitamin D (100 nM) on day 14 and infected with *M. tuberculosis* (MOI =

0.2) on day 16. Cells were lysed at different time points (day 0,1, 2 and 3). 2 donors are represented. **(J)** As in (**I**), but using BCG instead of *M. tuberculosis*.