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Supplemental Information

Stronger induction of trained immunity by mucosal BCG or MTBVAC vaccination compared to standard intradermal vaccination

Michel P.M. Vierboom, Karin Dijkman, Claudia C. Sombroek, Sam O. Hofman, Charelle Boot, Richard A.W. Vervenne, Krista G. Haanstra, Maarten van der Sande, Liesbeth van Emst, Jorge Domínguez-Andrés, Simone J.C.F.M. Moorlag, Clemens H.M. Kocken, Jelle Thole, Esteban Rodríguez, Eugenia Puentes, Joost H.A. Martens, Reinout van Crevel, Mihai G. Netea, Nacho Aguilo, Carlos Martin, and Frank A.W. Verreck

Stronger induction of trained immunity by mucosal BCG or MTBVAC vaccination compared to standard intradermal vaccination

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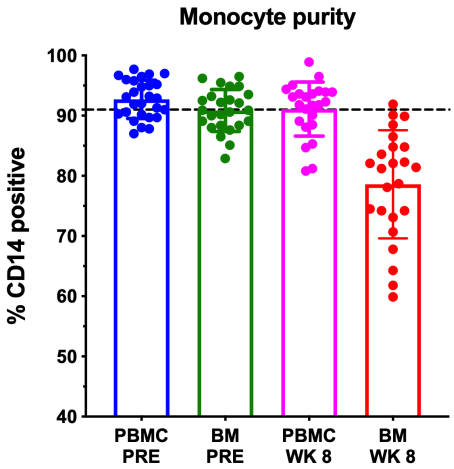
Supplemental Table 1. (Related to Figure 1) List of animal's identifiers, gender, and age and body weight at start of the experiment, per treatment group (including group averages with standard deviation between brackets)**.**

Treatment	Animal ID	Gender	Age (years)	Body Weight (kg)
BCG.id	R12050	M	6,4	12,8
	R12117	F	6,3	12,15
	R13046	F	5,5	6,25
	R13125	M	5,4	7,9
	R13130	M	5,3	11,1
	R13164	F	5,2	5,5
			[5.7 ± 0.5]	[9.3 ± 3.1]
BCG.muc	R12028	F	6,5	7,2
	R13073	F	5,4	6,1
	R13080	F	5,4	7,8
	R13154	M	5,3	10,6
	R13173	M	5,2	9,85
	R14034	M	4,4	7,8
			[5.4 ± 0.7]	[8.2 ± 1.7]
MTBVAC.id	R11024	F	7,5	5,55
	R13026	M	5,5	9,3
	R13067	M	5,4	12,8
	R13097	M	5,4	10,4
	R13141	F	5,3	9,3
	R14087	F	4,4	6,2
			[5.6 ± 1.0]	[8.9 ± 2.7]
MTBVAC.muc	R11015	F	7,5	7,5
	R11030	M	7,4	10,05
	R12157	F	6,2	7,75
	R13129	M	5,3	10,8
	R13139	F	5,3	6,3
	R14100	M	4,4	7,65
			[6.0 ± 1.3]	[8.3 ± 1.7]
BCG.iv	R13004	M	5,5	8,5
	R13150	M	4,2	5,75
	R14172	M	5,3	9,4
			[5.0 ± 0.7]	[7.7 ± 1.4]

A



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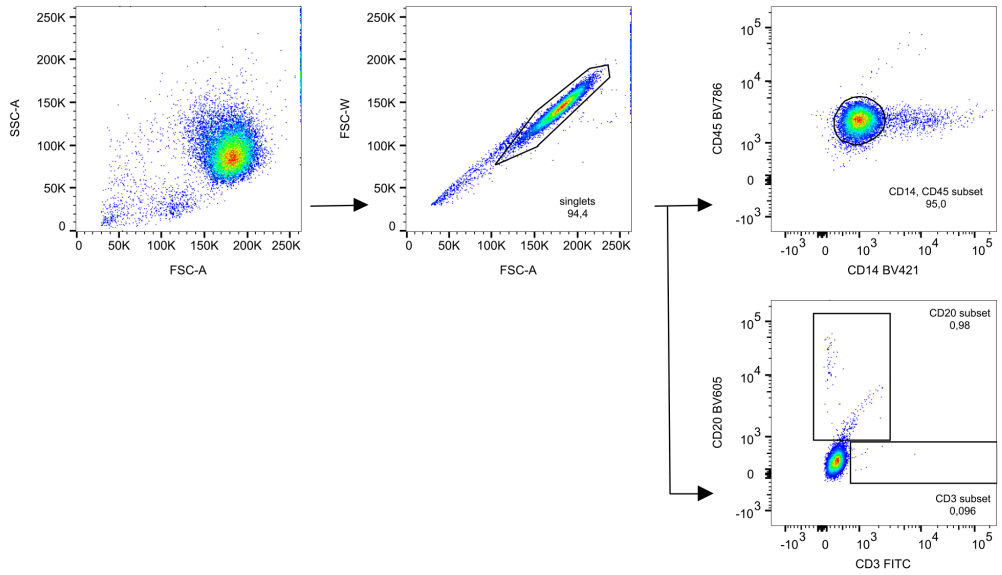
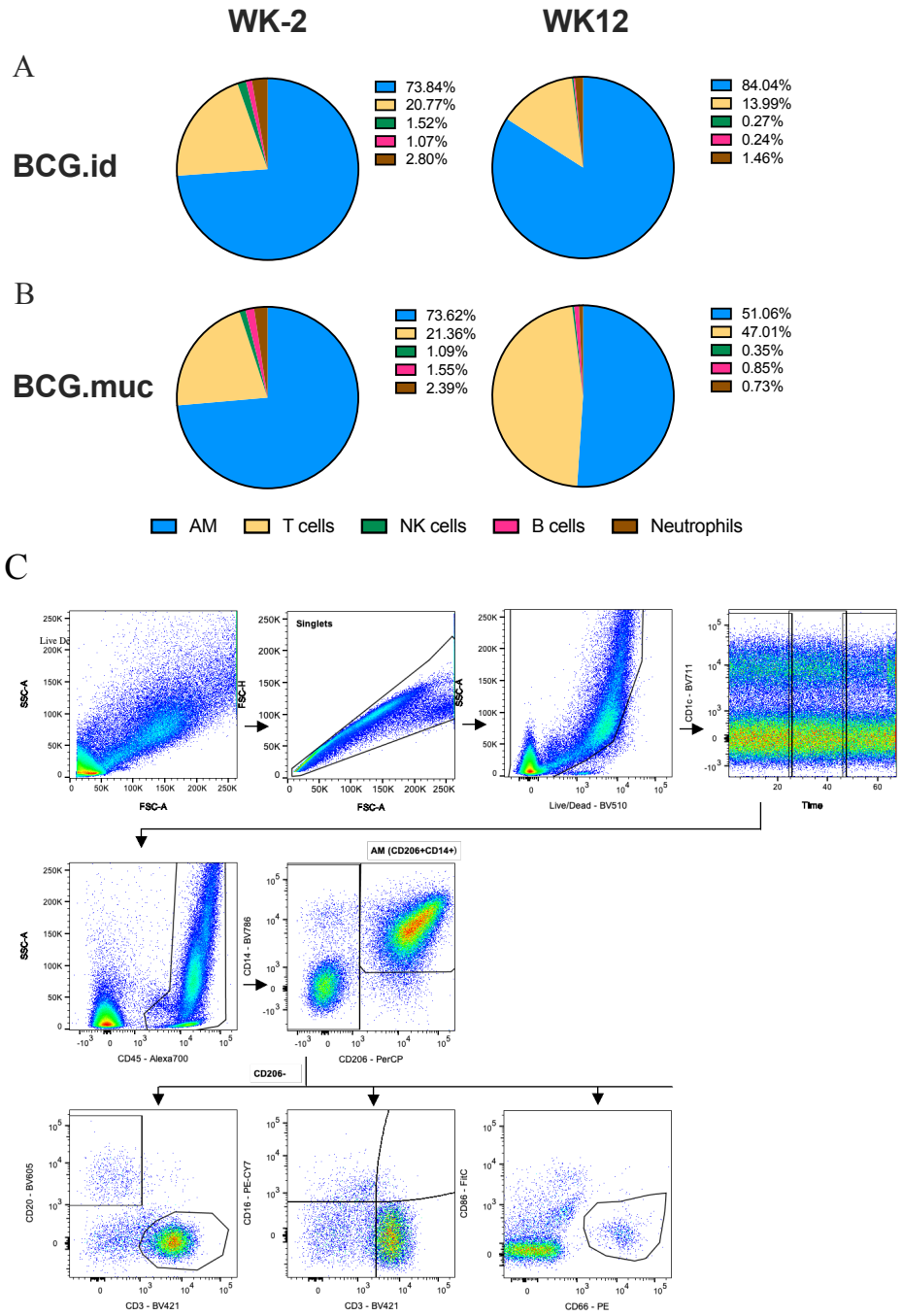
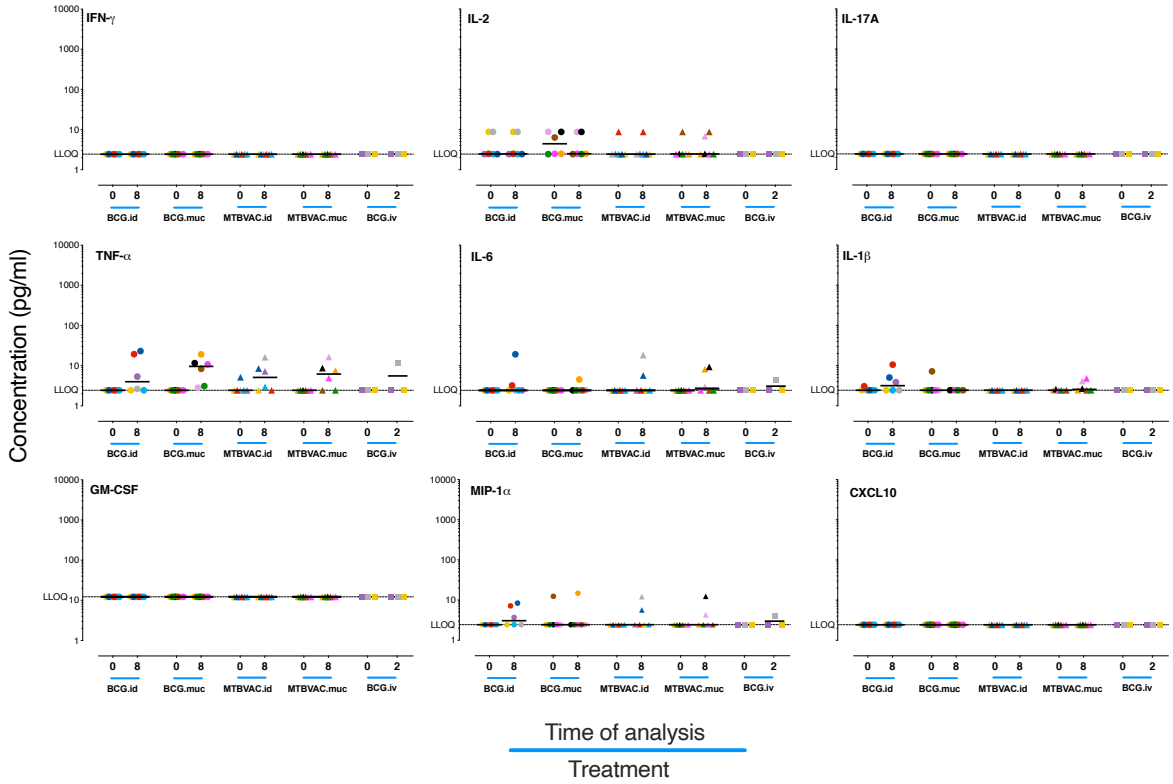


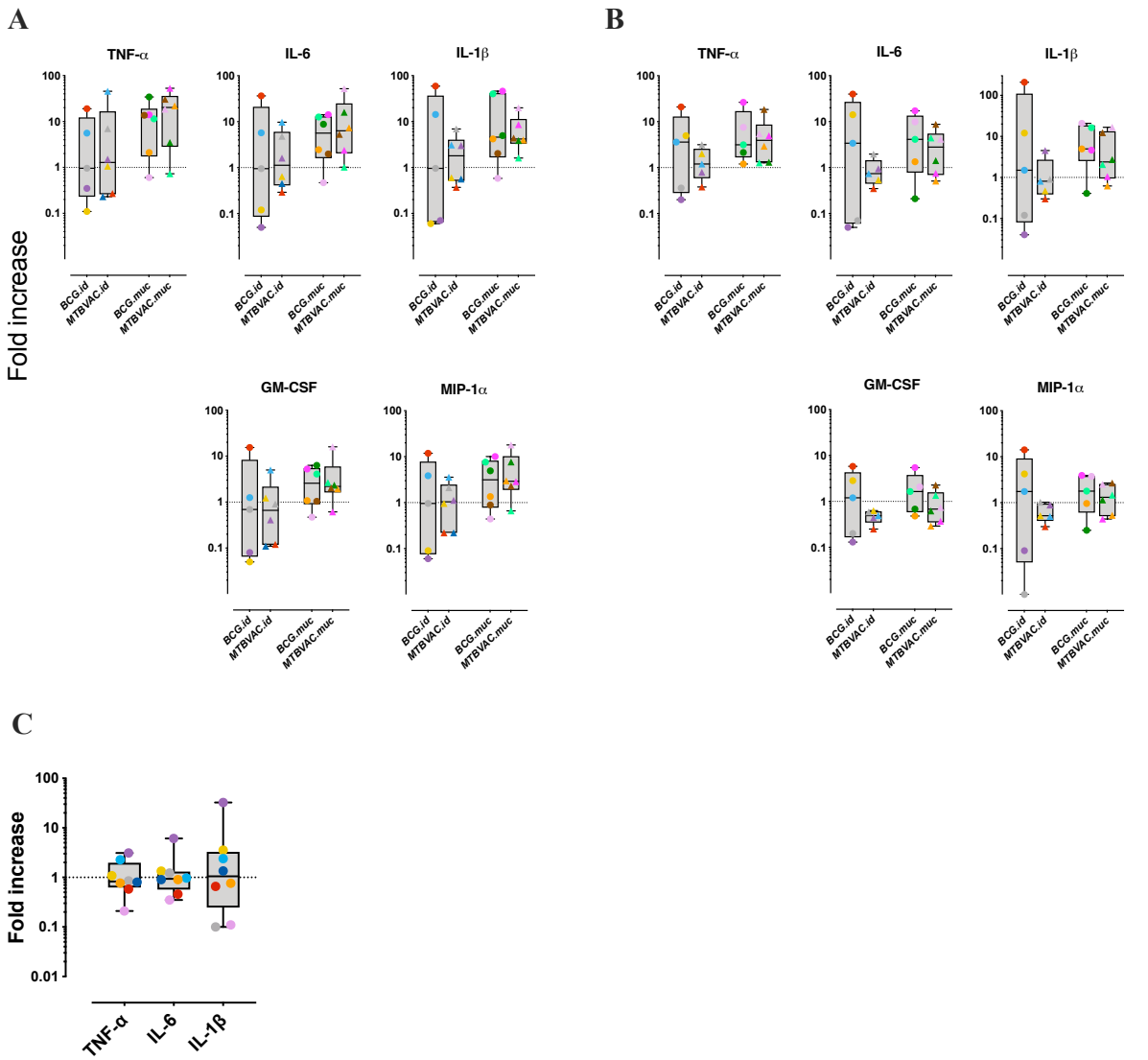
Supplemental Figure 1. Purity of CD14+ monocytes (Related to Figure 3, 5, 6 and 7) **A**) Monocytes from PBMC and BM were isolated by magnetic cell sorting and analyzed by flow cytometry before (PRE) and after (WK 8) vaccination. **B)** Gating strategies for the analysis of the purity of isolated CD14+ monocyte population. Singlets/CD45+/ Viable/CD14+. CD14+ cells are expressed as % from the CD45+/viable fraction.



Supplemental Figure 2. Composition of BAL cells pre and post vaccination (Related to Figure 4)
The relative abundance of major leukocyte populations in BAL samples was measured prior to and 12 weeks after intradermal or mucosal vaccination. **A)** Intradermal vaccination (BCG.id) had a minimal impact on the composition of the leukocytes. There is a relatively mild increase in alveolar macrophages (AM) and a minimal decrease in the T cells. **B)** Mucosal vaccination (BCG.muc), however, resulted in a marked increase in the number of T cells. Results on T cells have previously been reported as cell counts in Dijkman *et al*, 2019a, Extended Data Fig. 5a (Dijkman et al., 2019). **C)** Gating strategies for the analysis of the major subpopulation of BAL cells pre and post intradermal and mucosal vaccination with BCG (Dijkman et al., 2019). Singlets/Viable/Time gate/Leucocytes (CD45)/: CD206+/CD14+ (Alveolar Macrophages); CD206-: CD3 (T cells); CD20 (B cells); CD16+ (NK-cells) and CD66+ (Neutrophils). BAL cell composition is expressed as % from the CD45+/viable fraction.

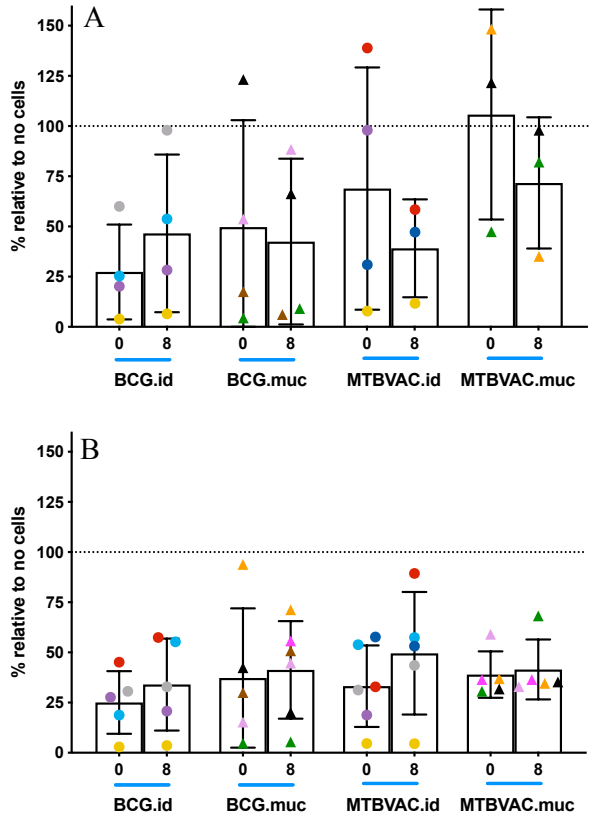


Supplemental Figure 3. Cytokine production of unstimulated (medium control) PBMC.mo. (Related to Figure 5) Cytokine production by freshly isolated monocytes purified from PBMC was measured after 24 hours of culture in medium alone. Indicated is the production for all the treatments both before (0) and after (week 2 for BCG.iv and week 8 for the remaining strategies) vaccination. Values below the range of the standard curve were set at the lower limit of quantification (LLOQ).

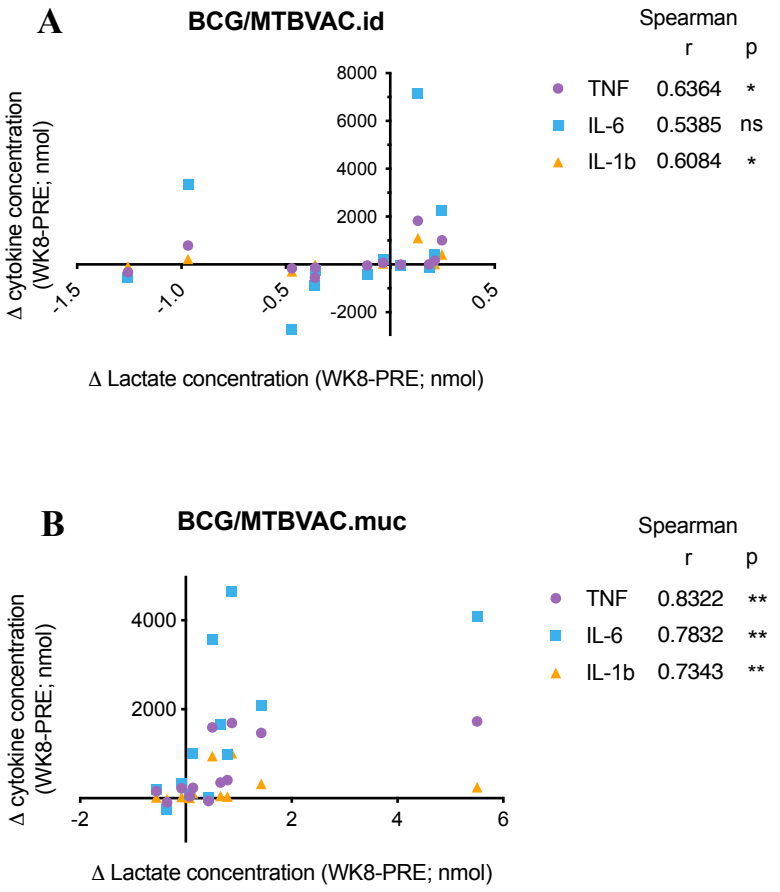


Supplemental Figure 4. MTBVAC is equally potent as BCG in the induction of trained immunity. (Related to Figure 5 and 6). Cytokine production was measured after 24 hours of stimulation with LPS (0.1µg/mL). The ratio of week 8 over week 0 is presented as fold increase for intradermal (id) and mucosal (muc) vaccination with either of the two vaccines for **(A)** PBMC.mo and **(B)** BM.mo.

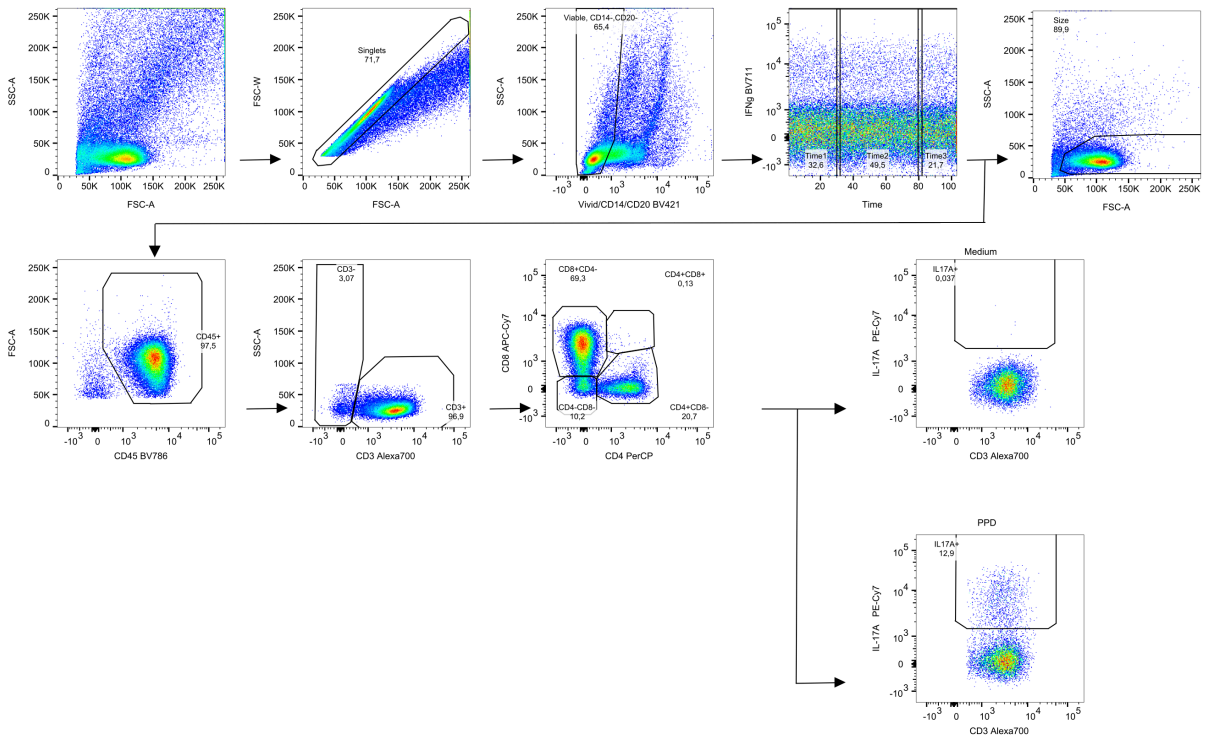
C) Non-vaccinated control animals show stable cytokine production over 8-week period. Cytokine production by PBMC.mo in unvaccinated control animals after 24 hours of stimulation with LPS (o.1µg/mL). Production of three signature cytokines, characteristic for trained immunity, was measured for 8 control animals on week 0 and week 8. The ratio of week 8 over week 0 is presented as fold increase.



Supplemental Figure 5. **No mycobactericidal activity by PBMC.mo after vaccination**. (Related to Figure 5). Evaluation of mycobactericidal activity by blood derived monocytes (PBMC.mo) before and 8 weeks after vaccination. (A) Percentage uptake of mycobacteria relative control growth where no monocytes were added. (B) Percentage killing of mycobacteria relative control growth where no monocytes were added.



Supplemental Figure 6. **Lactate production correlates with cytokine production** (Related to Figure 5, 6 and 7). Correlation of change in cytokine production versus change in lactate production after **(A)** intradermal and **(B)** mucosal vaccination with BCG/MTBVAC. Correlation is calculated as non-parametric Spearman's rho (r) and statistical significance indicated in p-values.



Supplemental Figure 7. **Gating strategy for the analysis of PPD specific polyfunctional Th17 population.** (Related to Figure 2D). T-cells were gated as Singlets/Lymphocytes/Viable/ CD14-CD20-/CD45+ /CD3+ before CD4 and CD8 gating was applied. After doublet exclusion, lymphocytes were gated based on size and granularity. Any anomaly indicative of unstable signal acquisition was excluded using the time parameter. Events from the combined time-gates were plotted against the dump channel containing the viability, CD14 and C20 markers and subsequently gated as viable, CD14- and CD20-. Cells were further selected for CD45 and CD3 positivity before CD4 and CD8 gating was applied. Boolean gating of any cytokine expression of IL-2/IFN-γ/TNFα/IL-17A