1   **The route of vaccine administration determines whether blood neutrophils undergo long-term** 2 **phenotypic modifications**

3

4   Yanis Feraoun¹, Jean-Louis Palgen¹, Candie Joly¹, Nicolas Tchitchek⁴, Ernesto Marcos-Lopez ¹, Nathalie
5 Dereuddre-Bosquet¹, Anne-Sophie Gallouet¹, Vanessa Contreras¹, Yves Lévy^{2,3}, Frédéric Martinon¹, Roger Le
6 Grand¹, and Anne-Sophie Beignon^{1*}

7

8   ¹Université Paris-Saclay, Inserm, CEA, Center for Immunology of Viral, Auto-immune, Hematological
9 and Bacterial Diseases (IMVA-HB/IDMIT), Fontenay-aux-Roses & Le Kremlin-Bicêtre, France

10   ²INSERM U955, Henri Mondor Hospital, University of Paris East, Créteil, France.

11   ³VRI, Créteil, France.

12   ⁴UMR_S 959, Immunology-Immunopathology-Immunotherapy (i3), Sorbonne Université and Inserm,
13 Paris, France.

14   *correspondance: anne-sophie.beignon@cea.fr

15

16   **Article type:** Original Research article

17   **Number of words:** 10 055 words

18   **Number of figures:** 5 figures

19   **Number of tables:** no table

20   **Supplementary information:** 2 supplementary figures and 1 supplementary table

21   **Keywords:** vaccine, innate immunity, innate immune memory, trained immunity, Modified vaccinia
22 virus Ankara (MVA), administration routes, mass cytometry (CyTOF)

23   **ABSTRACT**

24   Innate immunity modulates adaptive immunity and defines the magnitude, quality, and longevity of
25 antigen-specific T- and B- cell immune memory. Various vaccine and administration factors influence the
26 immune response to vaccination, including the route of vaccine delivery.

27   We studied the dynamics of innate cell responses in blood using a preclinical model of non-human
28 primates immunized with a live attenuated vaccinia virus, Modified vaccinia virus Ankara (MVA), and mass
29 cytometry. We previously showed that MVA induces a strong, early, and transient innate response, but also
30 late phenotypic modifications of blood myeloid cells after two months when injected subcutaneously. Here,
31 we show that the early innate effector cell responses and plasma inflammatory cytokine profiles differ
32 between subcutaneous and intradermal vaccine injection. Additionally, we show that the intradermal
33 administration fails to induce more highly activated/mature neutrophils long after immunization, in contrast
34 to subcutaneous administration.

35   Different batches of antibodies, staining protocols and generations of mass cytometers were used to
36 generate the two datasets that were compared. Mass cytometry data were analyzed in parallel using the
37 same analytical pipeline based on three successive clustering steps, including SPADE, and categorical
38 heatmaps were compared using the Manhattan distance to measure the similarity between cell cluster
39 phenotypes.

40   Overall, we show that the vaccine *per se* is not sufficient for the late phenotypic modifications of innate
41 myeloid cells, which are evocative of innate immune training. Its route of administration is also crucial, likely
42 by influencing the early innate response, and systemic inflammation, and the vaccine biodistribution.

43 INTRODUCTION

44 Vaccination is among the major advances in terms of public health by conferring protection against
45 many infectious diseases. However, there are still no vaccines against several pathogens, such as the human
46 immunodeficiency virus (HIV). Furthermore, certain vaccines are still insufficiently effective, such as flu
47 vaccines, which are not universal but seasonal, or intradermal vaccination with *Mycobacterium bovis* bacillus
48 Calmette-Guérin (BCG), which displays variable efficacy in preventing tuberculosis (TB) in adolescents and
49 adults. These examples highlight the importance of carrying out in-depth investigations on the modes of
50 action of vaccines to better understand the host and vaccine factors that influence innate and adaptive
51 immune responses to vaccination (1) to guide and improve vaccine design.

52 The early innate immune response is not only among the first lines of antiviral defense but also
53 orchestrates and modulates the antigen-specific effector and memory B- and T-cell responses by determining
54 the frequency, functions, and dynamics of antigen-specific T and B cells (2,3). This is indeed the principle of
55 vaccination.

56 The route of vaccine administration influences antigen/adjuvant trafficking, local and systemic
57 inflammation, innate effectors, and the resulting adaptive immune response (4–7). We have shown that, like
58 the antigen-specific antibody and T-cell responses, the early innate responses differ between subcutaneous
59 (SC) and intradermal (ID) immunization, with the recruitment of distinct cell populations and activation of
60 different immunomodulatory genes in skin and blood in response to a model live attenuated vaccine,
61 Modified vaccinia virus Ankara (MVA), in non-human primates (NHPs) (8).

62 Here, we investigated the long-term impact of the route of vaccine delivery on the innate myeloid cell
63 compartment. We hypothesized that the different early innate effector responses may lead to different
64 innate immunological imprints, which may last several weeks or months following vaccine injection. We
65 previously showed that a SC injection of MVA induced late changes in the phenotype of innate myeloid cells
66 in monkeys (9,10). They occurred between two weeks and two months after SC immunization, in spite of the
67 resolution of systemic inflammation, shown by a return to baseline blood leukocyte counts and inflammatory

68 cytokine levels. The innate myeloid response to a second MVA exposure two months later differed from the
 69 response to the first immunization, and to a second one two weeks later, in that it involved phenotypically
 70 more highly activated/mature neutrophils, monocytes, and dendritic cells (DCs). These late phenotypic
 71 modifications were associated with functional modifications as the *ex vivo* inflammatory response of PBMCs
 72 was enhanced short after the second immunization at two months compared to short after the first
 73 immunization and to a second immunization at two weeks (10).

74 We thus reused the mass cytometry dataset that originated from this previous preclinical study with
 75 macaques immunized with MVA SC (9,10) and compared the innate myeloid cell response with a newly
 76 generated dataset after ID injection. CYTOF data were analyzed similarly but independently, using sequential
 77 optimized clustering steps. They were compared using a strategy that we formerly developed that is based
 78 on categories of marker expression and Manhattan distance (10). Here, we demonstrate that ID
 79 administration of MVA failed to modify the phenotype of blood neutrophils long after immunization, contrary
 80 to SC injection, and although it mobilized neutrophils early after immunization.

81 MATERIALS AND METHODS

82 Animals

83 Adult male cynomolgus macaques (*Macaca fascicularis*) (n = 6, identified as 1BJR13, 1BJZ13, 1BLE13,
84 1GW14, AF103H and AN363H) were imported from Mauritius and housed in the CEA animal facility in
85 Fontenay-aux-Roses, France.

86 Vaccine, immunization and blood sampling

87 Animals were immunized twice, two months apart, with the ANRS recombinant MVA-HIV B vaccine
88 (Transgene) at a dose of 4×10^8 plaque forming units (PFU) by ID injections distributed over 10 injection
89 points (150 μ L/point of injections, all along the back, in two columns). Recombinant HIV-1 antigens consisted
90 of the complete sequence of gag, fused to fragments from pol (residues 172-219, 325-383 and 461-519) and
91 nef (residues 66-147 and 182-206) of the Bru/Lai isolate. Blood samples were taken longitudinally before and
92 after immunizations in either lithium-heparin (Greiner Bio-One) for mass cytometry analysis, or in
93 ethylenediaminetetraacetic acid (EDTA) (Greiner Bio-One) for complete blood counts (CBCs) and plasma
94 preparation. The same batch and dose of vaccine and vaccine schedule, but a different route of
95 administration, were used relative to a previous preclinical study that analyzed innate responses after SC
96 immunization (9), and for which the mass cytometry dataset was reused here.

97 Determination of plasma antibody titers

98 Direct enzyme-linked immunosorbent assays (ELISA) were performed according to a previously
99 published protocol (10) to determine total IgG titers specific to MVA in macaque EDTA-plasma. Antibody
100 titers were calculated by extrapolation from a five-parameter logistic curve representing optical density (OD)
101 versus plasma dilution and were defined as the reciprocal of the plasma dilution up to two times the OD of
102 the plasma taken before corresponding immunization and diluted to 1:50.

103 Neutralizing antibody titers were evaluated using a previously described cell-based assay (10) based
104 on the infection of HeLa cells with MVA-eGFP pre-incubated with serial dilutions of plasma-EDTA and flow

105 cytometry analysis The curve representing the percentage of living eGFP⁺ cells as a function of the dilution of
106 plasma-EDTA was plotted to calculate the neutralizing titer, equal to the reciprocal of the dilution of the
107 sample resulting in two times less infected cells than after incubation with the plasma of the same macaque
108 taken before immunization and diluted 1: 100.

109 **Complete blood count**

110 Blood cell counts were determined from EDTA blood using an HMX A/L analyzer (Beckman Coulter).

111 **Measurement of plasma cytokine concentrations**

112 The following cytokines, chemokines, and growth factors were quantified in plasma using a 23-plex
113 MAP NHP immunoassay kit (PCYTMG-40K-PX30, Millipore) following the manufacturer's recommendations:
114 IL-17, GM-CSF, IFN- γ , IL1- β , IL1RA, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-12/23(p40), IL-15, IL-18, MCP1,
115 MIP1- α , MIP1-b, scCD40L, TGF- α , TNF- α , VEGF, and G-CSF. Cytokine concentrations (in pg/mL) are plotted
116 as a function of time (in days). The area under the curves (AUCs) of the early cytokine response after the first
117 MVA immunization (H0 post-prime (PP) to D14PP) were calculated using GraphPad Prism 9 to represent the
118 kinetics and magnitude of cytokine release as a single value. GM-CSF could not be quantified in plasma from
119 SC-vaccinated animals. Heatmap was generated with DisplayR.

120 **Statistics**

121 Ab titers, CBC and cytokine concentrations after ID immunizations were compared with Wilcoxon tests,
122 whereas CBC after SC and ID injections were compared with unpaired two-tailed t tests, using GraphPad
123 Prism 9.

124 **Leukocyte staining and acquisition by mass cytometry**

125 A distinct, albeit highly similar, Ab panel (addition of 6 and suppression of 3 markers) and staining
126 protocol (with or without heparin and with or without barcoding) were used between the ID and SC studies
127 and the samples were acquired on a CyTOF 1 and Helios instrument for the SC and ID studies, respectively.
128 Leukocytes were fixed extemporaneously without *ex vivo* restimulation and frozen following a previously

described protocol that allows the analysis of all leukocytes and minimizes the batch effects inherent to the use of fresh cells (9). Briefly, three million fixed leukocytes were thawed and stained with a panel of Abs targeting innate myeloid cells (**Table S1**) in the presence of 300 U heparin to prevent nonspecific binding of metals by eosinophils (11). Purified Abs were conjugated to lanthanide metals using MAXPAR Lanthanide Staining kits (Fluidigm, South San Francisco, California, USA) following the recommendations of the supplier. Cells were barcoded using the Cell-ID 20-Plex Pd barcoding kit (Fluidigm). After washing in Barcode Perm Buffer, cells were incubated with one of the indicated combinations of Pd for 30 min at room temperature. Data were acquired using a Helios mass cytometer (Fluidigm) the day after staining after an overnight incubation in 0.1 μ M iridium in PBS +1,6% PFA.

Quality control and reproducibility

We controlled the quality and reproducibility of each staining and acquisition session (3 sessions in total with samples for all timepoints of interest from macaques 1BJR13 and 1BJZ13, 1BLE13 and 1GW14, and AF103H and AN363H) by staining and acquiring control samples consisting of aliquots of fixed and frozen blood leukocytes from a healthy macaque after *ex vivo* stimulation of whole blood for 2 hours with three TLR ligands: LPS (LPS *E. coli* 0111: B4, Invivogen) at 1 μ g/mL, Poly I-C (Invivogen) at 100 μ g/mL, and R848 (Mabtech) at 10 μ M in the presence of Brefeldin A (10 μ g/mL, SIGMA) for the last hour, in parallel with the tested samples. Comparison of the expression profiles of all markers by the control samples led us to exclude data from the first staining and acquisition session comprising the samples of two macaques (1BJR13 and 1BJZ13), as well as certain markers (CCR5, CXCR4, CD125, CD39, CD23, IL-1a) for which the expression profiles were too different from those of the two other staining and acquisitions sessions (**Figure S1**).

Mass cytometry data preprocessing

Zero mean signal intensity (MSI) values were first randomized between -1 and 0 to avoid a bias in the density estimation by the SPADE algorithm. The FCS files were then normalized using the MATLAB program by Rachel Finck et al (12). Tube replicates were concatenated using the Cytobank tool (Mountain View, California, USA). Samples were de-barcoded using Debarcoder software (Fluidigm, San Francisco, USA)

154 following the instructions of the user guide. The initial manual gating included the definition of singlets (based
155 on Ir191/cell length), intact cells (Ir191/Ir193), no beads (Ce140/ Gd155), and the exclusion of CD3+CD66+
156 cells using Cytobank, as previously described (9). Although the use of heparin strongly reduced the
157 nonspecific staining of eosinophils, a small number of CD3⁺CD66⁺ cells were still present and were excluded
158 (approximately 0.2% of all acquired events).

159 **Automatic identification of cell populations with SPADE**

160 The spanning-tree progression analysis of density-normalized events (SPADE) clustering algorithm (13)
161 was used to automatically identify cell clusters or groups of cell clusters composed of phenotypically similar
162 cells within the dataset consisting of singlet non-CD66⁺ CD3⁺ leukocytes from macaques (n = 4) collected at
163 various timepoints (H0PP, H6PP, D14PP, H0 post-boost (PB), H6PB, and D14PB). Uniform random
164 downsampling was used to select 100,000 cells from each sample (corresponding to the number of cells
165 contained in the smallest sample) before a final upsampling step. The optimal SPADE parameters were
166 determined using the SPADEVizR package (14) in R. The quality of the clustering was quantified as the
167 percentage of clusters with a unimodal and narrow distribution for all markers of the classification. Marker
168 distributions were assessed using the Hartigan's dip test (p-value < 0.05 to reject the unimodality hypothesis).
169 Markers distributions with an interquartile range < 2 were considered narrow.

170 **Heatmap representation of cell clusters**

171 The SPADE tree was manually annotated based on the expression of key markers (CD1c, CD3, CD4,
172 CD8, CD11c, CD14, CD16, CD20, CD66, CD123, CD125, CD172a, CADM1, and HLA-DR) to identify granulocyte
173 clusters (**Figure S2**). A heatmap allowing easy visualization of the complete phenotype of each cluster of
174 granulocytes was generated using the SPADEVizR by hierarchical clustering of clusters and markers based on
175 the Euclidean metric (14). The range of expression of markers was divided between the 5th and 95th percentile
176 into five categories for all clusters of cells. For each cluster, samples containing less than 50 cells were
177 excluded from phenotype inference.

178 **Definition of phenotypic families**

179 Clusters of cells sharing similar phenotypes were gathered into phenotypic families and superfamilies
 180 on the basis of the dendrogram resulting from the hierarchical clustering of the clusters, which were
 181 annotated manually. The number of cells for a given cell population was calculated as follows: $CBC \times$
 182 population frequency.

183 **Phenotypic comparison of cell clusters**

184 Both SC and ID datasets were analyzed independently using the same strategy. Clusters were
 185 compared two by two by calculating the Manhattan distance between the categories of expression of the
 186 clustering markers shared between the two datasets and visualized using the package CytoCompare in R (15).

RESULTS

MVA injected ID induces a strong specific humoral response

Adult macaques ($n = 6$) were immunized with 4×10^8 PFU of recMVA HIVB by ID injections twice, two months apart (**Figure 1A**). We assessed the humoral response to confirm the efficiency of vaccination, as Abs are the main immune correlate of protection for most vaccines (16), including vaccinia virus (VACV) and MVA against smallpox (17). As previously shown (8), MVA was highly immunogenic after ID injection. It induced high levels of MVA-specific IgGs (**Figure 1B**). As expected, the peak of the secondary response was higher than that of the primary response. Titers remained elevated, plateauing at $117,335 \pm 50,060$ 28 days post-boost. Neutralizing activity was only detected after the second immunization (**Figure 1C**). The analysis was performed using EDTA-plasma, precluding a direct comparison with the Ab response induced in macaques identically immunized previously but through the SC route (10), which were analyzed using sera. However, in a previous comparative study, we reported that the adaptive responses differed between ID and SC MVA immunizations, with SC delivery inducing higher levels of nAbs, and ID delivery inducing more polyfunctional CD8⁺ T cells (8).

We next analyzed the innate immune response in blood overtime, as it shapes and can even predict the magnitude, quality, and persistence of the Ab response, with myeloid cells playing a key role in capturing and presenting vaccine antigens to B and T lymphocytes, we next analyzed them in blood over time.

ID and SC immunizations with MVA induces strong but distinct inflammatory responses

ID injections induced a rapid and massive increase in the number of total leukocytes in blood (1.88 ± 0.29 times more leukocytes per milliliter of blood within 6 hours, $p < 0.0001$), but it was transient (the CBC decreased as soon as one day after immunization and returned to baseline 14 days post-immunization) and largely due to an increase in the number of granulocytes and, to a lesser extent, monocytes (**Figure 2A**). There was no difference between the first and second ID immunization ($p = 0.95$, paired t-test comparing the abundances at H6PP and H6PB). SC immunizations triggered a greater statistically significant increase in the total number of leukocytes, granulocytes, and monocytes (**Figure 2B**).

212 We next identified the systemic cytokine signature of MVA injected ID. The AUC, as an approximation
 213 of exposure over time, showed that, among 22 tested soluble factors, 16 cytokines were produced in
 214 response to the first ID injection: IL-12/23(p40), MCP-1, sCD40L, IL-1ra, VEGF, IL-10, IL-8, IL-13, G-CSF, IL-2,
 215 TNF- α , MIP-1 α , IL-5, TGF- α , IL-4, and IL-17 (from the highest to the lowest cumulative concentrations) (**Figure**
 216 **3A**). Only two cytokines differed significantly between the first and second ID immunization: MCP-1
 217 concentrations decreased ($p = 0.003$) and those of IL-1b increased ($p = 0.02$). The cytokine profiles after the
 218 first ID or SC immunization differed quantitatively and qualitatively (**Figures 3B and 3C**). Immunization by the
 219 ID route induced a 1.79 times greater production of cytokines than that by the SC route ($p = 0.009$; Wilcoxon
 220 tests), with five cytokines representing more than 90% of those produced (**Figure 3B**), whereas immunization
 221 by the SC route resulted in a more diverse cytokine production profile, with five additional cytokines: MIP-
 222 1 β , IL-15, IFN- γ , IL-6, and IL-1 β (**Figures 3B**). Unsupervised hierarchical clustering showed SC-immunized
 223 animals to be highly similar to each another in terms of cytokine production, in contrast to ID-immunized
 224 animals, which showed greater heterogeneity(**Figure 3C**). Based on the cytokine dendrogram, the following
 225 cytokines distinguished between the two routes of MVA delivery: IL-15, IFN- γ , MIP-1 β , IL-6, TGF- α , IL-1 β , IL-
 226 4, IL-5, IL-2, TNF- α , MIP-1 α , IL-17, and G-CSF (**Figure 3C**).

227 Overall, our results showed that MVA injected ID led to strong systemic inflammation, which was
 228 resolved rapidly. The inflammatory response was comparable between the first and second ID injection,
 229 except for IL-1b and MCP-1, but different from that following SC injection in terms of the magnitude of the
 230 cellular response and cytokine profile in blood. We next extensively characterized the phenotype of innate
 231 blood myeloid cells over time after MVA ID immunizations.

232 **Blood granulocytes are highly heterogeneous and diverse**

233 Blood leukocytes were stained with iridium and a panel of 35 titrated lanthanide-conjugated
 234 antibodies targeting innate myeloid cells, including granulocytes (**Figure 4A and Table S1**), and analyzed by
 235 mass cytometry. We used a previously described analysis pipeline (9) comprised of several successive
 236 clustering steps to identify cell clusters and groups of cell clusters with a similar phenotype and characterize

237 their dynamics in response to immunizations (**Figure 4B**). The SPADE algorithm was first applied to all samples
238 of the dataset. It consisted of 26 samples, corresponding to four of the six immunized macaques (1GW14,
239 AF163H, AN363H, 1BLE13) and six time points (H0PP, H6PP, D14PP, H0PB, H6PB, and D14PB) (**Figure 1A**),
240 minus two samples that were not available for technical reasons (H0PP for macaque 1BLE13 and D14PP for
241 macaque 1GW14).

242 We compared various parameters of SPADE, with the following parameters found to be optimal: 800
243 clusters, 22 clustering markers (CD66, HLA-DR, CD3, CD64, CD8, CD123, CD11a, CD11b, CD62L, CD4, FcεRI,
244 CD86, CD172a, CD1c, CD32, CD16, CD11c, CD14, CD141, CD20, CCR7, and CADM1), and a downsampling of
245 20%, enabling maximum quality clustering, with 58% of the clusters having a uniform and narrow distribution
246 of all clustering markers. As expected, these parameters differed from those of the SC dataset (9). Cell
247 clusters on the resulting SPADE tree cell were manually annotated based on the expression of several key
248 markers (CD66, CD3, HLA-DR, CD8, CD123, CD4, CD125, CD172a, CD1c, CD16, CD11c, CD14, CD141, CD20,
249 and CADM1) and the major leukocyte populations (B cells, T cells, NK cells, monocytes and DCs, and
250 granulocytes) were identified (**Figure S2**).

251 We next focused the analysis on the granulocyte compartment, as our panel was solely dedicated to
252 target innate myeloid cells, and because major phenotypic modifications occur mainly in granulocytes late
253 after MVA SC immunizations (18). The phenotypes of the granulocytes involved in the vaccine response were
254 organized in the form of a heatmap after hierarchical clustering of the cell clusters and markers, once the
255 levels of expression were categorized into five classes of signal intensity (**Figure 5A**), to visualize them more
256 easily. Based on the clusters dendrogram, clusters sharing a similar phenotype were grouped into so-called
257 “phenotypic families” and further grouped into “superfamilies”. Sixteen distinct phenotypic families (1 to 16)
258 were distinguishable and grouped into five superfamilies (A to E). Neutrophils (CD66^{hi}) were clustered within
259 three superfamilies (A, B and E) according to their relative level of activation within the dataset. Superfamily
260 A (phenotypic families 1, 2, and 3) comprised the least activated neutrophils of the dataset, with very low to
261 low expression of cytokines (especially IL-8) and activation markers (especially CD45). Superfamily B

262 (phenotypic families 4, 5, and 6) was composed of moderately activated neutrophils, with stronger
 263 expression of IL-8, CD86, CD39, CADM1, CD45, and CD11b, compared to superfamily A. Of note, among
 264 superfamily B, family 5 showed high expression of CD14, a characteristic already reported for neutrophils of
 265 cynomolgus macaques, without its function being clearly defined (19). Superfamily E (families 11, 12, 13, 14,
 266 and 15) contained the most highly activated neutrophils of this dataset, which expressed activation and
 267 migration markers and cytokines. This superfamily showed strong heterogeneity, from neutrophils of families
 268 11 and 12 with the highest expression of activation markers, including IL-8, CD14, CD11a, CD11c, CD141,
 269 CD16, CD86, and HLA-DR, to neutrophils from family 13 displaying a relatively less activated or mature profile.

270 Superfamily C (phenotypic families 7, 8, and 9) was clearly separated from the rest of the granulocytes.
 271 It included eosinophils with a CD66^{med} CD125⁺ phenotype and high expression of several markers, including
 272 CD45, CD62L, CD11a, CD125, CD23, IL-6, CXCR4, and FcεRI, suggesting strong activation, especially for family
 273 7. Nevertheless, these results must be interpreted with caution. The use of heparin during staining may have
 274 insufficiently inhibited interactions between the content of the eosinophilic granules and the metals
 275 conjugated to the Abs, and thus may have insufficiently prevented artefactual positive staining (11), making
 276 the phenotypic characterization of eosinophils by mass cytometry difficult.

277 Finally, basophils (CD123⁺ HLA-DR⁻) were found in superfamily D (phenotypic families 10 and 16).
 278 Family 10 gathered basophils with a classic expression profile (CD123⁺ IL-4⁺ FcεRI⁺), whereas family 16
 279 grouped basophils with more diverse expression profiles.

280 Thus, we show a large phenotypic heterogeneity and diversity of blood macaque granulocytes, as
 281 previously reported (9). We next investigated whether the phenotypic diversity within the ID dataset was
 282 qualitatively close to, or different from that of the SC dataset.

283 **The ID and SC datasets share similar poorly and moderately, but not highly, activated neutrophils**

284 The analytical challenge for the phenotypic comparison between our two datasets lay in the use of
 285 different, albeit similar, Ab panels, Ab batches, staining protocols, and generations of mass cytometer, with
 286 different detection sensitivity. In addition, independent analyses were performed, following the same

287 workflow, but using different SPADE parameters (cluster numbers, clustering markers, and downsampling),
288 which were optimally defined for each dataset. We opted for a phenotypic comparison of the cell clusters
289 based on the Manhattan distance between the expression categories of the clustering markers shared
290 between the two datasets to address this challenge. The proximity between cell cluster phenotypes is shown
291 in the form of a circular graph after calculating for the sum of the absolute value of the difference between
292 categories for each shared marker for each paired clusters. This distance was penalized when one of the
293 terms was greater than 1. Distances equal to or less than 11 were considered as significant, and are
294 represented by linking the two compared cell clusters (**Figure 5B**).

295   As expected, the eosinophils (superfamily C) from the ID dataset could not be associated with any
296 cluster from the SC dataset, as they were removed from the analysis due to of their nonspecific staining in
297 the absence of heparin during staining. Conversely, we found associations between basophils from the ID
298 and SC datasets, in particular from family 10. More importantly, only the least activated and moderately
299 activated neutrophils from the ID dataset were associated with neutrophils from the SC dataset, specifically
300 with the poorly and moderately activated neutrophil families. On the contrary, the vast majority of the most
301 highly activated neutrophils from the ID dataset were not associated with any neutrophil cluster of the SC
302 dataset. Only neutrophils from family 13 from the ID dataset were associated with neutrophils of the SC
303 dataset, either with moderately or highly activated neutrophils. As previously shown, these neutrophil
304 clusters did not exist prior to SC immunization. They were induced long after MVA prime immunization (9)
305 and responded to the second immunization. They were part of the SC boost signature defined in the LASSO-
306 LDA model (9).

307   Such a phenotype-limited comparison provides information about the presence of shared (basophils
308 and poorly and moderately activated neutrophils) or specific (highly activated neutrophils) cell populations
309 between the two datasets, both showing a high diversity of phenotypes, but does not indicate when, or in
310 what proportion these granulocytes circulate in blood before and after ID *versus* SC immunizations.

311 ID injection does not result in late modifications of the blood neutrophil phenotype, in contrast to 312 SC injection

313 We determined the impact of ID injections on granulocyte populations by investigating the differences
314 in their cell abundance over time represented as pie-charts (**Figure 5C**). As classically shown, eosinophils and
315 basophils were the least numerous granulocytes present at baseline and did not show major changes in
316 frequency after the first or second immunization. Among neutrophils, clusters from two superfamilies, the
317 least (families 1, 2, and 3, which matched families 5, 7, 3, 1, 11, 13, and 4 in the SC dataset, **Figure 5B**) and
318 most highly activated neutrophils (families 11 and 12 which were not phenotypically associated with
319 neutrophils within the SC dataset, **Figure 5B**) were found at baseline. They represented the majority of cell
320 types. Thus, the steady-state differed between studies, as neutrophils present before SC immunization were
321 poorly and moderately activated (9). Six hours after the first ID immunization, the number of granulocytes
322 increased, without major modifications in the proportion of the various subpopulations, still with a
323 predominance of the least and most highly activated neutrophils. On day 14, cell counts returned to baseline
324 values. Hence, the early response of granulocytes differed between ID and SC in magnitude and composition,
325 but from the beginning. Long after the first ID immunization, and immediately before the second, the
326 granulocyte composition showed slight changes, whereas the cell counts remained at the basal level. The
327 frequency of the least activated neutrophils was slightly lower, in favor of the most highly activated
328 neutrophils and, to a lesser extent, moderately activated neutrophils, which were almost absent at baseline,
329 although the difference was not statistically significant. This redistribution was not commensurate with the
330 major modifications of composition seen long after SC immunization, with the appearance of highly activated
331 neutrophils (9,10), which had no counterpart in the ID dataset (**Figure 5B**). Finally, the neutrophil responses
332 to the first and second ID immunizations highly resembled each other, in sharp contrast to what was observed
333 after SC immunizations (9,10).

DISCUSSION

ID administration of MVA fails to induce neutrophils that are more highly activated/mature long after immunization and that respond to a second immunization, in contrast to SC

In-depth phenotyping by mass cytometry combined with an analysis pipeline composed of successive clustering steps, specifically and previously developed for longitudinal multidimensional data, allowed us to analyze the quantitative and qualitative differences between the innate myeloid cell responses in blood after one or two ID immunizations of MVA and to compare them to those following another route of immunization, SC, using the Manhattan distance to measure the similarities of phenotype between cell clusters. MVA administration induced the substantial, rapid, and transient recruitment of granulocytes to blood, regardless of its route of delivery. However, such early mobilization was stronger after SC injection of the vaccine. Granulocyte counts increased, whereas the cell subset composition remained unchanged early after the first ID and SC immunization, mirroring that present before immunization. The early response to a second ID injection of the vaccine did not differ from the first in terms of magnitude and dynamics. Most importantly, it involved a similar distribution of cell subsets, in contrast to the response to a second SC injection, which engaged more highly mature/activated cells that were induced long after the first administration of the vaccine (9). Thus, depending on the route of MVA administration, not only the Ab and T cells responses (8), and the early inflammatory/innate responses to the first immunization (**Figures 2B, 3 and 5C**, and as previously shown (8,9)) differed, but also the long-term impact on neutrophils (**Figure 5C** and as previously shown (9)).

Comparing high dimensional cytometry datasets

High-dimensional cytometry, including mass cytometry and spectral flow cytometry, is a powerful technique to monitor immunity, identify cell subsets associated with diseases or potent responses to vaccines, and decipher the complex mechanisms of the immune response to vaccines (20–27). However, the comparison and integration of different datasets generated at multiple sites and on different days is challenging, even when using the same technology. This is due to technical differences, called batch effects,

359 that affect the signal intensity (on which commonly used unsupervised analytical methods, such as SPADE,
360 visNE, FlowSOM, CITRUS, and UMAP, are based) and need to be distinguished from true biological variability
361 (28). Several algorithms have been proposed to normalize signal intensity to reduce batch effects before
362 unsupervised cell cluster identification and to compare multiple datasets, such as Cytoviz (29), and JSOM
363 (30). iMUBAC can even compare different datasets in the absence of shared technical replicates, used as
364 reference samples, by overlaying cells from several healthy controls as anchors (31).

365   The issue here is somewhat distinct, as the two datasets were analyzed separately. The raw mass
366 cytometry data generated after the SC immunizations were not re-analyzed together with the mass
367 cytometry data newly generated after the ID immunizations, and after batch effect correction. High intra-
368 cluster homogeneity was achieved using the SPADEVizR package for each independent analysis. Then, cells
369 from the two vaccine immunogenicity studies (SC vs ID vaccine administration) were matched by comparing
370 their categories of marker expression, instead of the mean intensity, to mitigate the expected technical
371 differences in staining efficacy and cytometer sensitivity. Five categories were defined based on the range of
372 expression of each marker between the 5th and 95th percentiles for each dataset. Distances were calculated
373 as the sum of the absolute value of the Manhattan distance between the categorical values for each shared
374 marker of each cluster. Two clusters were associated if the sum was below a certain threshold, and a penalty
375 was applied, if the value of a term was too high. The threshold and penalty were set after trial and error and
376 manual inspection of the heatmap and marker expression profiles between clusters. Given the large
377 differences in the neutrophil response to MVA between the two delivery routes, such a simple method, and
378 admittedly not scalable nor benchmarked against other algorithms, such as overlaying cell clusters onto a
379 reference scaffold map (32), or measuring the quadratic form distance post-clustering (33), proved to be
380 sufficient to define a stringent cluster-wise comparison.

381   **Neutrophils and the humoral response to vaccines**

382   Neutrophils are the most numerous leukocytes in the blood. Their unexpected phenotypic and
383 functional heterogeneity, and plasticity have been recently reported (34). Apart from their key antimicrobial

activity, they shape adaptive immunity (35,36) by releasing cytokines and chemokines, granules, and NETs and by interacting with other immune cells and eventually acting as antigen presenting cells (APC) (37,38). Neutrophils can directly provide B-cell help through the production of BAFF and, hence, contribute to plasma-cell generation and antigen-specific Ab production (39–41). Whether and how neutrophil subsets, including highly activated neutrophils induced long after MVA SC injection, interact with B cells and modulate the primary and secondary MVA-specific Ab response is yet to be determined.

Innate immune training and vaccines

Certain vaccines, in particular certain live attenuated vaccines, such as BCG and oral poliomyelitis vaccine (OPV), also provide protection against unrelated pathogens beyond the pathogen they initially target by a process called nonspecific effects of vaccines (NSE) (42). Cross-reactive T and B cells and antibodies (Abs), bystander activation, and innate immune memory, also called trained immunity, contribute to NSE. By definition, trained cells respond faster and more strongly to a secondary challenge with homologous or even heterologous pathogens than a ‘naïve’ cells, long after the initial stimulation (43). The mechanisms of trained immunity involve the epigenetic reprogramming of innate cells, which are known to be short-lived, and that of their hematopoietic stem and progenitor cells (HSPCs) in the bone marrow (BM). Resting trained cells display an enhanced responsiveness to a challenge without having themselves encountered the trained immunity-inducing stimulus, by inheritance from their HSPCs. BCG is a canonical trained immunity stimulus. It induces trained monocytes and neutrophils (44–46) that contribute to its anti-TB and nonspecific protective effects (47). In addition to BCG, several microbial, as well as endogenous, ligands have been shown to imprint innate cells, with increased pro- or anti-inflammatory responsiveness that may be beneficial or detrimental to the host (18).

SC injection of MVA, but not ID, induced the late presence of highly activated neutrophils that were better equipped to respond to a second injection. This raises the questions of whether these cells are authentic trained cells, how they compare with BCG-induced innate memory cells in terms of features and origin, and how the route of MVA delivery influence their generation.

409 The literature has emphasized the enhanced responsiveness of blood trained cells to challenge through
410 the persistence of epigenetic marks inherited from trained HSPCs. However, the modulation of expression of
411 certain markers associated with increased activation by resting trained but unchallenged cells, including
412 neutrophils, has been reported after BCG given ID to humans (46,48). The phenotypic modifications of
413 neutrophils induced by MVA injected SC are yet to be associated with functional and epigenetic
414 modifications.

415 The state of neutrophil activation prior to immunization differed between animals from the two
416 cohorts, which were otherwise healthy, without any low-grade inflammation and with CBCs in the normal
417 range. This is likely the result of their different immunological history related to different environmental
418 exposure, although they were naive (*i.e.*, not previously experimentally immunized nor infected) (49). As
419 recently stated, to the degree that vaccine-induced trained immunity can provide heterologous protection,
420 the trained immune status at the time of vaccination may also modulate the immunogenicity of vaccines
421 (50), including their capacity to further train innate cells.

422 **Route of vaccine delivery and innate immune responses**

423 The route of injection determines the biodistribution of vaccines and their kinetics of expression,
424 including those of VACV and MVA. In mice, MVA-expressing neutrophils were shown to migrate from the skin
425 to the BM following ID injection (54). VACV can infect human BM hematopoietic stem cells *in vitro* (55).
426 Intraperitoneal VACV injection in mice was shown to induce the rapid and transient expansion of HSPCs, with
427 a bias towards common myeloid progenitors (CMP), which was MyD88 dependent. Finally, the route of MVA
428 delivery modulates the early systemic inflammatory cytokine response, as reported here (**Figures 3B and 3C**).
429 Not only did the profile of cytokines produced early in response to ID or SC delivery of MVA differ, but
430 interestingly, IL-1 β and IFN- γ , proposed to play a key role in the induction of trained immunity (44,46,57,58),
431 were differentially induced depending on the route of MVA delivery. Both were produced at low levels
432 relative to those of other cytokines, and exclusively after SC injection. Whether MVA needs to reach and

433 reside in the BM for a while, to be directly detected by HSPCs, or even infect them, to induce the late
434 modifications of neutrophil phenotype is not known.

435 BCG has previously been reported to induce trained monocytes in mice after intravenous (IV) injection,
436 , but not after SC injection. BCG was present in the BM for up to seven months, where it infected
437 monocytes/macrophages but not HSPCs after IV injection, whereas it was absent from the BM after SC
438 injection. The long-term persistence of BCG in the BM was not required for the induction of immune training.
439 The almost complete clearance of BCG from the BM by a four weeks antibiotic treatment with
440 antimycobacterial drugs four weeks after IV injection did not prevent BM Lin-Sca1+c-Kit+ (LSK) cells to expand
441 (44). In macaques, pulmonary mucosal delivery of BCG was also shown to induce trained monocytes in the
442 blood and BM in more vaccinated animals than “classical” ID injection (50). However, whether the early
443 systemic inflammatory/innate responses also contribute to the difference between IV and SC injection, or ID
444 and mucosal delivery for innate immune training was not addressed.

445 Future studies are needed to fully understand which vaccines/adjuvants imprint innate cells long after
446 their administration and how, in particular by defining the respective roles of the vaccine biodistribution and
447 persistence, and the early inflammatory/innate response, to harness this property to optimize protection
448 against infectious diseases, whether it is mediated by the innate or adaptive immune systems (18,59,60).

DATA AVAILABILITY STATEMENT

Mass cytometry data were deposited publicly. FCS files from the SC and ID studies are available on the FlowRepository database through ID FR-FCM-ZYBG and FR-FCM-Z4KJ, respectively.

ETHICS STATEMENT

The study was approved by the Ministry of National Education, Higher Education and Research and by the Ethics Committee in Animal Experimentation No. 44 under the reference A17003.

AUTHOR CONTRIBUTION

ASB, YL and RLG acquired funding; CJ, JLP, ASB, FM, and RLG designed the study; VC orchestrated the immunizations, longitudinal follow-up of the animals and biological sample collection and biobanking. CJ and JLP processed and biobanked biological samples; NDB generated plasma cytokine data; CJ and YF analyzed plasma cytokine data; YF stained samples for mass cytometry analysis; EML, and ASG acquired samples by mass cytometry; YF, JLP, NT, and ASB analyzed data; YF and JLP prepared the figures; YF and ASB wrote the original draft of the manuscript; YF, JLP, NT, RLG, and ASB reviewed and edited it. All the authors approved the submitted version.

FUNDING

This work was supported by the IDMIT infrastructure and funded by the ANR via grant No ANR-11-INBS-0008. N.T. held a fellowship from the ANRS (France Recherche Nord&Sud Sida-HIV Hépatites). This work was also supported by the “Investissements d’Avenir” programs managed by the ANR under reference ANR-10-LABX-77-01 funding the Vaccine Research Institute (VRI), Créteil (ImMemory research program) and ANR-10-EQPX-02-01 funding the FlowCyTech facility (IDMIT, Fontenay-aux-

Roses, France). Funds were also received from the European Commission: ADITEC, FP7-HEALTH-2011-280873; Transvac, EU H2020 GA 730964; EHVA, EU H2020 GA 681032.

ACKNOWLEDGEMENTS

We deeply thank all the members of IMVA-HB/IDMIT, in particular Laetitia Bossevot, Benoit Delache, Nina Dhooge, Karine Gombert-Rannou, Orianne Lacroix, Sébastien Langlois, Julie Morin, and Jean-Marie Robert, members of the IDMIT core facilities ASW (Animal Welfare and Science), L2I (Immunology and Infectiology), and LFC (FlowCyttech).

CONTRIBUTION TO THE FIELD STATEMENT

We have previously reported, using an animal model highly relevant for human immunology, that a vaccine, when injected subcutaneously, triggered the presence of neutrophils better equipped to respond to a second vaccination long after the first one. Here we show that the intradermal injection of the same vaccine failed to induce such late neutrophil phenotypic modifications, although it mobilized neutrophils early after immunization. Single cells were analyzed by mass cytometry, a technique allowing their detailed characterization, and data from two vaccine studies were matched. Our contribution is twofold: (i) we demonstrate that the route of vaccine injection, likely through the modulation of the vaccine biodistribution and/or of the magnitude and quality of the early inflammatory/innate effector responses plays a role in the long-term innate immunological imprinting; (ii) we propose a simple method to compare existing and newly generated mass cytometry datasets post-clustering.

REFERENCES

1. Zimmermann P, Curtis N. Factors That Influence the Immune Response to Vaccination. *Clin Microbiol Rev* (2019) 32:e00084-18. doi:10.1128/CMR.00084-18
2. Sallusto F, Lanzavecchia A, Araki K, Ahmed R. From vaccines to memory and back. *Immunity* (2010) 33:451–463. doi:10.1016/j.immuni.2010.10.008
3. Van Tilbeurgh M, Lemdani K, Beignon A-S, Chapon C, Tchitchek N, Cheraitia L, Marcos Lopez E, Pascal Q, Le Grand R, Maisonnasse P, et al. Predictive Markers of Immunogenicity and Efficacy for Human Vaccines. *Vaccines (Basel)* (2021) 9:579. doi:10.3390/vaccines9060579
4. Liard C, Munier S, Arias M, Joulin-Giet A, Bonduelle O, Duffy D, Shattock RJ, Verrier B, Combadière B. Targeting of HIV-p24 particle-based vaccine into differential skin layers induces distinct arms of the immune responses. *Vaccine* (2011) 29:6379–6391. doi:10.1016/j.vaccine.2011.04.080
5. Schmidt ST, Khadke S, Korsholm KS, Perrie Y, Rades T, Andersen P, Foged C, Christensen D. The administration route is decisive for the ability of the vaccine adjuvant CAF09 to induce antigen-specific CD8(+) T-cell responses: The immunological consequences of the biodistribution profile. *J Control Release* (2016) 239:107–117. doi:10.1016/j.jconrel.2016.08.034
6. Ols S, Yang L, Thompson EA, Pushparaj P, Tran K, Liang F, Lin A, Eriksson B, Karlsson Hedestam GB, Wyatt RT, et al. Route of Vaccine Administration Alters Antigen Trafficking but Not Innate or Adaptive Immunity. *Cell Rep* (2020) 30:3964-3971.e7. doi:10.1016/j.celrep.2020.02.111
7. Herzog C. Influence of parenteral administration routes and additional factors on vaccine safety and immunogenicity: a review of recent literature. *Expert Rev Vaccines* (2014) 13:399–415. doi:10.1586/14760584.2014.883285
8. Rosenbaum P, Tchitchek N, Joly C, Rodriguez Pozo A, Stimmer L, Langlois S, Hocini H, Gosse L, Pejowski D, Cosma A, et al. Vaccine Inoculation Route Modulates Early Immunity and Consequently Antigen-Specific Immune Response. *Front Immunol* (2021) 12:645210. doi:10.3389/fimmu.2021.645210
9. Palgen J-L, Tchitchek N, Elhmouzi-Younes J, Delandre S, Namet I, Rosenbaum P, Dereuddre-Bosquet N, Martinon F, Cosma A, Lévy Y, et al. Prime and Boost Vaccination Elicit a Distinct Innate Myeloid Cell Immune Response. *Scientific Reports* (2018) 8:3087. doi:10.1038/s41598-018-21222-2
10. Palgen J-L, Tchitchek N, Rodriguez-Pozo A, Jouhault Q, Abdelhouahab H, Dereuddre-Bosquet N, Contreras V, Martinon F, Cosma A, Lévy Y, et al. Innate and secondary humoral responses are improved by increasing the time between MVA vaccine immunizations. *npj Vaccines* (2020) 5:1–16. doi:10.1038/s41541-020-0175-8
11. Rahman AH, Tordesillas L, Berin MC. Heparin reduces nonspecific eosinophil staining artifacts in mass cytometry experiments. *Cytometry A* (2016) 89:601–607. doi:10.1002/cyto.a.22826
12. Finck R, Simonds EF, Jager A, Krishnaswamy S, Sachs K, Fantl W, Pe'er D, Nolan GP, Bendall SC. Normalization of mass cytometry data with bead standards. *Cytometry A* (2013) 83:483–494. doi:10.1002/cyto.a.22271

- 531 13. Qiu P, Simonds EF, Bendall SC, Gibbs KD, Bruggner RV, Linderman MD, Sachs K, Nolan GP, Plevritis
532 SK. Extracting a cellular hierarchy from high-dimensional cytometry data with SPADE. *Nat*
533 *Biotechnol* (2011) 29:886–891. doi:10.1038/nbt.1991
- 534 14. Gautreau G, Pejowski D, Le Grand R, Cosma A, Beignon A-S, Tchitchek N. SPADEVizR: an R package
535 for visualization, analysis and integration of SPADE results. *Bioinformatics* (2017) 33:779–781.
536 doi:10.1093/bioinformatics/btw708
- 537 15. Platon L, Pejowski D, Gautreau G, Targat B, Le Grand R, Beignon A-S, Tchitchek N. A computational
538 approach for phenotypic comparisons of cell populations in high-dimensional cytometry data.
539 *Methods* (2018) 132:66–75. doi:10.1016/j.ymeth.2017.09.005
- 540 16. Plotkin SA. Correlates of protection induced by vaccination. *Clin Vaccine Immunol* (2010) 17:1055–
541 1065. doi:10.1128/CVI.00131-10
- 542 17. Pittman PR, Hahn M, Lee HS, Koca C, Samy N, Schmidt D, Hornung J, Weidenthaler H, Heery CR,
543 Meyer TPH, et al. Phase 3 Efficacy Trial of Modified Vaccinia Ankara as a Vaccine against Smallpox.
544 *N Engl J Med* (2019) 381:1897–1908. doi:10.1056/NEJMoa1817307
- 545 18. Palgen J-L, Feraoun Y, Dzangué-Tchoupou G, Joly C, Martinon F, Le Grand R, Beignon A-S. Optimize
546 Prime/Boost Vaccine Strategies: Trained Immunity as a New Player in the Game. *Front Immunol*
547 (2021) 12:612747. doi:10.3389/fimmu.2021.612747
- 548 19. Antal-Szalmas P, Strijp JA, Weersink AJ, Verhoef J, Van Kessel KP. Quantitation of surface CD14 on
549 human monocytes and neutrophils. *J Leukoc Biol* (1997) 61:721–728. doi:10.1002/jlb.61.6.721
- 550 20. Arunachalam PS, Scott MKD, Hagan T, Li C, Feng Y, Wimmers F, Grigoryan L, Trisal M, Edara VV, Lai
551 L, et al. Systems vaccinology of the BNT162b2 mRNA vaccine in humans. *Nature* (2021) 596:410–
552 416. doi:10.1038/s41586-021-03791-x
- 553 21. Cross DL, Verheul MK, Leipold MD, Obermoser G, Jin C, Jones E, Starr JS, Mohorianu I, Blohmke CJ,
554 Maecker HT, et al. Vi-Vaccinations Induce Heterogeneous Plasma Cell Responses That Associate
555 With Protection From Typhoid Fever. *Front Immunol* (2020) 11:574057.
556 doi:10.3389/fimmu.2020.574057
- 557 22. DeGottardi Q, Gates TJ, Yang J, James EA, Malhotra U, Chow I-T, Simoni Y, Fehlings M, Newell EW,
558 DeBerg HA, et al. Ontogeny of different subsets of yellow fever virus-specific circulatory CXCR5+
559 CD4+ T cells after yellow fever vaccination. *Sci Rep* (2020) 10:15686. doi:10.1038/s41598-020-
560 72610-6
- 561 23. Mueller S, Taitt JM, Villanueva-Meyer JE, Bonner ER, Nejo T, Lulla RR, Goldman S, Banerjee A, Chi
562 SN, Whipple NS, et al. Mass cytometry detects H3.3K27M-specific vaccine responses in diffuse
563 midline glioma. *J Clin Invest* (2020) 130:6325–6337. doi:10.1172/JCI140378
- 564 24. Reeves PM, Raju Paul S, Baeten L, Korek SE, Yi Y, Hess J, Sobell D, Scholzen A, Garritsen A, De Groot
565 AS, et al. Novel multiparameter correlates of Coxiella burnetii infection and vaccination identified
566 by longitudinal deep immune profiling. *Sci Rep* (2020) 10:13311. doi:10.1038/s41598-020-69327-
567 x
- 568 25. Lingblom CMD, Kowli S, Swaminathan N, Maecker HT, Lambert SL. Baseline immune profile by
569 CyTOF can predict response to an investigational adjuvanted vaccine in elderly adults. *J Transl Med*
570 (2018) 16:153. doi:10.1186/s12967-018-1528-1

- 571 26. Pejowski D, Tchitchek N, Rodriguez Pozo A, Elhrouzi-Younes J, Yousfi-Bogniaho R, Rogez-Kreuz C,
572 Clayette P, Dereuddre-Bosquet N, Lévy Y, Cosma A, et al. Identification of Vaccine-Altered
573 Circulating B Cell Phenotypes Using Mass Cytometry and a Two-Step Clustering Analysis. *J Immunol*
574 (2016) 196:4814–4831. doi:10.4049/jimmunol.1502005
- 575 27. Swadling L, Capone S, Antrobus RD, Brown A, Richardson R, Newell EW, Halliday J, Kelly C, Bowen
576 D, Fergusson J, et al. A human vaccine strategy based on chimpanzee adenoviral and MVA vectors
577 that primes, boosts, and sustains functional HCV-specific T cell memory. *Sci Transl Med* (2014)
578 6:261ra153. doi:10.1126/scitranslmed.3009185
- 579 28. Olsen LR, Leipold MD, Pedersen CB, Maecker HT. The anatomy of single cell mass cytometry data.
580 *Cytometry A* (2019) 95:156–172. doi:10.1002/cyto.a.23621
- 581 29. Trussart M, Teh CE, Tan T, Leong L, Gray DH, Speed TP. Removing unwanted variation with
582 CytoFURV to integrate multiple CyTOF datasets. *Elife* (2020) 9:e59630. doi:10.7554/eLife.59630
- 583 30. Lim HS, Qiu P. JSOM: Jointly-evolving self-organizing maps for alignment of biological datasets and
584 identification of related clusters. *PLoS Comput Biol* (2021) 17:e1008804.
585 doi:10.1371/journal.pcbi.1008804
- 586 31. Ogishi M, Yang R, Gruber C, Zhang P, Pelham SJ, Spaan AN, Rosain J, Chbihi M, Han JE, Rao VK, et
587 al. Multibatch Cytometry Data Integration for Optimal Immunophenotyping. *J Immunol* (2021)
588 206:206–213. doi:10.4049/jimmunol.2000854
- 589 32. Spitzer MH, Gherardini PF, Fragiadakis GK, Bhattacharya N, Yuan RT, Hotson AN, Finck R, Carmi Y,
590 Zunder ER, Fantl WJ, et al. IMMUNOLOGY. An interactive reference framework for modeling a
591 dynamic immune system. *Science* (2015) 349:1259425. doi:10.1126/science.1259425
- 592 33. Orlova DY, Meehan S, Parks D, Moore WA, Meehan C, Zhao Q, Ghosn EEB, Herzenberg LA, Walther
593 G. QFMatch: multidimensional flow and mass cytometry samples alignment. *Sci Rep* (2018)
594 8:3291. doi:10.1038/s41598-018-21444-4
- 595 34. Ng LG, Ostuni R, Hidalgo A. Heterogeneity of neutrophils. *Nat Rev Immunol* (2019) 19:255–265.
596 doi:10.1038/s41577-019-0141-8
- 597 35. Costa S, Bevilacqua D, Cassatella MA, Scapini P. Recent advances on the crosstalk between
598 neutrophils and B or T lymphocytes. *Immunology* (2019) 156:23–32. doi:10.1111/imm.13005
- 599 36. Leliefeld PHC, Koenderman L, Pillay J. How Neutrophils Shape Adaptive Immune Responses. *Front*
600 *Immunol* (2015) 6:471. doi:10.3389/fimmu.2015.00471
- 601 37. Lin A, Loré K. Granulocytes: New Members of the Antigen-Presenting Cell Family. *Front Immunol*
602 (2017) 8:1781. doi:10.3389/fimmu.2017.01781
- 603 38. Vono M, Lin A, Norrby-Teglund A, Koup RA, Liang F, Loré K. Neutrophils acquire the capacity for
604 antigen presentation to memory CD4+ T cells in vitro and ex vivo. *Blood* (2017) 129:1991–2001.
605 doi:10.1182/blood-2016-10-744441
- 606 39. Naranjo-Gomez M, Lambour J, Piechaczyk M, Pelegri M. Neutrophils are essential for induction
607 of vaccine-like effects by antiviral monoclonal antibody immunotherapies. *JCI Insight* (2018) 3:
608 doi:10.1172/jci.insight.97339

- 609 40. Parsa R, Lund H, Georgoudaki A-M, Zhang X-M, Ortlieb Guerreiro-Cacais A, Grommisch D,
610 Warnecke A, Croxford AL, Jagodic M, Becher B, et al. BAFF-secreting neutrophils drive plasma cell
611 responses during emergency granulopoiesis. *J Exp Med* (2016) 213:1537–1553.
612 doi:10.1084/jem.20150577
- 613 41. Musich T, Rahman MA, Mohanram V, Miller-Novak L, Demberg T, Venzon DJ, Felber BK, Franchini
614 G, Pavlakis GN, Robert-Guroff M. Neutrophil Vaccination Dynamics and Their Capacity To Mediate
615 B Cell Help in Rhesus Macaques. *The Journal of Immunology* (2018)
616 doi:10.4049/jimmunol.1800677
- 617 42. de Bree LCJ, Koeken VACM, Joosten LAB, Aaby P, Benn CS, van Crevel R, Netea MG. Non-specific
618 effects of vaccines: Current evidence and potential implications. *Semin Immunol* (2018) 39:35–43.
619 doi:10.1016/j.smim.2018.06.002
- 620 43. Divangahi M, Aaby P, Khader SA, Barreiro LB, Bekkering S, Chavakis T, van Crevel R, Curtis N,
621 DiNardo AR, Dominguez-Andres J, et al. Trained immunity, tolerance, priming and differentiation:
622 distinct immunological processes. *Nat Immunol* (2021) 22:2–6. doi:10.1038/s41590-020-00845-6
- 623 44. Kaufmann E, Sanz J, Dunn JL, Khan N, Mendonça LE, Pacis A, Tzelepis F, Pernet E, Dumaine A,
624 Grenier J-C, et al. BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity
625 against Tuberculosis. *Cell* (2018) 172:176-190.e19. doi:10.1016/j.cell.2017.12.031
- 626 45. Cirovic B, de Bree LCJ, Groh L, Blok BA, Chan J, van der Velden WJFM, Bremmers MEJ, van Crevel
627 R, Händler K, Picelli S, et al. BCG Vaccination in Humans Elicits Trained Immunity via the
628 Hematopoietic Progenitor Compartment. *Cell Host & Microbe* (2020) 28:322-334.e5.
629 doi:10.1016/j.chom.2020.05.014
- 630 46. Moorlag SJCFM, Rodriguez-Rosales YA, Gillard J, Fanucchi S, Theunissen K, Novakovic B, de Bont
631 CM, Negishi Y, Fok ET, Kalafati L, et al. BCG Vaccination Induces Long-Term Functional
632 Reprogramming of Human Neutrophils. *Cell Rep* (2020) 33:108387.
633 doi:10.1016/j.celrep.2020.108387
- 634 47. Khader SA, Divangahi M, Hanekom W, Hill PC, Maeurer M, Makar KW, Mayer-Barber KD, Mhlanga
635 MM, Nemes E, Schlesinger LS, et al. Targeting innate immunity for tuberculosis vaccination. *J Clin*
636 *Invest* (2019) 129:3482–3491. doi:10.1172/JCI128877
- 637 48. Kleinnijenhuis J, Quintin J, Preijers F, Joosten LAB, Ifrim DC, Saeed S, Jacobs C, van Loenhout J, de
638 Jong D, Stunnenberg HG, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific
639 protection from reinfection via epigenetic reprogramming of monocytes. *Proceedings of the*
640 *National Academy of Sciences* (2012) 109:17537–17542. doi:10.1073/pnas.1202870109
- 641 49. Hamilton SE, Badovinac VP, Beura LK, Pierson M, Jameson SC, Masopust D, Griffith TS. New
642 Insights into the Immune System Using Dirty Mice. *J Immunol* (2020) 205:3–11.
643 doi:10.4049/jimmunol.2000171
- 644 50. Vierboom MPM, Dijkman K, Sombroek CC, Hofman SO, Boot C, Vervenne RAW, Haanstra KG, van
645 der Sande M, van Emst L, Domínguez-Andrés J, et al. Stronger induction of trained immunity by
646 mucosal BCG or MTBVAC vaccination compared to standard intradermal vaccination. *Cell Rep Med*
647 (2021) 2:100185. doi:10.1016/j.xcrm.2020.100185

- 648 51. Liu H, Yu W, Tang X, Wang H, Ouyang W, Zhou J, Chen Z. The route of inoculation determines the
649 tissue tropism of modified vaccinia Tiantan expressing the spike glycoprotein of SARS-CoV in mice.
650 *J Med Virol* (2010) 82:727–734. doi:10.1002/jmv.21667
- 651 52. Abadie V, Bonduelle O, Duffy D, Parizot C, Verrier B, Combadière B. Original encounter with
652 antigen determines antigen-presenting cell imprinting of the quality of the immune response in
653 mice. *PLoS ONE* (2009) 4:e8159. doi:10.1371/journal.pone.0008159
- 654 53. Gómez CE, Nájera JL, Domingo-Gil E, Ochoa-Callejero L, González-Aseguinolaza G, Esteban M. Virus
655 distribution of the attenuated MVA and NYVAC poxvirus strains in mice. *J Gen Virol* (2007)
656 88:2473–2478. doi:10.1099/vir.0.83018-0
- 657 54. Duffy D, Perrin H, Abadie V, Benhabiles N, Boissonnas A, Liard C, Descours B, Reboulleau D,
658 Bonduelle O, Verrier B, et al. Neutrophils transport antigen from the dermis to the bone marrow,
659 initiating a source of memory CD8+ T cells. *Immunity* (2012) 37:917–929.
660 doi:10.1016/j.immuni.2012.07.015
- 661 55. Yu Q, Hu N, Ostrowski M. Poxvirus tropism for primary human leukocytes and hematopoietic cells.
662 *Methods Mol Biol* (2009) 515:309–328. doi:10.1007/978-1-59745-559-6_22
- 663 56. Singh P, Yao Y, Weliver A, Broxmeyer HE, Hong S-C, Chang C-H. Vaccinia virus infection modulates
664 the hematopoietic cell compartments in the bone marrow. *Stem Cells* (2008) 26:1009–1016.
665 doi:10.1634/stemcells.2007-0461
- 666 57. Mitroulis I, Ruppova K, Wang B, Chen L-S, Grzybek M, Grinenko T, Eugster A, Troullinaki M,
667 Palladini A, Kourtzelis I, et al. Modulation of Myelopoiesis Progenitors Is an Integral Component of
668 Trained Immunity. *Cell* (2018) 172:147–161.e12. doi:10.1016/j.cell.2017.11.034
- 669 58. Arts RJW, Moorlag SJCFM, Novakovic B, Li Y, Wang S-Y, Oosting M, Kumar V, Xavier RJ, Wijmenga
670 C, Joosten LAB, et al. BCG Vaccination Protects against Experimental Viral Infection in Humans
671 through the Induction of Cytokines Associated with Trained Immunity. *Cell Host & Microbe* (2018)
672 23:89–100.e5. doi:10.1016/j.chom.2017.12.010
- 673 59. Sánchez-Ramón S, Conejero L, Netea MG, Sancho D, Palomares Ó, Subiza JL. Trained Immunity-
674 Based Vaccines: A New Paradigm for the Development of Broad-Spectrum Anti-infectious
675 Formulations. *Front Immunol* (2018) 9:2936. doi:10.3389/fimmu.2018.02936
- 676 60. Xing Z, Afkhami S, Bavananthasivam J, Fritz DK, D’Agostino MR, Vaseghi-Shanjani M, Yao Y,
677 Jeyanathan M. Innate immune memory of tissue-resident macrophages and trained innate
678 immunity: Re-vamping vaccine concept and strategies. *J Leukoc Biol* (2020) 108:825–834.
679 doi:10.1002/JLB.4MR0220-446R

680 **FIGURE LEGENDS**

681 **Figure 1. Experimental approach. (A)** Experimental design. Six male and adult cynomolgus macaques
682 were immunized two months apart with MVA HIV-B at a dose of 4×10^8 PFU injected intradermally. Blood
683 was drawn longitudinally at hours (H for hour), days (D for day), or months (M for month) before and after
684 the first (PP for post-prime) and second (PB for post-boost) immunization to assess the inflammatory, innate,
685 and humoral responses and the phenotype of blood innate myeloid cells by mass cytometry. Immunizations
686 are indicated by the blue dotted lines **(B)** Immunogenicity of MVA injected ID. Individual (black) and mean
687 (green, with standard deviation) titers of anti-MVA IgG (left) and nAb (right) in EDTA plasma were plotted
688 over time. Anti-MVA IgG titers were measured by ELISA. The MVA neutralizing capacity was quantified using
689 a cell-based assay and MVA-eGFP. Immunizations are indicated by the blue dotted lines. Titers were
690 compared by Wilcoxon tests. Statistically significant p values ($p < 0.05$) are indicated by an asterisk (*).

691 **Figure 2. Leukocyte counts in blood after ID or SC immunizations. (A)** Longitudinal monitoring of total
692 leukocyte, granulocyte (including neutrophils, eosinophils and basophils), and monocyte counts (in
693 thousands per μl of blood) before and after ID immunizations for each animal. Immunizations are indicated
694 by the blue dotted lines. Counts were compared to baseline using Wilcoxon tests. p-values < 0.05 were
695 considered statistically significant and are indicated by an asterisk (*). **(B)** Comparison of leukocyte counts
696 (in thousands per μl of blood) before and after immunization by the ID (in green) or SC (in black) route.
697 Immunizations are indicated by the blue dotted lines. Counts were compared between routes of vaccine
698 delivery using unpaired two-tailed t tests. Statistically significant p values ($p < 0.05$) are indicated by an
699 asterisk (*).

700 **Figure 3. Comparison of cytokine and chemokine expression profiles in blood after ID or SC MVA**
701 **immunizations.** The plasma concentration of 22 cytokines was measured before, 1 day, 7 days, and 14 days
702 after MVA injected either ID ($n = 6$) or SC ($n = 5$). Areas under the curve from H0 to D14 post-immunization
703 were calculated to represent an approximation of exposure over time, in $\text{pg} \times \text{ml}^{-1} \times \text{day}$. **(A)** The mean AUC of
704 each cytokine after the first and second MVA ID injection is shown as a heatmap. Colors represent the AUC

705 values from the lowest (blue) to the highest (red), standardized for each row. Statistically significant
 706 differences ($p < 0.05$, Wilcoxon tests) between the 1st and 2nd injection are indicated by an asterisk (*). **(B)**
 707 The mean AUC of each cytokine produced after the first ID (left) or SC (right) MVA injection is displayed as a
 708 pie-chart. Differentially expressed cytokines according to the vaccine delivery route are colored in dark green
 709 for cytokines with a higher concentration after ID injection than SC, in light green for those with a higher
 710 concentration after SC injection than ID, and in yellow for those produced only after SC injection. The size of
 711 the inner pie is proportional to the sum of the AUC of all cytokines. **(C)** Heatmap representation of individual
 712 AUC for each cytokine after the first ID or SC injection of MVA. Each row corresponds to a cytokine and each
 713 column to an animal. Dendrograms represent the hierarchal clustering of animals (upper) and cytokines (left)
 714 based on the Euclidean distance using the Ward2 clustering method. Colors represent the AUC values from
 715 the lowest (blue) to the highest (orange), standardized for each row.

716 **Figure 4. Analytical approach for mass cytometry characterization of granulocytes. (A)** Panel of
 717 antibodies for mass cytometry analysis. Fixed leukocytes were stained with a panel of metal-conjugated
 718 antibodies (**Table S1**). Markers in black were used to compare the phenotype of clusters between datasets.
 719 Markers in light grey were excluded from the analysis, not selected as SPADE clustering markers for the ID
 720 dataset, or not shared with the SC dataset. **(B)** Analysis pipeline of mass cytometry data. After ID
 721 immunizations, cells sharing a similar phenotype were clustered using the SPADE algorithm. Clusters were
 722 manually annotated on the SPADE tree, and granulocytes clusters identified (**Figure S2**), and further grouped
 723 into “phenotypic families” and “superfamilies” after categorization of their marker expression and
 724 hierarchical clustering. Finally, a comparison of marker expression categories based on the Manhattan
 725 distance was used to associate granulocytes from the two datasets analyzed separately that display a similar
 726 phenotype.

727 **Figure 5. Phenotypic diversity and dynamics in granulocytes after ID and SC MVA immunizations. (A)**
 728 Hierarchical clustering of granulocyte clusters represented as a heatmap. Each row corresponds to a cell
 729 cluster and each column to a marker. The level of expression of the markers was divided into five categories

730 ranging from white to brown. The dendrogram allowed the grouping of clusters with similar phenotypes into
731 16 “phenotypic families” (numbered from 1 to 16) and “superfamilies” (named with letters between brackets,
732 from A to E), colored based on the manual annotation as in (9). Neutro.: neutrophils. Ba.: basophils. **(B)**
733 Comparisons of the phenotypes of granulocytes present in the ID (green) and SC (black) datasets. Black lines
734 inside the circle connect phenotypically similar clusters after calculation of the Manhattan distance. The color
735 code is identical to that of the heatmap. Neutro.: neutrophil. Mod.: moderately. Baso: basophils. **(C)** Pie
736 charts representing the composition in granulocyte phenotypic families over time for each macaque after
737 MVA immunizations by the ID route. Each slice represents a phenotypic family, for which the color is identical
738 to that of the heatmap. The size of the pie is proportional to the cell concentration in the blood. Unavailable
739 data are represented by empty circles.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Control samples. The same fixed and frozen control samples were stained and acquired with the samples from the vaccinated animals after *ex vivo* restimulation with a mixture of TLR ligands. **(A)** Gating strategy to define the CD66^{high} HLA-DR⁻, CD66^{-/mid} HLA-DR⁻, and CD66^{-/mid} HLADR⁺ cell populations. The non-stimulated control sample for the first staining/acquisition session is shown. **(B)** Comparison of the staining profiles using overlaid histograms.

Figure S2. SPADE tree annotation. The topology of the SPADE tree is shown, with the color of each node of the SPADE tree representing the median expression of the indicated markers among all cells from the entire dataset with a scale adapted for each marker. This allowed the identification of the major blood cell populations, including granulocytes. The size of the node is not proportional to the number of cells it contains.

Metal	Marker	Clone	Supplier	Catalog number	$\mu\text{g}/3$ millions leukocytes	Surface	Intra-cellular
141Pr	CD66abce	TET2	Miltenyi	120-014-229	0.3	●	
142Nd	HLA-DR	L243	Ozyme	307651	0.2	●	
143Nd	CD3	SP34.2	BD	551916	2.5	●	
144Nd	CD64	10.1	Miltenyi	120-014-229	1.5	●	
145Nd	CD8	RPAT8	BD	557084	1	●	
146Nd	IL-6	MQ2.13A5	BD	554543	2		●
147Sm	CD123	7G3	BD	554527	1	●	
148Nd	IL-4	8D48	BD	554515	2		●
149Sm	CD11a	HI111	Miltenyi	120-014-229	2	●	
150Nd	CD11b	ICRF144	BD	555386	0.5	●	
151Eu	CD62L	SK11	Miltenyi	120-014-229	2	●	
152Sm	CD4	L200	BD	550625	2	●	
153Eu	FcεRI	AER37 (CRA1)	eBioscience (ThermoFisher Scientific)	14-5899-82	1.5	●	
154Sm	CD86	IT2.2	BD	555663	1	●	
155Gd	CD125	A14	BD	624084	0.5	●	
156Gd	CD172a	REA144	Miltenyi	120-014-229	1.5	●	
158Gd	IP-10	6D4	Miltenyi	120-014-229	1		●
159Tb	CD45	D058-1283	BD	552566	0.2	●	
160Gd	IL-1α	364/3B3	Miltenyi	120-014-229	2		●
161Dy	CD1c	AF5910	R&D systems (Biotechne)	AF5910	0.5	●	
162Dy	IL-12	C8.6	Miltenyi	120-014-229	1		●
163Dy	CD32	FLI8.26	BD	555447	0.5	●	
164Dy	IFNα	LT27/295	Miltenyi	120-014-229	0.5		●
165Ho	CD39	eBioA1	Biolegend	328002	1	●	
166Er	CCR5	3A9	BD	556041	0.5	●	
167Er	CD16	3G8	Miltenyi	120-012-311	2	●	
168Er	CD11c	3.9	Biolegend	301639	1.5	●	
169Tm	CXCR4	12G5	BD	555971	0.75	●	
170Er	CD14	M5E2	BD	555396	1	●	
171Yb	IL-8	G265.8	BD	554717	0.5		●
172Yb	CD23	9P25	Beckman	IMBULK1	2.5	●	

173Yb	CD141	1A4	Fluidigm	3173002B	1.5	●
174Yb	CD20	2H7	BD	556631	1	●
175Lu	CCR7	G043H7	Miltenyi	120-014-229	1	●
176Yb	CADM1	3 E1	Clinisciences MBL	CM004-3	0.5	●

751

752 **Supplementary Table 1. Antibody panel for mass cytometry.**

FIGURE 1

FIGURE 2

A

B

FIGURE 3

FIGURE 4

A

B

FIGURE 5