**SUPPLEMENTAL INFORMATION**

**Title:**

# Plant-derived virus-like particle vaccines drive cross-presentation of influenza A hemagglutinin peptides by human monocyte-derived macrophages

**Authors:**

Alexander I. Makarkov, Makan Golizeh, Elizabeth Ruiz-Lancheros, Angelica A. Gopal, Ian N. Costas-Cancelas, Sabrina Chierzi, Stephane Pillet, Nathalie Charland, Nathalie Landry, Isabelle Rouiller, Paul W. Wiseman, Momar Ndao, Brian J. Ward

## Supplemental Methods

### *Electron microscopy (EM)*

For negative stain preparation, the VLP samples were diluted with PBS pH 7.4 – 100 µg/mL based on estimated influenza hemagglutinin (HA) content. 5 µL of the diluted VLP solution was placed for 45 sec on 200 Hex grids (EMS Inc., Hatfield, PA), formerly carbon coated and glow-discharged. The grids were washed twice with 5µL of distilled water for 45 sec followed by 45 sec incubation with 2% uranyl acetate (EMS Inc.). Excess fluid was removed by blotting with a filter paper and samples were left to air dry. The grids were imaged commonly on a Tecnai T12 (FEI Inc., Hillsboro, OR). The MDM samples processing for EM is described in the manuscript, Methods section.

### *Endocytosis inhibitors screening*

A number of endocytosis inhibitors were screened based on their effect on virus-like particles (VLPs) internalization measured by 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD - Thermo Fisher Scientific, Eugene, OR) fluorescence dequenching upon VLP fusion with cell membranes. H1-VLPs (influenza HA based on the sequence of A/California/07/2009 H1N1 virus, Medicago Inc., Quebec, QC) were labelled with DiD (see Methods section of the manuscript). B10R cell culture (an immortalized murine bone marrow-derived macrophage cell line) was maintained in RPMI-1640 with 50 IU/mL penicillin, 50 µg/mL streptomycin and 10 mM HEPES (medium) supplemented with 10% fetal bovine serum (FBS, all from Wisent, Saint-Jean-Baptiste, QC) until reaching cell confluency. B10R cells were detached from plastic flask surface using 0.25% trypsin/2.21 mM EDTA in HBSS (Wisent) and plated on 96-well Nunclon Delta black flat-bottom plates (Thermo Fisher Scientific, Roskilde, Denmark) at 5 x $10^4$ cells per well. The following day, B10R cells in

triplicate wells were exposed to DiD-labelled VLPs in the medium (HA concentration 15.0 µg/mL); plates were kept at 4°C for one hour and then washed with ice-cold medium twice. Endocytosis inhibitors were applied in ice-cold medium supplemented with 10% FBS (100 µL/well): dynasore hydrate (50 µM), chlorpromazine hydrochloride (10 µg/mL), sucrose (0.45 M), pitstop 2 (25 µM), genistein (200 µM), filipin III from Streptomyces filipinensis (5 µg/mL), amiloride hydrochloride (1 mM), cytochalasin D (4 µM) (all from Sigma-Aldrich, St. Louis, MO). Pitstop 2 was also tested at the same concentration in serum-free medium. The plates were placed into a pre-heated (37°C) spectrophotometer (Infinite 200 PRO, Tecan, Männedorf, Switzerland), and DiD fluorescence was measured at 15-min intervals over 2 h. Fusion efficiency (%) was determined following addition of Triton X-100 (Sigma-Aldrich) to each well (final concentration 1%) to obtain full DiD dequenching.

***Toxicity assessment of endocytosis inhibitors***

B10R cell culture was maintained in the medium supplemented with 10% FBS until reaching cell confluency. B10R cells were detached from plastic flask surface using 0.25% trypsin/2.21 mM EDTA in HBSS and plated on 96-well Nunclon Delta black flat-bottom plates at 5 x $10^4$ cells per well. The following day, endocytosis inhibitors (see the endocytosis inhibitors screening section and the Supplemental Table 1) in the medium supplemented with 10% FBS were applied to B10R cells in triplicate wells in a volume 100 µL/well (5% $CO_2$, 37°C for 2 h). Chlorpromazine hydrochloride was applied at concentrations 10 and 100 µg/mL. Pitstop 2 was also tested in serum-free medium. Triton X-100 0.01% solution served as a positive control. The effects of endocytosis inhibitors on B10R cells viability were evaluated in parallel experiments with the CytoTox-ONE™ homogeneous membrane integrity assay and the CellTiter-Glo® 2.0 assay (both from Promega, Madison, WI). Cell membrane integrity was assessed by lactate

dehydrogenase (LDH) release. CytoTox-ONE™ reagent (100 µL/well) was applied for 10 min at RT. Stop solution (50µl) was then added to each well, and the fluorescence was measured on Infinite 200 PRO spectrophotometer at excitation and emission wavelengths 560 and 600 nm, respectively. The results were reported as a % of the maximum LDH release caused by adding lysis solution to the control wells. The effects of endocytosis inhibitors on metabolically active cells were quantitated by the amount of ATP with CellTiter-Glo® luminescent cell viability assay. CellTiter-Glo® 2.0 reagent (100 µl/well) was applied for 10 min at RT. Luciferase luminescence was measured on Infinite 200 PRO spectrophotometer. The results were reported as a % of ATP level reduction compared to control wells unexposed to any endocytosis inhibitor.

***Conventional image analysis***

Confocal microscopy images were analyzed with ImageJ software[1] for the purpose of evaluation the fluorescence intensity of immunolabelled HA or fluorophore-conjugated transferrin, or for the assessment of colocalization of HA with endosomal markers Rab5, Rab7 and Rab11.[2] In brief, to analyze the HA or transferrin fluorescence intensity per cell area we identified cellular boundaries on the brightfield channel, and used them to establish the regions of interest (ROIs) on the fluorescent channel(s). Then we determined the background fluorescence intensity in each experiment by averaging the values obtained from cells in the control sample (for HA: monocyte-derived macrophages (MDMs) unexposed to HA but stained with anti-HA primary and fluorescent secondary antibody; for transferrin: MDMs unexposed to transferrin). The average background fluorescence intensity was subtracted from the fluorescence values measured from HA or transferrin-exposed cells. For "cell-based" colocalization analysis, both "green" and "red" channels were denoised with ImageJ PureDenoise plugin,[3] then image background was subtracted with the "rolling ball" algorithm.[4]

HA colocalization with endosomal markers was determined within cell boundaries-defined ROIs (see above) using Colocalization Threshold plugin;[5] Costes thresholding approach was applied.[6] Pearson correlation coefficient R, Pearson coefficient for pixels whose intensity falls above a threshold value R (>t), and Manders above threshold colocalization coefficients tM1 and tM2 were analyzed.[2,7,8]

***Statistical analysis***

Statistical analysis was performed using GraphPad Prism 6.0 software. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post-test, two-way ANOVA followed by Tukey's multiple comparisons post-test or Mann-Whitney test were used to examine the differences between samples. P values $< 0.05$ were considered statistically significant.

**Supplemental tables**

**Supplemental Table 1**

**Endocytosis inhibitors used in screening experiments and their suggested mechanism of action**

| Inhibitor of endocytosis | Tested concentration / condition | Suggested mechanism of action | Toxicity |
|---|---|---|---|
| Dynamin-dependent endocytosis | | | |
| Dynasore | 50 µM | Non-competitive and reversible inhibitor of GTPase activity of dynamin.[9] Dynasore suppresses both CME and CIE.[10,11] | Non-toxic |
| Clathrin-mediated endocytosis (CME) | | | |
| Chlorpromazine | 10 – 100 µg/mL | Not well understood. It has been suggested that chlorpromazine causes AP-2 and clathrin relocation from plasma membrane to endosomal membranes and therefore depletes AP-2 and clathrin from the plasma membrane and prevents clathrin-coated endocytic vesicles formation.[12] Chlorpromazine probably affects dynamin activity.[13] | Non-toxic at 10 µg/mL. Toxicity observed at higher concentrations |
| Hyperosmotic sucrose | 0.45 M | Not well understood. It has been suggested that hyperosmolarity leads to trapping clathrin in "microcages" and depleting it from plasma membrane.[14] | Greatly reduced ATP level in cells |
| Pitstop 2 | 25 µM (applied in serum-free or 10% FBS supplemented medium) | Not well understood. Pitstop 2 was developed as cell-permeable selective CME inhibitor.[15] However, later pitstop 2 has been shown to potently inhibit CIE.[16,17] | Toxic in serum-free medium (recommended use due to sequestering by serum albumins) |
| Clathrin-Independent Endocytosis | | | |
| Genistein | 200 µM | Tyrosine-kinase inhibitor.[18] Phosphorylation of tyrosine at caveolin-1 is the prerequisite for pinching off caveolar vesicles from plasma membrane.[19] | Non-toxic |
| Filipin III | 5 µg/mL | Cholesterol depleting and lipid-raft disrupting agent.[20] | Moderate cytotoxicity and |

| | | | |
|---|---|---|---|
| | | | massive ATP level reduction. |
| Macropinocytosis | | | |
| Amiloride | 1 mM | Inhibition of $Na^+/H^+$ exchange leads to lowering submembranous pH and preventing Rac1 and Cdc42 signaling that is essential for actin remodeling.[21] | Non-toxic |
| Phagocytosis / macropinocytosis | | | |
| Cytochalasin D | 4 μM | Blocking of actin polymerization, disassembly of actin cytoskeleton.[22,23] | Non-toxic |

**Supplemental Table 2**

**HA-derived peptides associated with major histocompatibility complex (MHC) I identified in MDM lysates using high-resolution tandem mass spectrometry**

| ## | Sequence * | Length | Mass | Start position | End position | Charge state | PEP | MS/MS Count | Sample |
|---|---|---|---|---|---|---|---|---|---|
| 1 | AILVVLLYTFATANADTLCI | 20 | 2124.1541 | 3 | 22 | 2 | 0.01 | 2 | D |
| 2 | VVLLYTFATANADTLCIGYH | 20 | 2184.0925 | 6 | 25 | 3 | 0.01 | 1 | D |
| 3 | LYTFATANADTLCIGYHANN | 20 | 2171.9946 | 9 | 28 | 3 | 0.01 | 1 | D |
| 4 | LCIGYHA | 7 | 775.3687 | 20 | 26 | 2 | 0.01 | 1 | B |
| 5 | LCIGYHANNSTDTVDTVLEK | 20 | 2192.0419 | 20 | 39 | 3 | 0.01 | 1 | D |
| 6 | CIGYHAN | 7 | 776.3276 | 21 | 27 | 2 | 0.01 | 1 | B |
| **7, 8** | **GYHANNSTDTVDTVLEKNVT** | **20** | **2177.0237** | **23** | **42** | **3** | **0.01** | **3(A), 2(D)** | **A, D** |
| **9, 10** | **DTVLEKNVTVTHSVNLLEDK** | **20** | **2253.1852** | **34** | **53** | **3(A), 2(D)** | **0.01** | **1** | **A, D** |
| 11 | LEDKHNGKLCKLRGVAPLHL | 20 | 2240.2576 | 50 | 69 | 3 | 0.01 | 1 | D |
| 12 | GKLCKLRGVAPLHLGK | 16 | 1689.0236 | 56 | 71 | 3 | 0.01 | 1 | C |
| 13 | LRGVAPLHLGKCNIAGWILG | 20 | 2087.1826 | 61 | 80 | 2 | 0.01 | 1 | A |
| 14 | PLHLGKC | 7 | 766.4160 | 66 | 72 | 2 | 0.01 | 1 | B |
| 15 | LGNPECESLSTASSWSYIVE | 20 | 2170.9729 | 79 | 98 | 3 | 0.01 | 1 | D |
| 16 | LSTASSWSY | 9 | 1000.4502 | 87 | 95 | 2 | 0.00 | 1 | A |
| 17 | TASSWSYIVETPSSDNGTCY | 20 | 2166.9052 | 89 | 108 | 3 | 0.01 | 1 | D |
| 18 | SWSYIVETPSSDNGTCYPGD | 20 | 2176.8895 | 92 | 111 | 2 | 0.01 | 2 | A |
| 19 | VETPSSDNGTCYPGDFIDYE | 20 | 2207.8841 | 97 | 116 | 3 | 0.01 | 1 | D |
| 20 | ETPSSDNGTCYPGDFIDYEE | 20 | 2237.8583 | 98 | 117 | 3 | 0.01 | 1 | A |
| 21 | PSSDNGT | 7 | 676.2664 | 100 | 106 | 2 | 0.01 | 1 | B |
| 22 | PSSDNGTCYPGDFIDYEELR | 20 | 2276.9532 | 100 | 119 | 3 | 0.01 | 1 | D |
| 23 | CYPGDFI | 7 | 813.3367 | 107 | 113 | 2 | 0.01 | 1 | B |
| 24 | YEELREQLSSVSSFERFEIF | 20 | 2494.2016 | 115 | 134 | 3 | 0.01 | 1 | A |
| 25 | VSSFERFEIFPKTSSWPNHD | 20 | 2409.1390 | 125 | 144 | 3 | 0.01 | 1 | D |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 26 | RFEIFPKTSSWPNHDSNKGV | 20 | 2345.1553 | 130 | 149 | 2 | 0.01 | 1 | D |
| 27 | SSWPNHDSNKGVTAACPHAG | 20 | 2034.8966 | 138 | 157 | 3 | 0.01 | 2 | D |
| 28 | SNKGVTAACPHAGAKSFYKN | 20 | 2050.0054 | 145 | 164 | 3 | 0.01 | 1 | D |
| 29 | KGVTAACPHAGAKSFYKNLI | 20 | 2075.0986 | 147 | 166 | 3 | 0.01 | 1 | D |
| 30 | HAGAKSF | 7 | 716.3606 | 155 | 161 | 2 | 0.01 | 2 | B |
| 31 | AKSFYKNLIWLVKKGNSYPK | 20 | 2383.3416 | 158 | 177 | 3 | 0.01 | 2 | D |
| 32 | NLIWLVKKGNSYPKLSKSYI | 20 | 2350.3413 | 164 | 183 | 3 | 0.01 | 2 | D |
| 33 | IWLVKKGNSYPKLSKS | 16 | 1847.0669 | 166 | 181 | 2 | 0.01 | 1 | C |
| 34 | WLVKKGNSYPKLSKSYINDK | 20 | 2367.2951 | 167 | 186 | 3 | 0.01 | 1 | D |
| 35 | VKKGNSYPKLSKSYINDKGK | 20 | 2253.2481 | 169 | 188 | 3 | 0.01 | 1 | A |
| 36 | KLSKSYINDKGKEVLVLWGI | 20 | 2289.3097 | 177 | 196 | 3 | 0.01 | 2 | D |
| 37 | SKSYINDKGKEVLVLWGIHH | 20 | 2322.2485 | 179 | 198 | 3 | 0.01 | 1 | D |
| 38 | NDKGKEVLVLWGIHHPPTSA | 20 | 2197.1644 | 184 | 203 | 3 | 0.01 | 1 | A |
| 39 | DKGKEVLVLWGIHHPSTSAD | 20 | 2188.1277 | 185 | 204 | 3 | 0.01 | 1 | A |
| 40 | EVLVLWGIHHPSTSADQQSL | 20 | 2216.1226 | 189 | 208 | 3 | 0.01 | 1 | D |
| 41 | LWGIHHPSTSADQQSLYQNA | 20 | 2252.0610 | 193 | 212 | 3 | 0.01 | 1 | D |
| 42 | PSTSADQ | 7 | 704.2977 | 199 | 205 | 2 | 0.01 | 1 | B |
| 43 | LYQNADAYVFVGSSRY | 16 | 1851.8792 | 208 | 223 | 3 | 0.01 | 1 | C |
| 44 | YVFVGSSRYSKKFKPEIAIR | 20 | 2374.3161 | 215 | 234 | 3 | 0.01 | 2 | D |
| 45 | VFVGSSRYSKKFKPEIAIRP | 20 | 2308.3056 | 216 | 235 | 3 | 0.01 | 3 | D |
| 46 | SSRYSKKFKPEIAIRPKVRD | 20 | 2404.3703 | 220 | 239 | 3 | 0.01 | 1 | D |
| 47 | SRYSKKFKPEIAIRPKVRDR | 20 | 2473.4394 | 221 | 240 | 3 | 0.01 | 1 | A |
| 48 | RYSKKFKPEIAIRPKVRDRE | 20 | 2515.4499 | 222 | 241 | 3 | 0.01 | 1 | D |
| 49 | SKKFKPEIAIRPKVRD | 16 | 1911.1418 | 224 | 239 | 3 | 0.01 | 3 | C |
| 50 | KFKPEIAIRPKVRDREGRMN | 20 | 2439.3645 | 226 | 245 | 3 | 0.01 | 1 | D |
| 51 | KPEIAIRPKVRDQEGR | 16 | 1891.0752 | 228 | 243 | 3 | 0.01 | 1 | C |
| 52 | IRPKVRDREGRMNYYWTLVE | 20 | 2580.3383 | 233 | 252 | 3 | 0.01 | 1 | D |
| 53 | RDREGRMNYYWTLVEPGDKI | 20 | 2497.2172 | 238 | 257 | 3 | 0.01 | 1 | D |
| 54 | PGDKITFEATG | 11 | 1134.5557 | 253 | 263 | 3 | 0.00 | 1 | B |
| 55 | ATGNLVVPRYAFAMERNAGS | 20 | 2123.0582 | 261 | 280 | 2 | 0.01 | 1 | A |

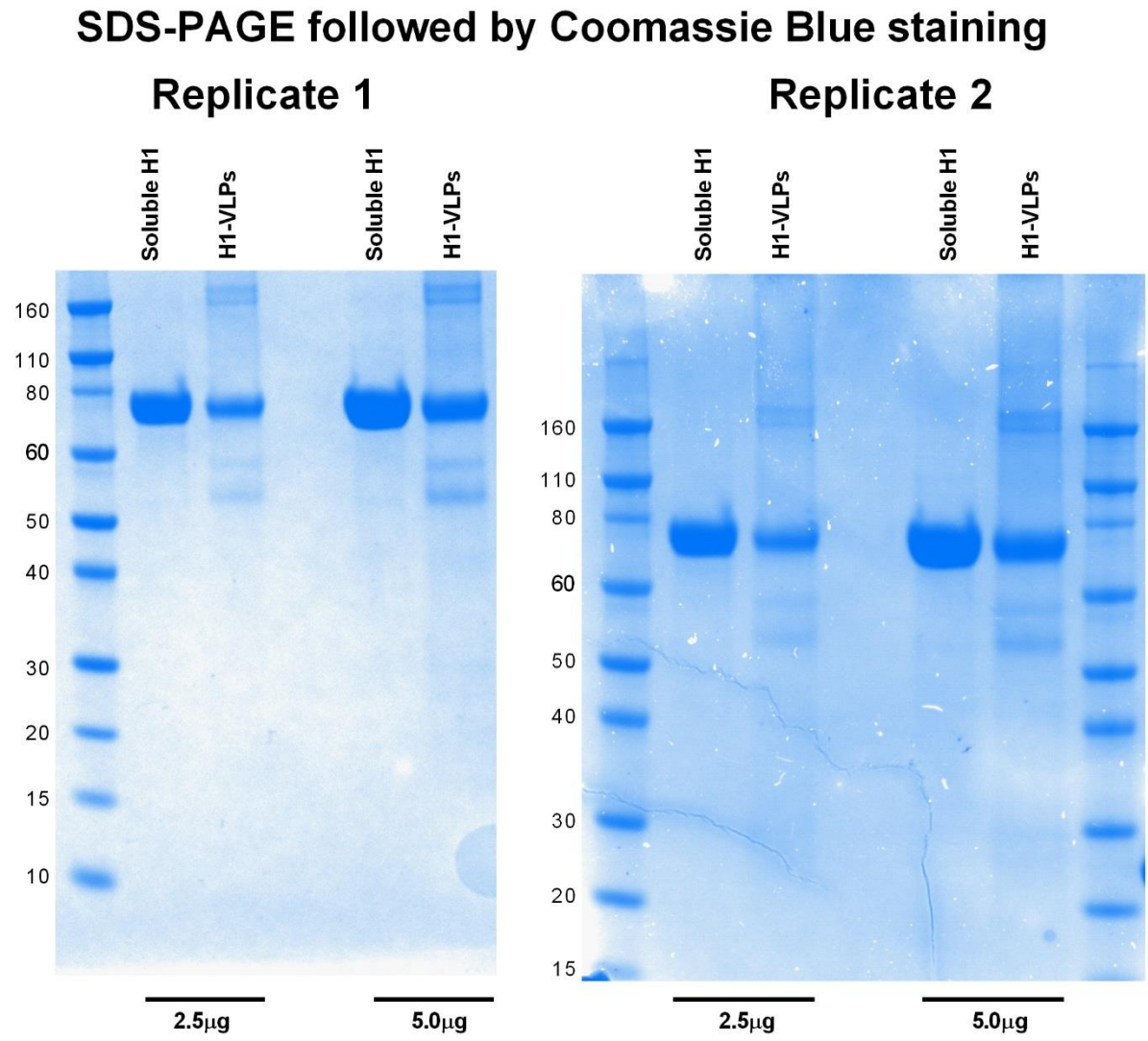
| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **56, 57** | **YAFAMERNAGSGIIISDTPV** | **20** | **2111.0357** | **270** | **289** | **2** | **0.01** | **1** | **A, D** |
| 58 | FAMERNAGSGIIISDTPVHD | 20 | 2129.0212 | 272 | 291 | 2 | 0.01 | 1 | D |
| 59 | AGSGIIISDTPVHDCNTTCQ | 20 | 2030.9037 | 278 | 297 | 3 | 0.01 | 1 | D |
| 60 | SGIIISDTPVHDCNTTCQTP | 20 | 2100.9456 | 280 | 299 | 3 | 0.01 | 1 | A |
| **61, 62** | **SDTPVHDCNTTCQTPKGAIN** | **20** | **2100.9205** | **285** | **304** | **2(A), 3(D)** | **0.01** | **1** | **A, D** |
| 63 | TPVHDCNTTCQTPKGAINTS | 20 | 2086.9412 | 287 | 306 | 3 | 0.01 | 1 | A |
| 64 | KGAINTSLPFQNIHPI | 16 | 1748.9574 | 300 | 315 | 2 | 0.01 | 1 | C |
| 65 | GAINTSLPFQNIHPITIGKC | 20 | 2123.1197 | 301 | 320 | 3 | 0.01 | 1 | A |
| 66 | PITIGKCPKYVKSTKL | 16 | 1775.0379 | 314 | 329 | 3 | 0.01 | 1 | C |
| 67 | YVKSTKLRLATGLRNI | 16 | 1832.0996 | 323 | 338 | 3 | 0.01 | 1 | C |
| 68 | YVKSTKLRLATGLRNIPSIQ | 20 | 2257.3270 | 323 | 342 | 3 | 0.01 | 1 | D |
| 69 | TKLRLATGLRNIPSIQ | 16 | 1780.0683 | 327 | 342 | 3 | 0.01 | 1 | C |
| 70 | RLATGLRNIP | 10 | 1109.6669 | 330 | 339 | 2 | 0.00 | 1 | C |
| 71 | LATGLRNIPSIQSRGLFGAI | 20 | 2083.1902 | 331 | 350 | 3 | 0.01 | 1 | D |
| 72 | RNIPSIQSRGLFGAIAGFIE | 20 | 2145.1695 | 336 | 355 | 3 | 0.01 | 1 | D |
| 73 | IPSIQSRGLFGAIAGFIEGG | 20 | 1989.0684 | 338 | 357 | 2 | 0.01 | 1 | D |
| 74 | SIQSRGLFGAIAGFIEGGWT | 20 | 2066.0585 | 340 | 359 | 3 | 0.01 | 1 | D |
| **75, 76** | **LFGAIAGFIEGGWTGMVDGW** | **20** | **2082.9873** | **346** | **365** | **3** | **0.01** | **1(A), 6(D)** | **A, D** |
| 77 | IAGFIEGGWTGMVDGWYGYH | 20 | 2214.9833 | 350 | 369 | 3 | 0.01 | 1 | D |
| 78 | GFIEGGWTGMVDGWYGYHHQ | 20 | 2295.9796 | 352 | 371 | 3 | 0.01 | 1 | D |
| 79 | GYHHQNEQGSGYAADLKSTQ | 20 | 2189.9726 | 367 | 386 | 3 | 0.01 | 1 | A |
| 80 | STQNAIDEITNKVNSVIEKM | 20 | 2233.1260 | 384 | 403 | 3 | 0.01 | 1 | D |
| 81 | FTAVGKEFNHLEKRIENLNK | 20 | 2386.2757 | 407 | 426 | 3 | 0.01 | 1 | D |
| 82 | EFNHLEKRIENLNKKVDDGF | 20 | 2444.2448 | 413 | 432 | 3 | 0.01 | 1 | D |
| 83 | FNHLEKRIENLNKKVDDGFL | 20 | 2428.2863 | 414 | 433 | 3 | 0.01 | 1 | D |
| 84 | LEKRIEN | 7 | 900.5029 | 417 | 423 | 3 | 0.01 | 1 | B |
| **85, 86** | **NKKVDDGFLDIWTYNAELLV** | **20** | **2352.2002** | **425** | **444** | **3** | **0.01** | **1** | **A, D** |
| 87 | VDDGFLDIWTYNAELLVLLE | 20 | 2337.1780 | 428 | 447 | 3 | 0.01 | 1 | D |
| 88 | DDGFLDIWTYNAELLVLLEN | 20 | 2352.1525 | 429 | 448 | 2;3 | 0.01 | 2 | D |
| 89 | DIWTYNAELLVLLENERTLD | 20 | 2419.2271 | 434 | 453 | 3 | 0.01 | 1 | D |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 90 | LVLLENERTLDYHDSN | 16 | 1929.9432 | 443 | 458 | 3 | 0.01 | 1 | C |
| **91, 92** | **LLENERTLDYHDSNVKNLYE** | **20** | **2464.1870** | **445** | **464** | **2** | **0.01** | **1** | **A, D** |
| 93 | LDYHDSNVKNLYEKVRSQLK | 20 | 2448.2761 | 452 | 471 | 3 | 0.01 | 1 | D |
| 94 | SNVKNLYEKVRSQLKNNAKE | 20 | 2361.2765 | 457 | 476 | 2 | 0.01 | 1 | D |
| 95 | VKNLYEKVRSQLKNNA | 16 | 1903.0639 | 459 | 474 | 2 | 0.01 | 1 | C |
| 96 | RSQLKNNAKEIGNGCFEFYH | 20 | 2354.1226 | 467 | 486 | 3 | 0.01 | 1 | D |
| 97 | NNAKEIGNGCFEFYHKCDNT | 20 | 2302.9736 | 472 | 491 | 3 | 0.01 | 2 | D |
| 98 | GNGCFEFYHKCDNTCMESVK | 20 | 2310.9166 | 478 | 497 | 2 | 0.01 | 1 | D |
| 99 | EFYHKCDNTCMESVKNGTYD | 20 | 2382.9555 | 483 | 502 | 2 | 0.01 | 1 | D |
| 100 | CDNTCMESVKNGTYDYPKYS | 20 | 2316.9337 | 488 | 507 | 3 | 0.01 | 1 | A |
| 101 | TCMESVK | 7 | 796.3459 | 491 | 497 | 2 | 0.01 | 1 | B |
| 102 | KNGTYDYPKYSEEAKLNREE | 20 | 2433.1448 | 497 | 516 | 3 | 0.01 | 1 | D |
| 103 | REEIDGVKLESTRIYQILAI | 20 | 2345.2955 | 514 | 533 | 2 | 0.01 | 1 | D |
| 104 | DGVKLESTRIYQILAIYSTV | 20 | 2268.2365 | 518 | 537 | 2 | 0.01 | 1 | D |
| 105 | STRIYQILAIYSTVASSLVL | 20 | 2197.2358 | 524 | 543 | 3 | 0.01 | 1 | D |
| 106 | RIYQILAIYSTVASSL | 16 | 1797.0036 | 526 | 541 | 2 | 0.01 | 1 | C |
| **107, 108** | **QILAIYSTVASSLVLVVSLG** | **20** | **2032.1820** | **529** | **548** | **3** | **0.01** | **3(A), 2(D)** | **A, D** |
| 109 | AIYSTVASSLVLVVSLGAIS | 20 | 1949.1085 | 532 | 551 | 3 | 0.01 | 1 | D |
| 110 | IYSTVASSLVLVVSLGAISF | 20 | 2025.1398 | 533 | 552 | 3 | 0.01 | 1 | D |
| 111 | STVASSLVLVVSLGAISFWM | 20 | 2066.1122 | 535 | 554 | 2 | 0.01 | 1 | D |
| 112 | VASSLVLVVSLGAISFWMCS | 20 | 2068.0737 | 537 | 556 | 3 | 0.01 | 1 | A |
| 113 | VASSLVLVVSLGAISF | 16 | 1560.9127 | 537 | 552 | 3 | 0.01 | 1 | C |
| 114 | LVLVVSLGAISFWMCSNGSL | 20 | 2095.0846 | 541 | 560 | 3 | 0.01 | 1 | A |
| 115 | SLGAISFWMCSNGSLQCRIC | 20 | 2174.9734 | 546 | 565 | 3 | 0.01 | 2 | D |

*** sequences found in more than one sample highlighted in bold font**

**Supplemental Figures**

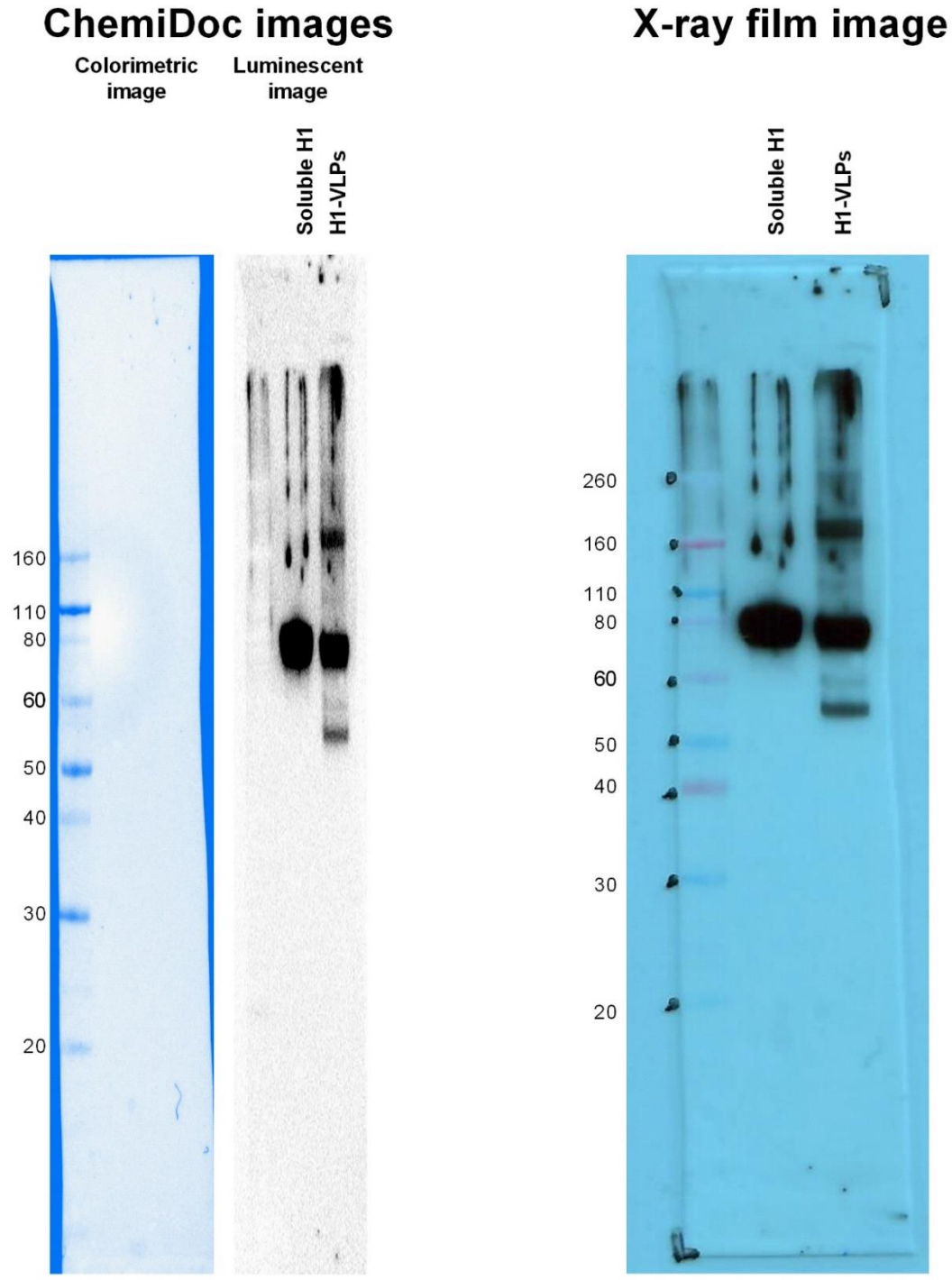
**a**




*(continued on next page)*

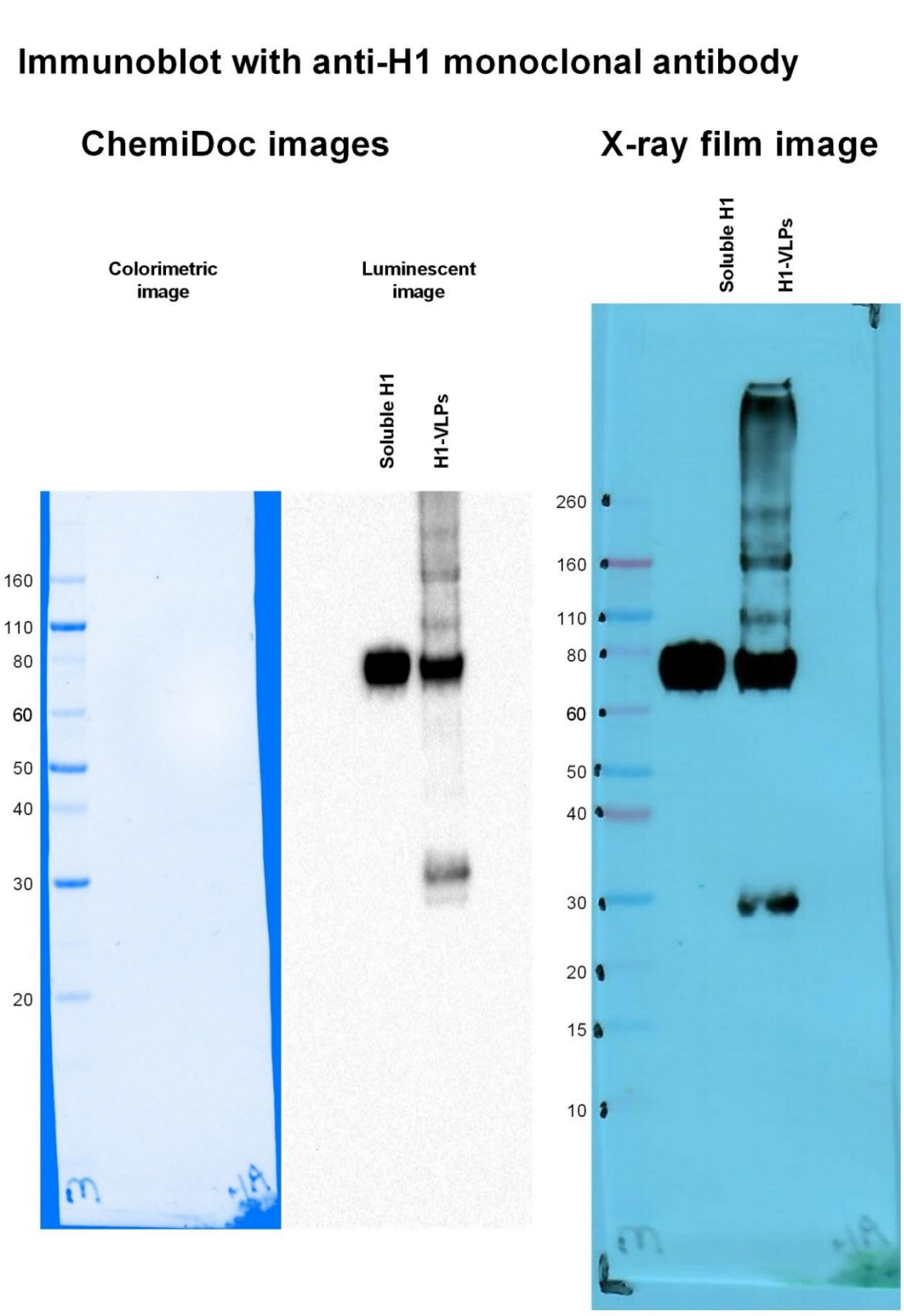
**b**

## Immunoblot with anti-H1 polyclonal antibody



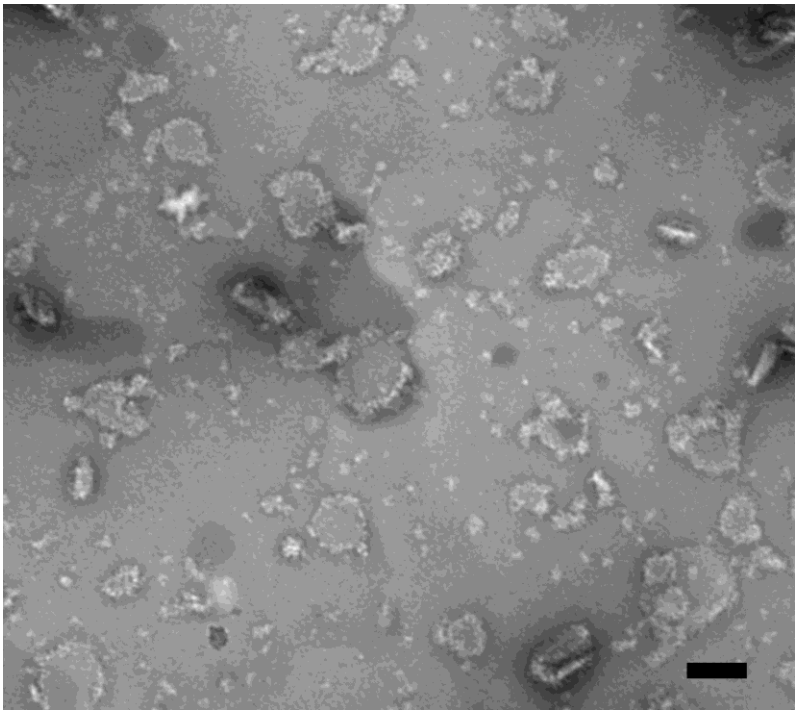

*(continued on next page)*

c



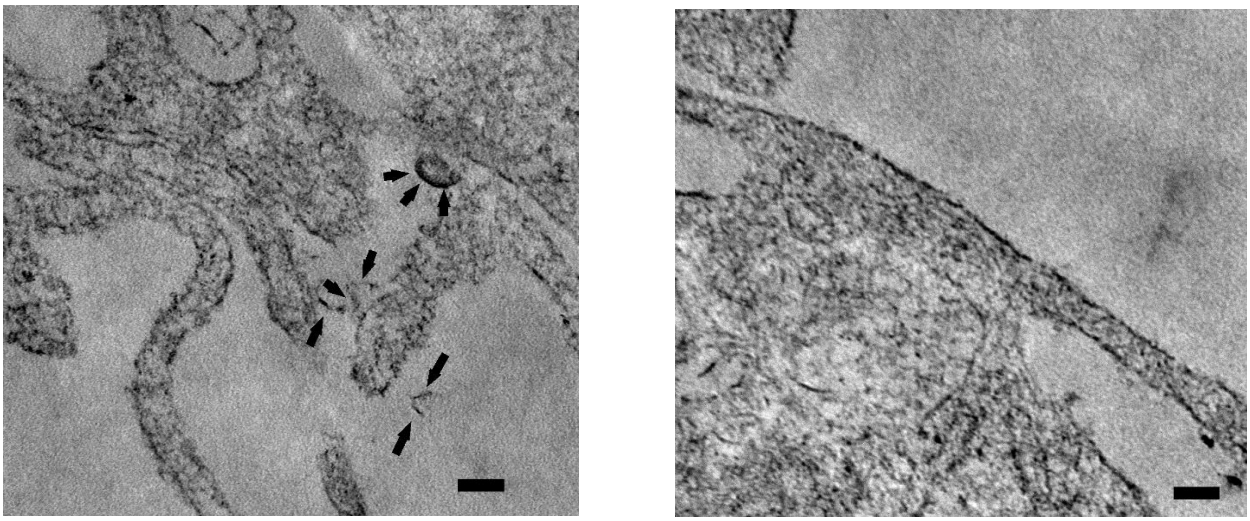

*(continued on next page)*

**d**



**e**

**H1-VLPs** **Soluble H1**



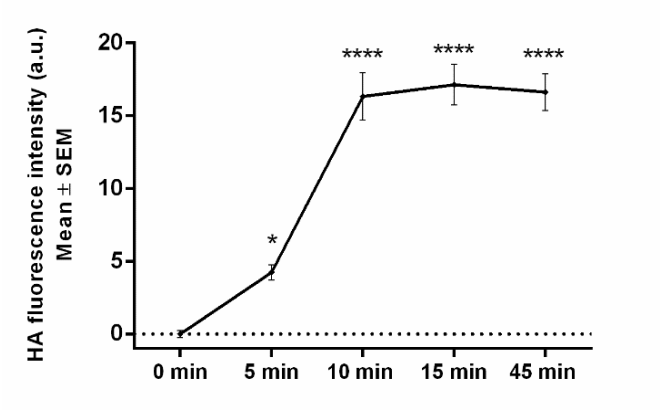
**Supplemental Figure 1. Gel Electrophoresis, Immunoblot and EM characterization of plant-derived H1-VLPs and soluble recombinant H1 (comparator).**

**(a-c)** Full, un-cropped images of SDS-PAGE with Coomassi blue staining and immunoblots,

***(legend continued on next page)***

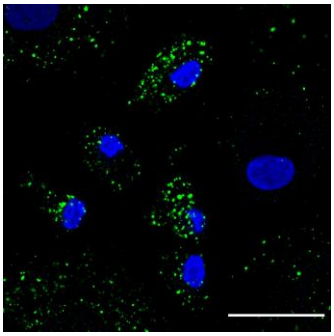
including full molecular weight markers. **(a)** Two representative examples of SDS-PAGE gels stained with Coomassie blue. The H1-VLPs and soluble recombinant H1 (comparator) were applied at 2.5 and 5.0 µg per sample (by HA content). The predominant protein observed in both samples corresponded to the molecular weight of monomeric H1 (~ 75 kDa). No additional bands suggesting HA cleavage or degradation products were found in the soluble H1 sample (comparator). The H1-VLPs sample had a small number of less prominent bands at ~160 kDa (probably HA dimers) and at ~55 kDa (probably HA1 subunit). Immunoblot images of H1-VLPs and soluble H1 (both applied at 0.5 µg per sample by HA content) with polyclonal (**b**) or monoclonal (**c**) anti-H1 antibodies. In both cases, images were acquired using the ChemiDoc™ XRS+ System (ChemiDoc) with molecular weight markers (left) or immunostained for H1 (middle). X-ray images of the same membranes are also presented (right). The predominant protein observed in both samples was again monomeric H1 (~ 75 kDa) with no additional bands suggesting HA cleavage or degradation products in the soluble H1 sample (comparator). For the VLP sample, a small number of fainter bands were seen on both immunoblots as well as the X-ray images: ~160 kDa (HA dimers) and ~55 kDa (HA1 subunit). On some blots immunostained with the monoclonal anti-H1, faint bands were present at ~240 kDa (likely HA trimmers) and at 30 kDA (HA2 subunit). The low evident band at ~ 105 kDa probably represents the uncleaved H1-HA2 subunit complexes. **(d)** Negative stain EM of H1-VLP sample. Representative image shows pleomorphic particles ~ 100 nm average diameter with spikes on their surface. Scale bar – 100 nm. **(e)** Representative images of human MDMs exposed to H1-VLPs (left) or soluble H1 (right) at HA concentration 15 µg/mL. Arrows indicate VLP-like structures on the surface on VLP-treated MDM. Scale bar – 100 nm.

**a**

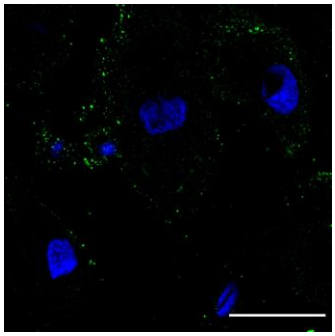



**b**

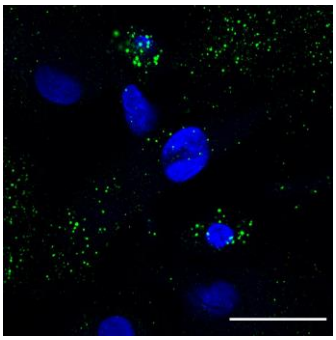
H1-VLPs, 15 µg/mL



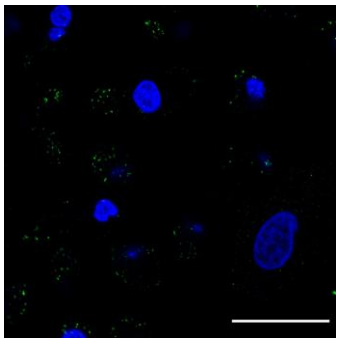
Split H1N1 vaccine, 15 µg/mL

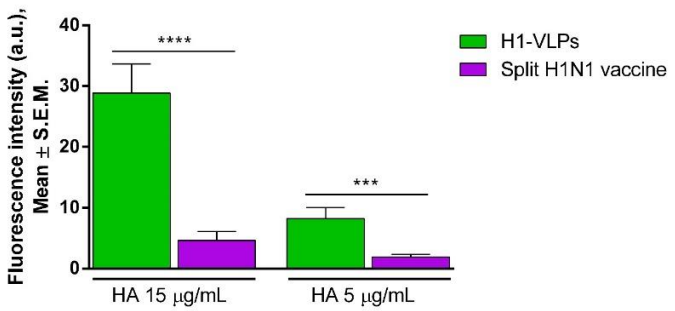


H1-VLPs, 5 µg/mL



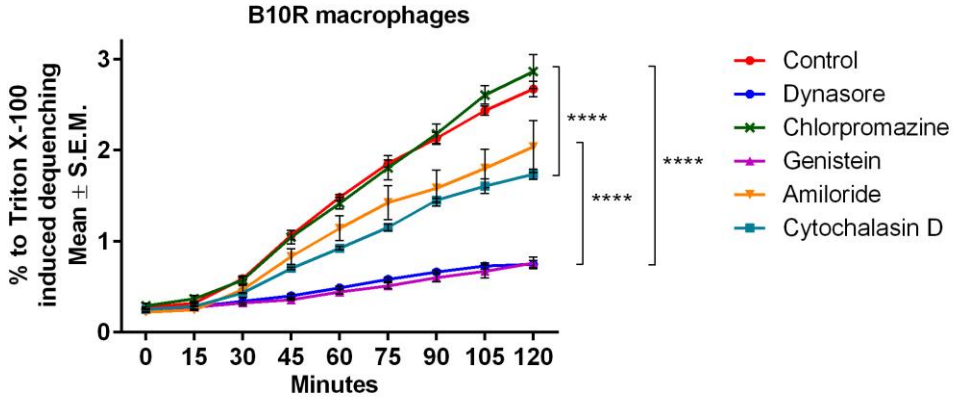
Split H1N1 vaccine, 5 µg/mL



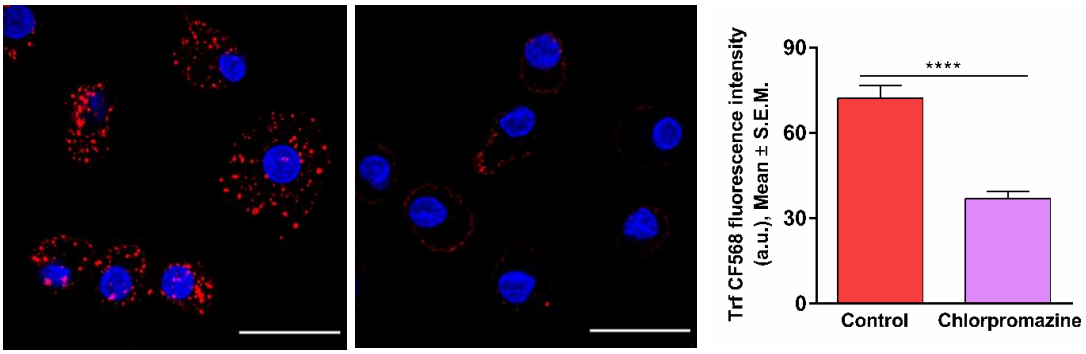



***(continued on next page)***

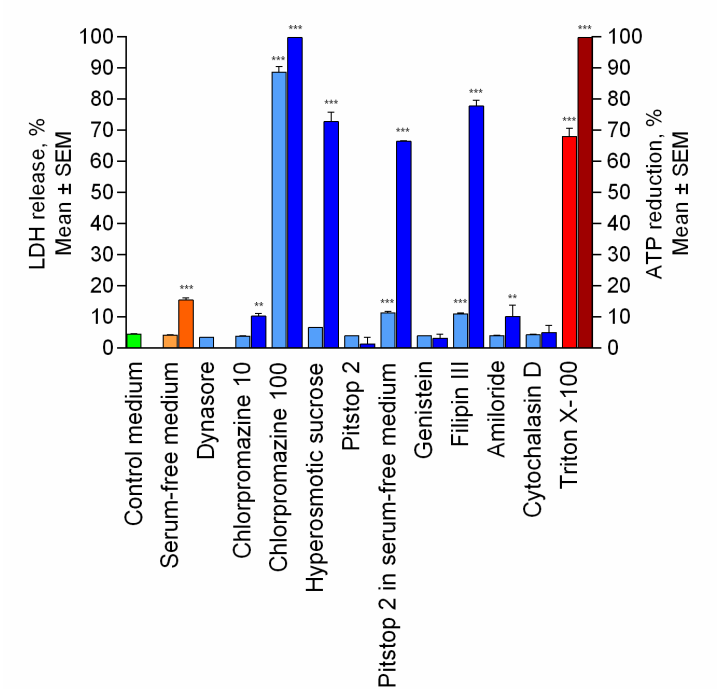
**c**




**d**




**e**



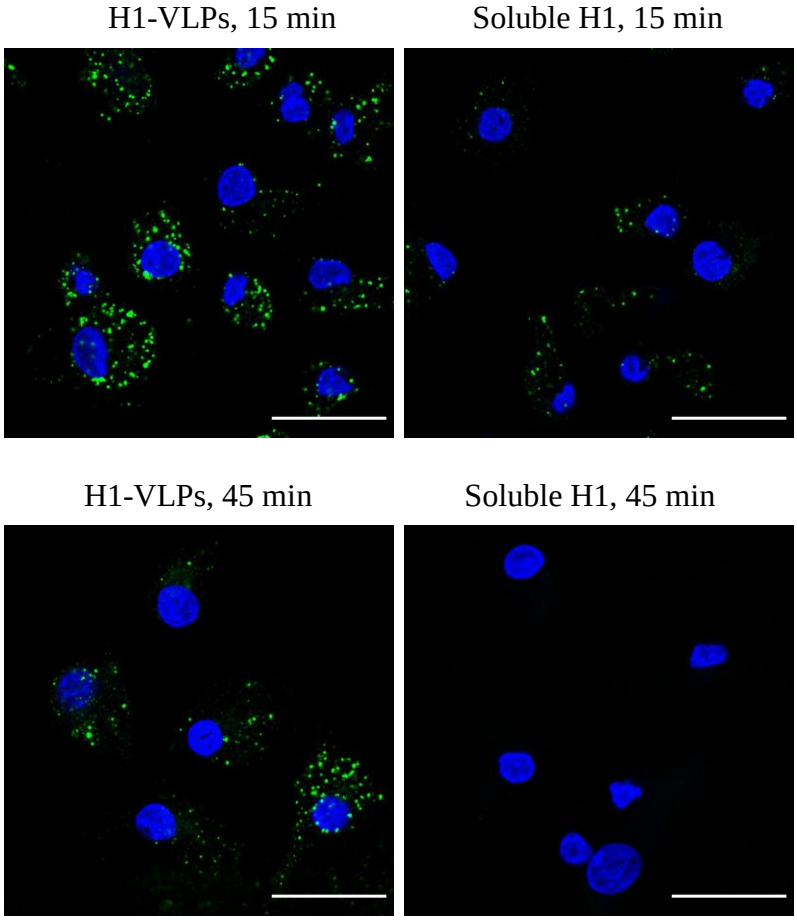

***(legend on next page)***

**Supplemental Figure 2. Endocytic pathways in human and murine macrophages.**

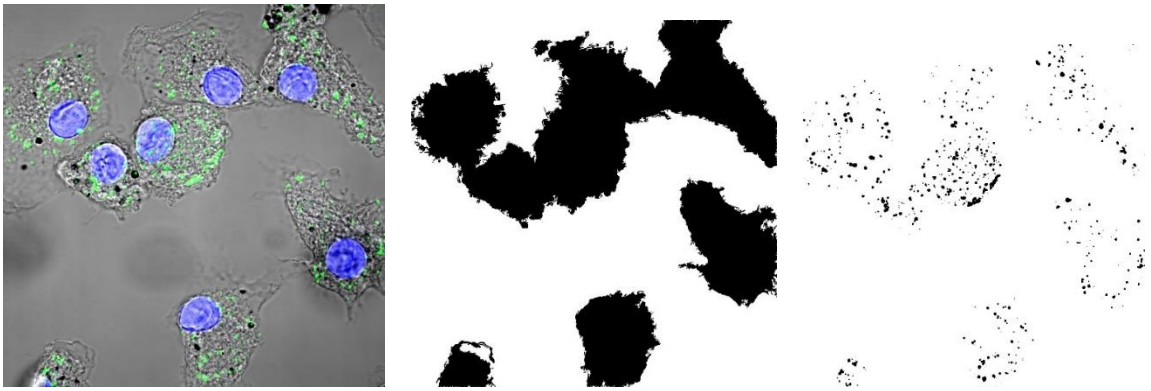
**(a)** Time-course of HA internalization by human MDMs. The amount of internalized protein was evaluated by the intensity of HA immunofluorescence per cell area on confocal microscopy images. The fluorescence intensity increased by 10 min and then remained at the same level up to 45 min. Based on three experiments. **(b)** Comparison of HA internalization by human MDMs exposed to either H1-VLPs or influenza A (H1N1) 2009 monovalent split vaccine for 30 min. Representative images of MDMs treated with H1-VLPs or monovalent H1N1 split vaccine at HA concentration 15 µg/mL (top) or 5 µg/mL (middle). Green: fluorescently labeled HA, blue: nuclei stained with DAPI. The amount of internalized protein was evaluated by the intensity of HA immunofluorescence per cell area on confocal microscopy images (bottom). Scale bar – 25 µm. **(c)** Effects of selected endocytosis inhibitors on DiD fluorescence dequenching by murine B10R macrophages loaded with DiD-labeled H1-VLPs (n=3). Chlorpromazine (10 µg/mL) did not affect DiD fluorescence. **(d)** Chlorpromazine (10 µg/mL) reduced transferrin (Trf) uptake by human MDMs. Representative images of the control sample treated with fluorescently-labelled transferrin (left), cell exposed to transferrin in the presence of chlorpromazine (middle) and the analysis of transference fluorescence intensities (right) are presented; based on three experiments. Red: transferrin conjugated with CF568 fluorophore, blue: nuclei stained with DAPI. Scale bar – 25 µm. **(e)** Cytotoxic effects of endocytosis inhibitors on murine B10R macrophages assessed by LDH release (left Y axis) and ATP level reduction (right Y axis) (n=3). Chlorpromazine hydrochloride (100 µg/mL), sucrose (0.45 M), pitstop 2 (25 µM) applied in serum-free medium, and filipin III from *Streptomyces filipinensis* (5 µg/mL) caused prominent LDH release and/or ATP level reduction in B10R macrophages. Mean ± Standard Error of the Mean (S.E.M.) presented on all graphs; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ (a, b,

e - one-way ANOVA followed by Tukey's multiple comparisons post-test; c - two-way ANOVA followed by Tukey's multiple comparisons post-test; d - Mann-Whitney test).

**a**




**b**



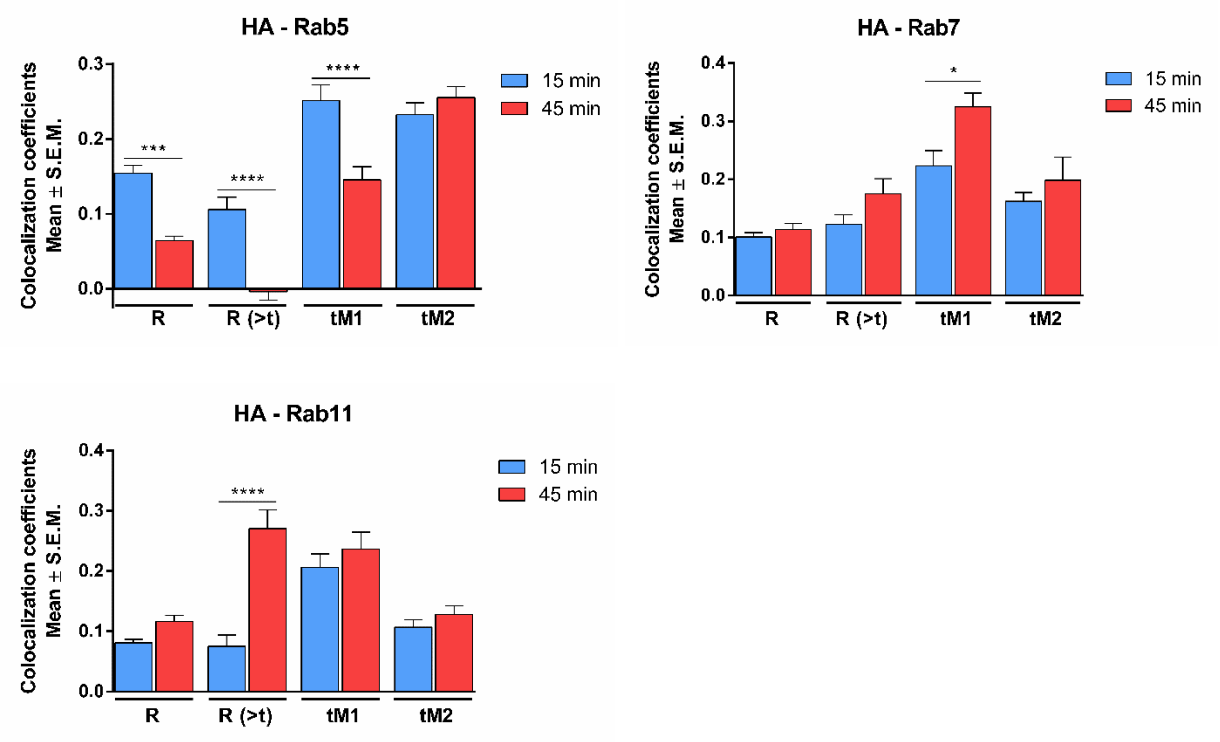
**Supplemental Figure 3. HA internalization by human MDMs exposed to H1-VLPs or soluble H1.**

**(a)** Representative images of MDMs pulsed (15 min) with H1-VLPs or soluble H1 at 15 min and

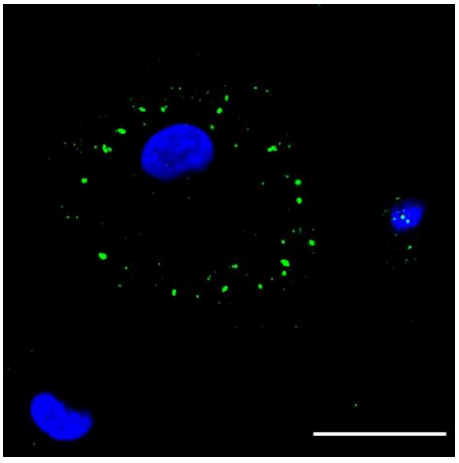
***(legend continued on next page)***

45 min. Green: fluorescently labeled HA, blue: nuclei stained with DAPI. Scale bar – 25 μm. **(b)** Examples of image segmentation strategies. Left - bright-field image of MDMs with internalized HA (green) and nuclei stained with DAPI (blue). Image segmentation based on identification of cell boundaries – entire cell area (middle). Image segmentation based on detecting HA-positive endosomes (right).

**a**




**b**



**Supplemental Figure 4. Endosomal trafficking of HA in MDMs pulsed (15 min) with H1-VLPs or soluble H1.**

**(a)** Conventional analysis of HA colocalization with Rab5, Rab7 or Rab11. Based on three or more experiments for each condition. Mean ± Standard Error of the Mean (S.E.M.) presented;

***(legend continued on next page)***

* $p<0.05$, *** $p<0.001$, **** $p<0.0001$ (one-way ANOVA followed by Tukey's multiple comparisons post-test). **(b)** Representative image of MDM pulsed with H1-VLPs at 45 min. Peripheral (towards the plasma membrane) re-distribution (recycling) of non-degraded HA can be seen. Green: fluorescently labeled HA, blue: nuclei stained with DAPI. Scale bar – 25 μm.

## Supplemental references


1. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9,** 671–675 (2012).
2. Pike, J. A., Styles, I. B., Rappoport, J. Z. & Heath, J. K. Quantifying receptor trafficking and colocalization with confocal microscopy. *Methods* **115,** 42–54 (2017).
3. Luisier, F. High-quality denoising of multidimensional fluorescence microscopy images (2D+t, 3D or color). *Biomedical Imaging Group (BIG), EPFL, Switzerland* (2014). Available at: http://bigwww.epfl.ch/algorithms/denoise/. (Accessed: 28th June 2018)
4. Castle, M. & Keller, J. Rolling Ball Background Subtraction. *Mental Health Research Institute, University of Michigan* (2015). Available at: https://imagej.net/Rolling_Ball_Background_Subtraction. (Accessed: 28th June 2018)
5. Collins, T. Colocalization Threshold. (2015). Available at: https://imagej.net/Colocalization_Threshold. (Accessed: 28th June 2018)
6. Costes, S. V *et al.* Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophys. J.* **86,** 3993–4003 (2004).
7. Dunn, K. W., Kamocka, M. M. & McDonald, J. H. A practical guide to evaluating colocalization in biological microscopy. *AJP Cell Physiol.* **300,** C723–C742 (2011).
8. Bolte, S. & Cordelières, F. P. A guided tour into subcellular colocalization analysis in light microscopy. *Journal of Microscopy* **224,** 213–232 (2006).
9. Macia, E. *et al.* Dynasore, a Cell-Permeable Inhibitor of Dynamin. *Dev. Cell* **10,** 839–850 (2006).
10. Delvendahl, I., Vyleta, N. P., von Gersdorff, H. & Hallermann, S. Fast, Temperature-Sensitive and Clathrin-Independent Endocytosis at Central Synapses. *Neuron* **90,** 492–498

(2016).

11. Preta, G., Cronin, J. G. & Sheldon, I. M. Dynasore - Not just a dynamin inhibitor. *Cell Communication and Signaling* **13,** 24 (2015).

12. Wang, L. H., Rothberg, K. G. & Anderson, R. G. W. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J. Cell Biol.* **123,** 1107–1117 (1993).

13. Daniel, J. A. *et al.* Phenothiazine-Derived Antipsychotic Drugs Inhibit Dynamin and Clathrin-Mediated Endocytosis. *Traffic* **16,** 635–654 (2015).

14. Heuser, J. E. & Anderson, R. G. W. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J. Cell Biol.* **108,** 389–400 (1989).

15. Von Kleist, L. *et al.* Role of the clathrin terminal domain in regulating coated pit dynamics revealed by small molecule inhibition. *Cell* **146,** 471–484 (2011).

16. Willox, A. K., Sahraoui, Y. M. E. & Royle, S. J. Non-specificity of Pitstop 2 in clathrin-mediated endocytosis. *Biol. Open* **3,** 326–331 (2014).

17. Dutta, D., Williamson, C. D., Cole, N. B. & Donaldson, J. G. Pitstop 2 Is a Potent Inhibitor of Clathrin-Independent Endocytosis. *PLoS One* **7,** e45799 (2012).

18. Akiyama, T. *et al.* Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* **262,** 5592–5595 (1987).

19. Tiruppathi, C., Song, W., Bergenfeldt, M., Sass, P. & Malik, A. B. Gp60 activation mediates albumin transcytosis in endothelial cells by tyrosine kinase-dependent pathway. *J. Biol. Chem.* **272,** 25968–25975 (1997).

20. Orlandi, P. A. & Fishman, P. H. Filipin-dependent inhibition of cholera toxin: Evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.* **141,**

905–915 (1998).

21. Koivusalo, M. *et al.* Amiloride inhibits macropinocytosis by lowering submembranous pH and preventing Rac1 and Cdc42 signaling. *J. Cell Biol.* **188,** 547–563 (2010).

22. Elliott, J. A. & Winn, W. C. Treatment of alveolar macrophages with cytochalasin D inhibits uptake and subsequent growth of Legionella pneumophila. *Infect. Immun.* **51,** 31–36 (1986).

23. Kuhn, D. A. *et al.* Different endocytotic uptake mechanisms for nanoparticles in epithelial cells and macrophages. *Beilstein J. Nanotechnol.* **5,** 1625–1636 (2014).