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Supplemental Information

Targeting Antigen to the Surface of EVs Improves the *In Vivo* Immunogenicity of Human and Non-human Adenoviral Vaccines in Mice

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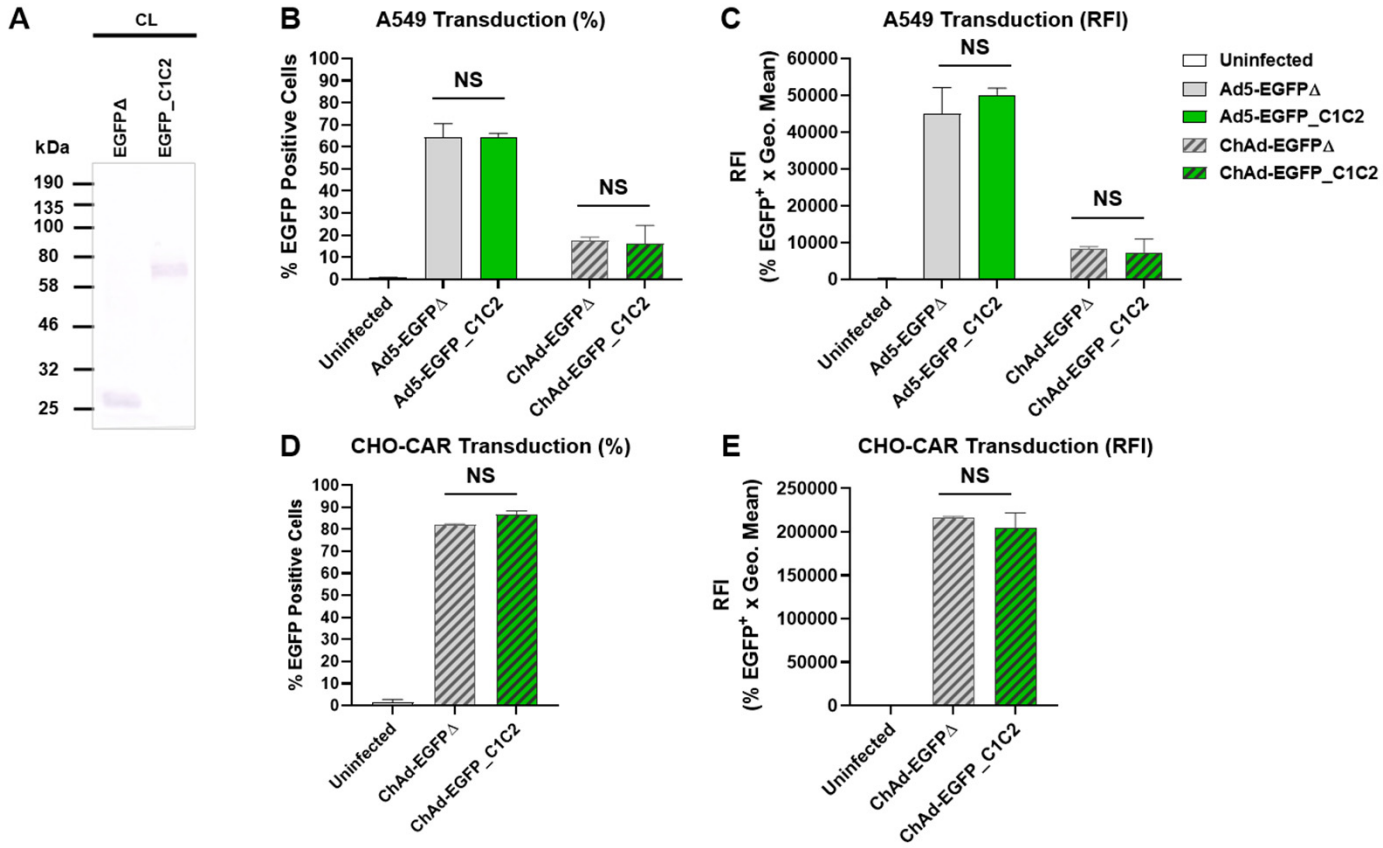
SUPPLEMENTARY TABLE.1 Exosome Validation

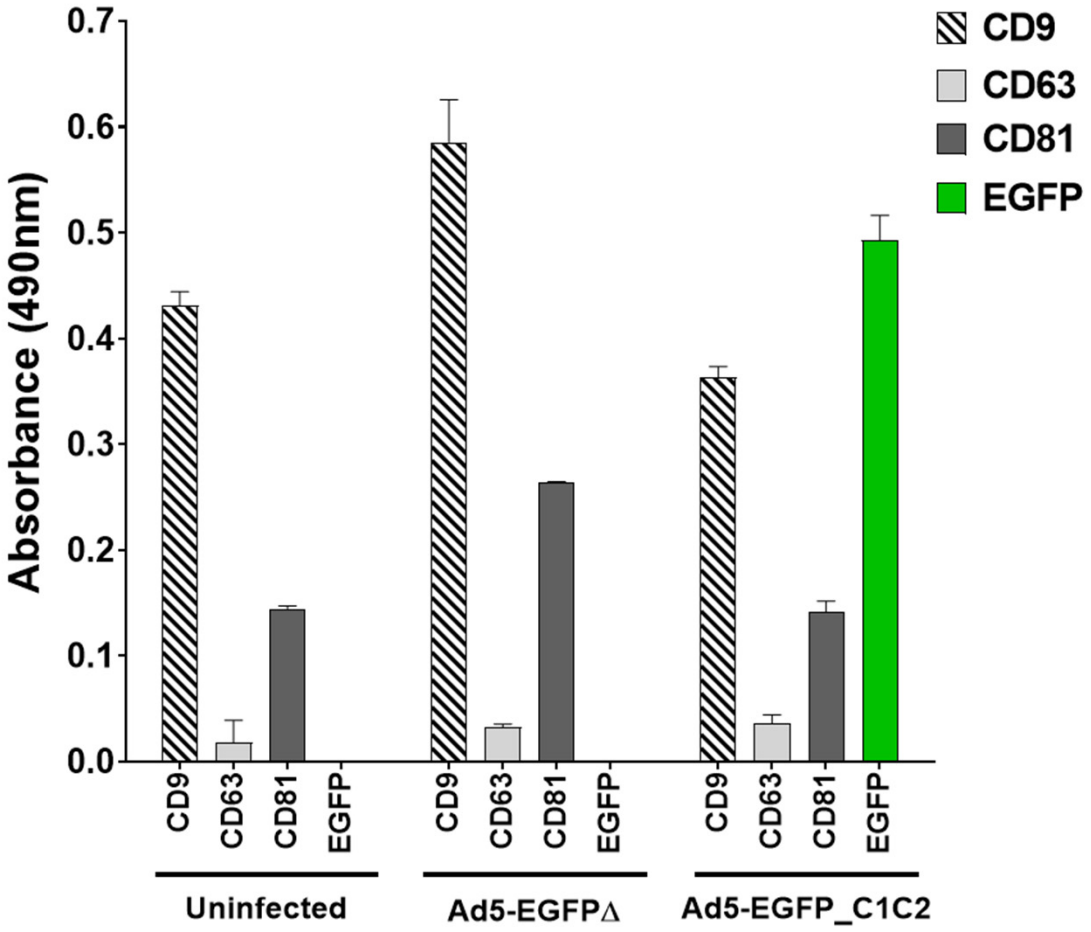
Plasmid name	Volume of Transfection (mL)	Transfection Efficiency (%)	# Cells at Harvest (96h)	Protein Conc [µg/mL]	NTA Particle conc $x10^{10}$/mL	Particle Mode (nm)
pcDNA-EGFPΔ	250mL	53.6	$6.66x10^{6}$/mL	694ug/mL	12.6 $x10^{10}$	161nm
pcDNA-EGFP_C1C2	250mL	53.7	$7.2x10^{6}$/mL	594ug/mL	10.5 $x10^{10}$	157nm

SUPPLEMENTARY FIGURE.1 - EGFP Transgene Expression



Supplementary Figure.1. Quantification of EGFP transgene expression for EGFPΔ and EGFP_C1C2. (A) Anti-HIS tag W.blot on 20µg cleared cell lysate of Expi293F cells transfected with pcDNA3.1SSmut-EGFPΔ or EGFP_C1C2 plasmid. The transfection efficiency of these samples, as measured by flow cytometry quantification of EGFP expression was also equivalent (*Supplementary Table.1*). **(B, C)** The transduction efficiency in A549 cells with Ad5 and ChAd vectors expressing EGFPΔ or EGFP_C1C2 transgene cassette as quantified by flow cytometry. Percentage EGFP$^+$ cells *(A)* and relative fluorescence intensity (RFI), generated by multiplying % EGFP$^+$ cells by the geometry mean fluorescence *(B)* are shown. An *n=3-6* samples were analyzed and >20,000 gated events acquired per sample. **(D, E)** The transduction efficiency of CHO-CAR cells with Ad5- and ChAd- vectors expressing EGFPΔ or EGFP_C1C2 transgene cassette as quantified by flow cytometry. ChAd vectors were tested in this additional cell line which better supports the entry of this vector *in vitro*. As above, % EGFP$^+$ cells and RFI data are shown. Data represent the mean ±S.D. No significant differences were detected when comparing EGFPΔ or EGFP_C1C2, as determined using non-parametric Mann-Whitney test. *NS = p>0.05*.

SUPPLEMENTARY FIGURE.2 – Ad Exosome Targeting *In Vitro*



Supplementary Fig.2. Exosome-display targeting of EGFP to the surface of EVs derived from A549 cells infected with Ad5-EGFP_C1C2 *in vitro*. ELISA plates were coated with 4µg/well of EVs precipitated from the SN of A549 cells grown in media with exosome-depleted FBS 72h post-infection with Ad5-EGFPΔ or Ad5-EGFP_C1C2 at a MOI 250 IFU/cell. An uninfected control was run in parallel. Tetraspanin ELISAs (CD9/CD63/CD81) were performed in parallel with an EGFP ELISA. Data represent OD490nm values following subtraction of matched isotype control Ab responses. Samples are representative of duplicate infection samples which were combined for EV precipitation and ELISAs were performed in duplicate wells. Data show mean ± S.D and are representative of two repeat experiments.

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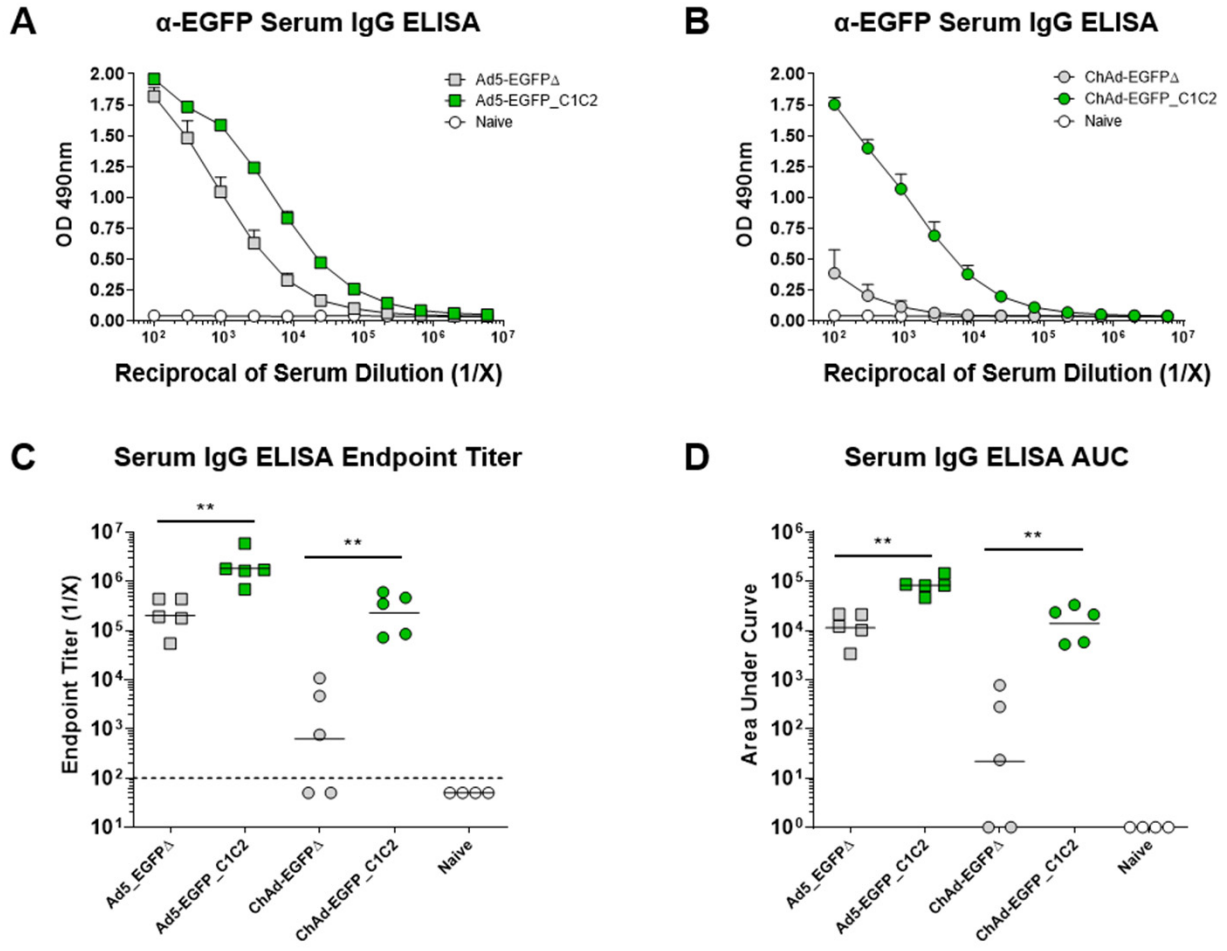
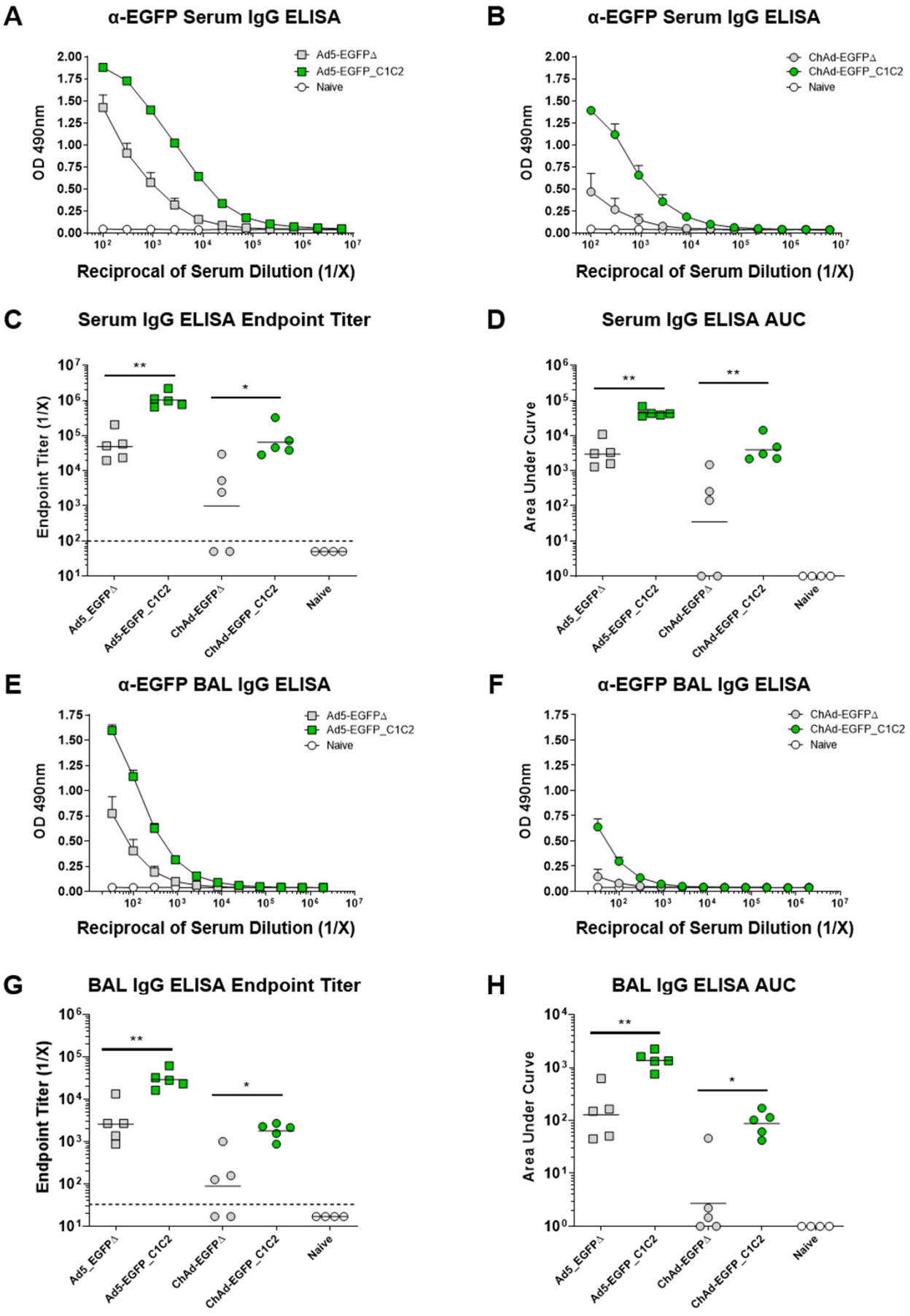
Supplementary Fig.3 D28 Serum *Intramuscular* Vaccination



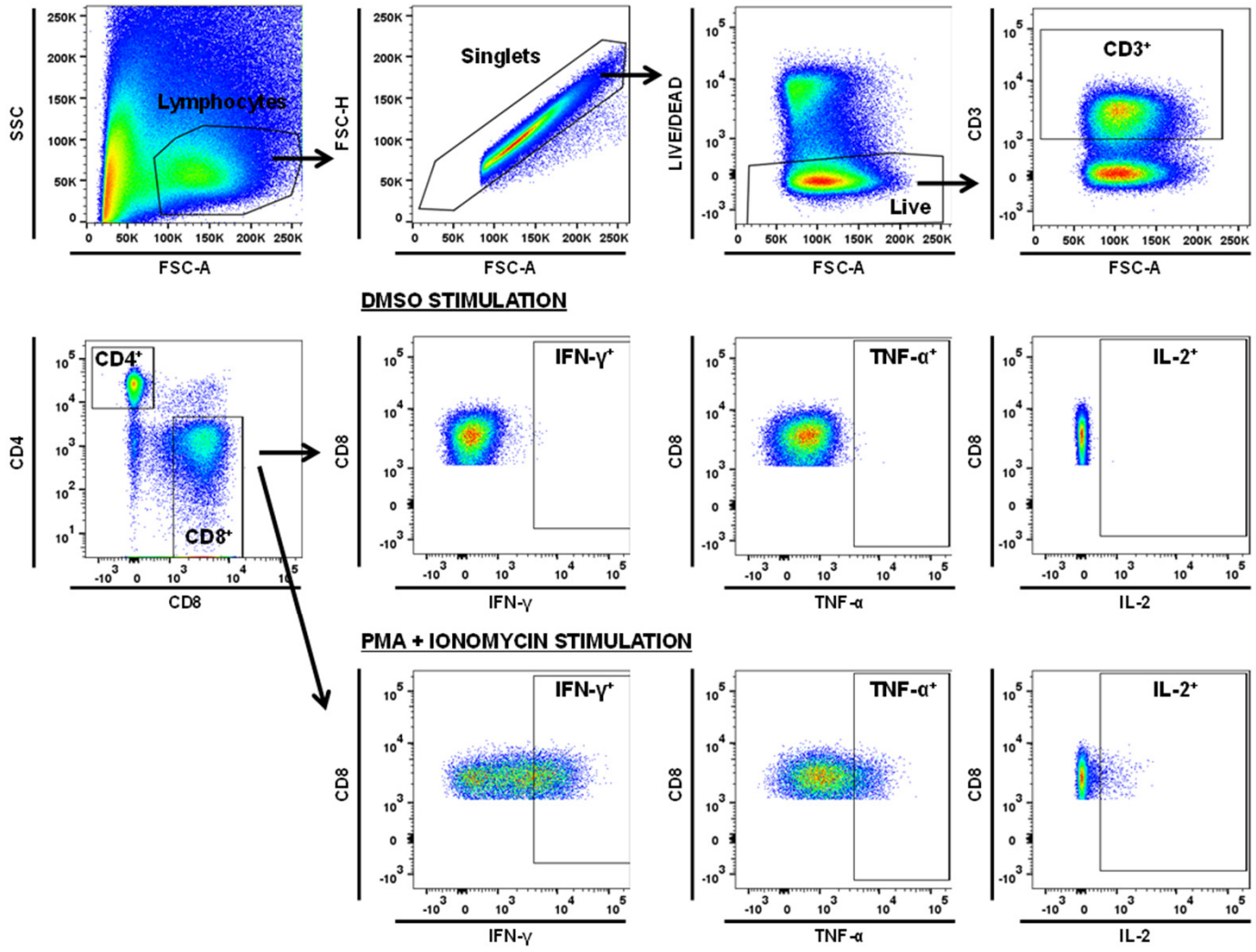
Supplementary Figure.3. Expression of EGFP_C1C2 fusion Ag by an adenoviral vaccine results in improved antigen-specific humoral immune responses in serum at D28 following intramuscular delivery in mice. (A) Mice were vaccinated intramuscularly (*i.m.)* with 1x10^8 IFU Ad5-EGFPΔ (*gray box*), Ad5-EGFP_C1C2 (*green box*) or PBS (naive) in a final volume of 50µL. Anti-EGFP IgG responses in sera were measured 28-days post-immunization by ELISA using plates coated with 1µg/mL recombinant EGFP protein. **(B)** Mice were vaccinated with 1x10^8 IFU ChAd-EGFPΔ (*gray circle*) or ChAd-EGFP_C1C2 (*green circle*) and ELISA assay performed exactly as described in A. Data show mean ±S.E.M (*n=5* mice/group) of duplicates. *Note:* Where S.E.M error bars are not visible, this is due to the error bar being shorter than the size of the symbol. **(C)** Endpoint titers were determined as the reciprocal dilution greater than x3 standard deviations (S.D.) plus the average of the mean of naive sera controls. Solid line indicates the geometric mean. Dashed line indicates starting dilution of sera (1:100), and therefore the lower limit of detection for the assay. Values below this line are estimated at half the input dilution (i.e. 1:50 dilution is estimated to represent endpoint). **(D)** Area under the curve (AUC) represents the total peak area calculated from ELISA values, where the baseline was set to the OD of the naive serum sample. Samples where no AUC could be calculated were set arbitrarily to a value of 1.0. Line indicates the geometric mean. Statistical significance was determined using a non-parametric Mann-Whitney test comparing EGFP_C1C2 with EGFPΔ Ad vaccines. ** $p<0.01$.

Supplementary Figure.4. Expression of EGFP_C1C2 fusion Ag by an adenoviral vaccine results in improved antigen-specific humoral immune responses in serum at D28 following intranasal delivery in mice. (A) Mice were vaccinated intranasally (*i.n.*) with $1x10^8$ IFU Ad5-EGFPΔ (*gray box*), Ad5-EGFP_C1C2 (*green box*) or PBS (naive) in a final volume of 50µL PBS. Anti-EGFP IgG responses in sera were measured 28-days post-immunization by ELISA. **(B)** Mice were vaccinated with $1x10^8$ IFU ChAd-EGFPΔ (*gray circle*) or ChAd-EGFP_C1C2 (*green circle*) and assay performed exactly as described in A. Data show mean ±S.E.M (*n=5* mice/group) of duplicates. *Note:* Where S.E.M error bars are not visible, this is due to the error bar being shorter than the size of the symbol. **(C)** Endpoint titers were determined from ELISA OD values as the reciprocal dilution greater than x3 standard deviations (S.D.) plus the average of the mean of naive sera controls. Line indicates the geometric mean. Dashed line indicates starting dilution of sera (1:100), values below this line are estimated at half the input dilution (i.e. 1:50 dilution is estimated to represent endpoint). Line indicates the geometric mean. **(D)** Area under the curve (AUC) represents the total peak area calculated from ELISA values, where the baseline was set to the OD of the naïve serum sample. Samples where no AUC could be calculated were set arbitrarily to a value of 1.0. Solid line indicates the geometric mean. **(E)** EGFP IgG responses in BAL at D28 post-immunization with Ad5 expressing EGFPΔ or EGFP_C1C2. **(F)** EGFP IgG responses in BAL at D28 following immunization with ChAd expressing EGFPΔ or EGFP_C1C2. **(G)** Endpoint titers were determined from ELISA OD values as described above. Solid line indicates the geometric mean. Dashed line indicates starting dilution of sera (1:33), values below this line are estimated at half the input dilution (i.e. 1:17 dilution is estimated to represent endpoint). **(H)** Area under the curve (AUC) values were calculated as described above. Solid line indicates the geometric mean. Statistical significance was determined using a non-parametric Mann-Whitney test. ** $p<0.05$, ** $p<0.01$.*

Supplementary Fig.4 D28 Serum *Intranasal* Vaccination



Supplementary Fig.5 Flow Cytometry Gating Strategy



Supplementary Figure.5. Schematic gating strategy for flow cytometry. An example of the gating strategy for flow cytometry surface staining with intracellular cytokine staining. Lymphocytes in lung or BAL were gated on lymphocytes, singlets and $CD3^+$ live cells. $CD8^+$ T-cells were identified and individual cytokine-negative and -positive populations gated (e.g. IFN-γ, TNF-α or IL-2). Mock stimulated cells (DMSO, no antigen) and positive control stimulated cells (PMA/ionomycin) are shown and were used to identify negative and positive populations of cells responding to the vaccine antigen, EGFP. Identical gates were applied for each sample being compared (i.e. gates applied to all groups for lung, or all groups for BAL).