

Supplemental material

$\alpha$ -Galactosylceramide protects swine against influenza infection when administered as a vaccine adjuvant

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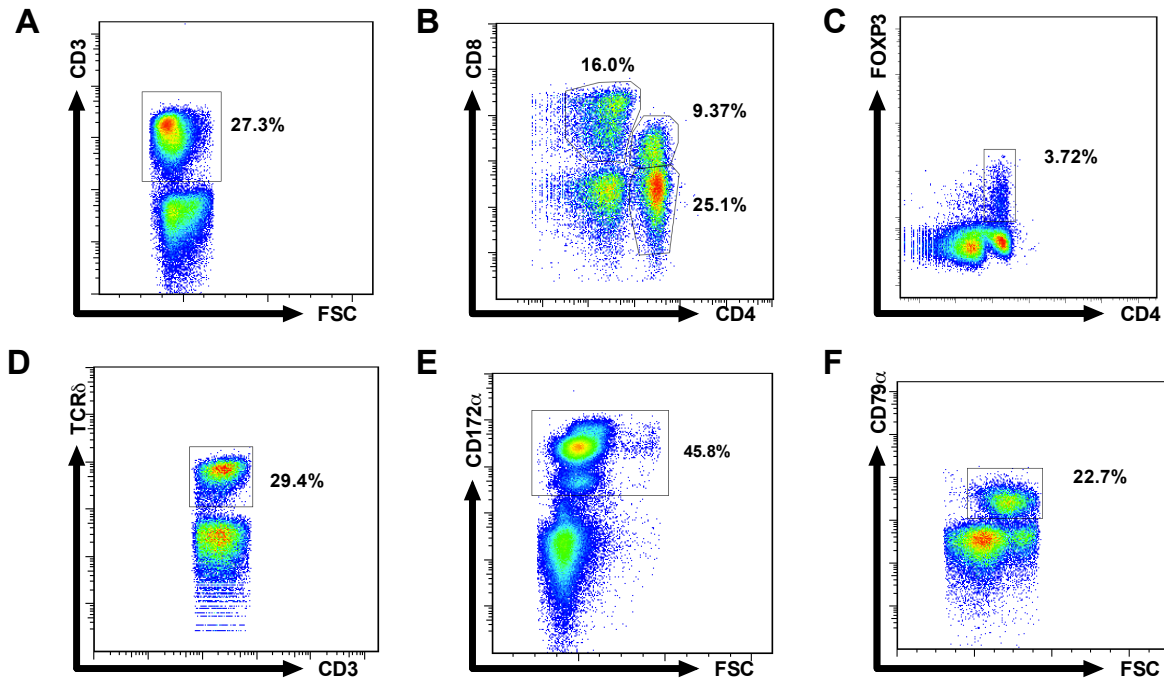
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Running Head: NKT-cells enhance swine influenza vaccines in pigs

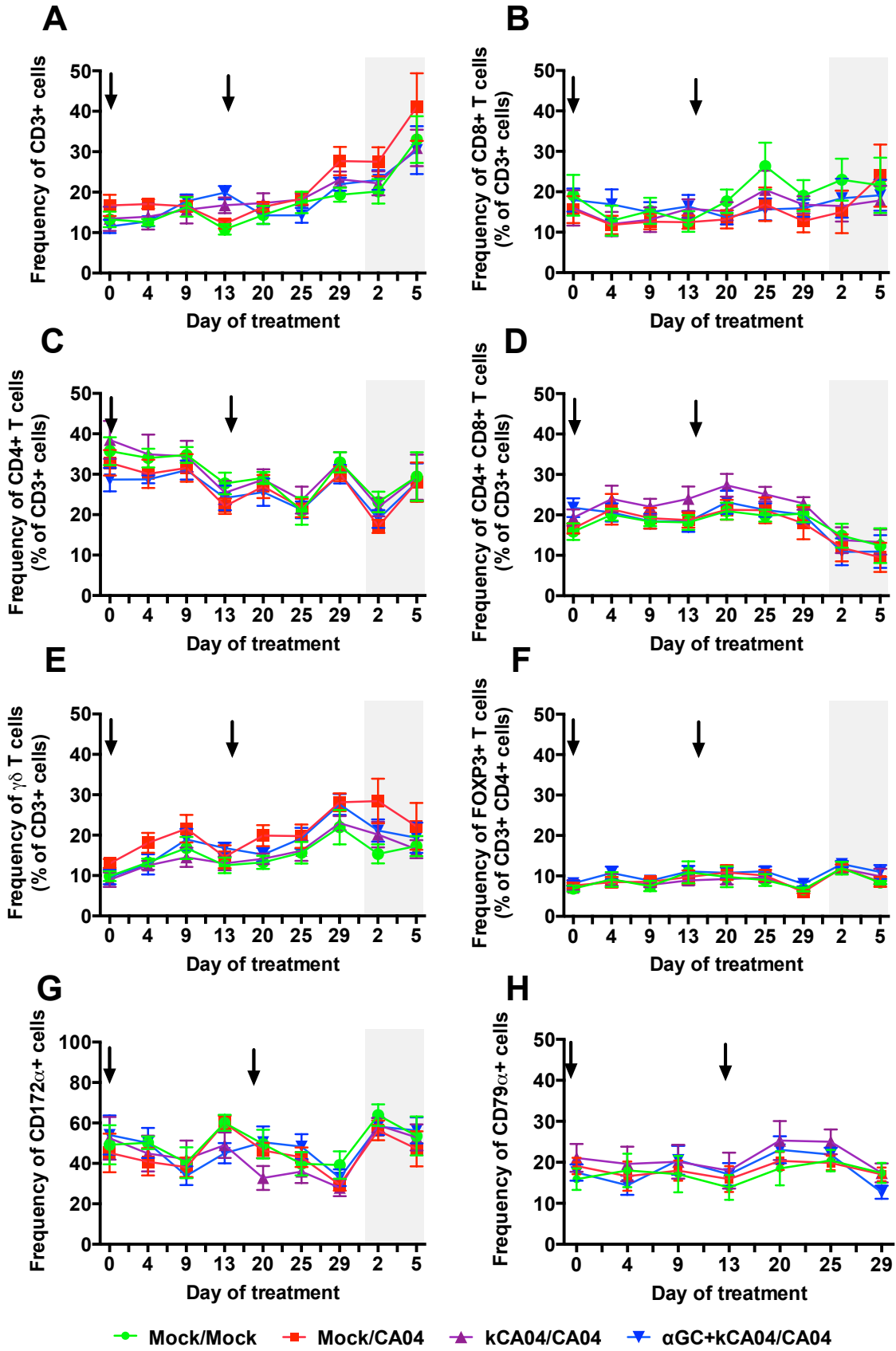
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Supplemental Figure S1. Gating strategy to identify immune cell populations in pigs. Single cell suspensions from blood, spleen, tracheobronchial lymph node (TBLN), lungs and bronchoalveolar lavage fluid (BALF) were labeled with mAb cocktails to differentiate (A) CD3<sup>+</sup> T cells, (B) CD4 and CD8 T cell subsets, (C) FoxP3<sup>+</sup> regulatory T cells, (D)  $\gamma\delta$  T cells, (E) monocytes and granulocytes, (F) and B cells. Conventional T cells were identified using anti-CD3 mAb after gating on lymphocyte-sized cells, single cells and live cells. CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and CD4<sup>+</sup> CD8<sup>+</sup> T cells were identified after gating on lymphocyte sized cells, single cells and live cells, as the percentage of CD3<sup>+</sup> cells that are positive for either one or both markers. Regulatory T cells were identified as a percentage of CD4<sup>+</sup> T cells that express the transcription factor FOXP3.  $\gamma\delta$  T cells were identified, after gating on lymphocyte-sized cells, single cells and live cells, as the percentage of CD3<sup>+</sup> cells positive for TCR $\delta$  mAb. Monocytes and granulocytes were identified as CD172 $\alpha$  positive cells after gating on single cells and live cells. B cells were identified as CD79 $\alpha$  positive cells after gating on lymphocyte-sized cells and single cells. One representative peripheral blood sample is shown.



Supplemental Figure S2. Frequency of immune cell populations during the vaccination period and post infection (shaded region). (A) CD3<sup>+</sup> cells as a proportion of live lymphocytes, (B) CD8<sup>+</sup> T cells, (C) CD4<sup>+</sup> T cells, (D) double positive CD8<sup>+</sup> CD4<sup>+</sup> T cells, (E)  $\gamma\delta$  T cells, (F) regulatory T cells, (G) monocytes and granulocytes, (H) B cells (H). Differences in PB immune cell populations were analyzed using the SAS PROC MIXED procedure and no treatment differences were identified. Data are represented as mean  $\pm$  SEM. Arrows indicate when vaccinations were administered.

Supplemental Table S1. Bronchoalveolar lavage fluid (BALF) leukocyte populations from pigs euthanized on day 3 p.i.

Immune cell population	Mock/Mock	Mock/CA04	kCA04/CA04	$\alpha$ GC+kCA04/CA04
CD8 <sup>+</sup> cells	20.85 $\pm$ 5.30	13.59 $\pm$ 4.90	13.33 $\pm$ 3.57	15.75 $\pm$ 2.65
CD4 <sup>+</sup> cells	9.01 $\pm$ 2.79	5.73 $\pm$ 1.68	5.88 $\pm$ 1.32	10.07 $\pm$ 1.74
CD4 <sup>+</sup> CD8 <sup>+</sup> cells	1.70 $\pm$ 0.43	1.77 $\pm$ 0.48	2.10 $\pm$ 1.03	3.06 $\pm$ 0.76
FoxP3 <sup>+</sup> T cells (% of CD4 <sup>+</sup> )	7.52 $\pm$ 0.82	9.07 $\pm$ 1.80	10.38 $\pm$ 1.32	8.85 $\pm$ 1.84
$\gamma\delta$ T cells	18.28 $\pm$ 2.45	18.15 $\pm$ 0.95	15.50 $\pm$ 1.51	16.73 $\pm$ 2.98
Monocytes and Granulocytes	80.88 $\pm$ 6.12	73.93 $\pm$ 8.37	86.75 $\pm$ 4.86	78.45 $\pm$ 3.99

Values represent mean  $\pm$  SEM for lymphocyte-sized single cells and cell population indicated in parenthesis. Four pigs from each treatment were compared at day 3 post infection. No significant difference ( $p > 0.05$ ) was detected between treatments for any of the immune cell populations tested when analyzed by the Kruskal-Wallis test. Mock vaccinated and mock challenged (Group 1; Mock/Mock); mock vaccinated and CA04 challenged (Group 2; Mock/CA04), kCA04 vaccinated and CA04 challenged (Group 3; kCA04/CA04);  $\alpha$ -GalCer+kCA04 vaccinated and CA04 challenged (Group 4;  $\alpha$ GC+kCA04/CA04).

Supplemental Table S2. Lung leukocyte populations from pigs euthanized on day 3 p.i.

Immune cell population	Mock/Mock	Mock/CA04	kCA04/CA04	$\alpha$ GC+kCA04/CA04
CD8 <sup>+</sup> cells	23.73 $\pm$ 5.34	23.13 $\pm$ 6.37	22.28 $\pm$ 2.84	23.98 $\pm$ 5.75
CD4 <sup>+</sup> cells	14.61 $\pm$ 3.19	11.10 $\pm$ 1.73	18.68 $\pm$ 2.23	14.17 $\pm$ 3.12
CD4 <sup>+</sup> CD8 <sup>+</sup> cells	1.34 $\pm$ 0.28	2.24 $\pm$ 0.86	2.79 $\pm$ 1.13	1.82 $\pm$ 0.35
FoxP3 <sup>+</sup> T cells (% of CD4 <sup>+</sup> )	6.75 $\pm$ 1.01	6.16 $\pm$ 1.09	5.38 $\pm$ 0.65	6.36 $\pm$ 1.41
$\gamma\delta$ T cells	19.73 $\pm$ 1.40	26.90 $\pm$ 6.12	20.65 $\pm$ 3.88	26.18 $\pm$ 4.88
Monocytes and Granulocytes	72.20 $\pm$ 3.63	68.25 $\pm$ 2.32	67.08 $\pm$ 2.96	65.93 $\pm$ 4.78

Values represent mean  $\pm$  SEM for lymphocyte-sized single cells and cell population indicated in parenthesis. Four pigs from each treatment were compared at day 3 post infection. No significant difference ( $p > 0.05$ ) was detected between treatments for any of the immune cell populations tested when analyzed by the Kruskal-Wallis test. Mock vaccinated and mock challenged (Group 1; Mock/Mock); mock vaccinated and CA04 challenged (Group 2; Mock/CA04), kCA04 vaccinated and CA04 challenged (Group 3; kCA04/CA04);  $\alpha$ -GalCer+kCA04 vaccinated and CA04 challenged (Group 4;  $\alpha$ GC+kCA04/CA04).

Supplemental Table S3. Tracheobronchial lymph node (TBLN) leukocyte populations from pigs euthanized on day 3 p.i.

Immune cell population	Mock/Mock	Mock/CA04	kCA04/CA04	$\alpha$ GC+kCA04/CA04
CD8 <sup>+</sup> cells	15.43 $\pm$ 2.04	17.22 $\pm$ 4.05	12.19 $\pm$ 1.14	15.10 $\pm$ 1.35
CD4 <sup>+</sup> cells	36.13 $\pm$ 7.31	35.03 $\pm$ 5.27	40.73 $\pm$ 7.58	35.23 $\pm$ 2.59
CD4 <sup>+</sup> CD8 <sup>+</sup> cells	5.55 $\pm$ 2.77	7.00 $\pm$ 3.77	8.40 $\pm$ 4.16	5.76 $\pm$ 2.75
FoxP3 <sup>+</sup> T cells (% of CD4 <sup>+</sup> )	7.60 $\pm$ 1.88	6.79 $\pm$ 1.04	9.89 $\pm$ 0.78	8.90 $\pm$ 1.16
$\gamma\delta$ T cells	5.48 $\pm$ 0.49	5.04 $\pm$ 0.64	5.56 $\pm$ 0.76	6.43 $\pm$ 1.22
Monocytes and Granulocytes	5.58 $\pm$ 2.18	9.34 $\pm$ 3.78	5.62 $\pm$ 1.25	8.55 $\pm$ 2.36

Values represent mean  $\pm$  SEM for lymphocyte-sized single cells and cell population indicated in parenthesis. Four pigs from each treatment were compared at day 3 post infection. No significant difference ( $p > 0.05$ ) was detected between treatments for any of the immune cell populations tested when analyzed by the Kruskal-Wallis test. Mock vaccinated and mock challenged (Group 1; Mock/Mock); mock vaccinated and CA04 challenged (Group 2; Mock/CA04), kCA04 vaccinated and CA04 challenged (Group 3; kCA04/CA04);  $\alpha$ -GalCer+kCA04 vaccinated and CA04 challenged (Group 4;  $\alpha$ GC+kCA04/CA04).

Supplemental Table S4. Splenic leukocyte populations from pigs euthanized on day 3 p.i.

Immune cell population	Mock/Mock	Mock/CA04	kCA04/CA04	$\alpha$ GC+kCA04/CA04
CD8 <sup>+</sup> cells	15.19 $\pm$ 3.12	16.03 $\pm$ 2.21	17.60 $\pm$ 1.06	20.88 $\pm$ 2.08
CD4 <sup>+</sup> cells	18.85 $\pm$ 1.27	15.70 $\pm$ 1.44	18.30 $\pm$ 1.98	16.18 $\pm$ 0.80
CD4 <sup>+</sup> CD8 <sup>+</sup> cells	2.47 $\pm$ 0.95	3.23 $\pm$ 0.85	2.11 $\pm$ 0.59	2.97 $\pm$ 0.32
FoxP3 <sup>+</sup> T cells (% of CD4 <sup>+</sup> )	6.90 $\pm$ 0.55	6.26 $\pm$ 0.71	7.28 $\pm$ 0.27	7.48 $\pm$ 0.45
$\gamma\delta$ T cells	29.58 $\pm$ 2.38	40.63 $\pm$ 6.15	35.83 $\pm$ 3.45	34.10 $\pm$ 5.31
Monocytes and Granulocytes	43.78 $\pm$ 3.22	37.08 $\pm$ 6.16	32.68 $\pm$ 2.91	28.15 $\pm$ 3.16

Values represent mean  $\pm$  SEM for lymphocyte-sized single cells and cell population indicated in parenthesis. Four pigs from each treatment were compared at day 3 post infection. No significant difference ( $p > 0.05$ ) was detected between treatments for any of the immune cell populations tested when analyzed by the Kruskal-Wallis test. Mock vaccinated and mock challenged (Group 1; Mock/Mock); mock vaccinated and CA04 challenged (Group 2; Mock/CA04), kCA04 vaccinated and CA04 challenged (Group 3; kCA04/CA04);  $\alpha$ -GalCer+kCA04 vaccinated and CA04 challenged (Group 4;  $\alpha$ GC+kCA04/CA04).



Supplemental Table S5. Immunohistochemical distribution of H1N1 antigen in bronchial and bronchiolar epithelium and alveolar type II cells

Treatment group	Bronchial Epithelium	Bronchiolar Epithelium	Alveolar type II Cells
Mock/Mock (n=1)	Neg	Neg	Neg
Mock/CA04 (n=2)	++	+++	++
kCA04/CA04 (n=2)	++	++	++
$\alpha$ GC+kCA04/CA04 (n=2)	Neg	Neg	Neg

Neg = Negative for virus antigen  
 + = Rare, focal distribution of virus antigen  
 ++ = Multifocal distribution of virus antigen  
 +++ = Diffuse distribution of virus antigen