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Supplementary Materials for

Induction of ICOS^{+}CXCR3^{+}CXCR5^{+} Th Cells Correlates with Antibody Responses to Influenza Vaccination

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Supplementary Materials and Methods

Clinical samples

Blood samples were obtained from healthy control subjects before and after the administration of a single intramuscular dose of a non-adjuvanted trivalent split seasonal influenza vaccine (Fluzone®, Sanofi Pasteur). A cohort of 12 healthy adults (M 6, F 6; Age 34 ± 9) received vaccination in 2009/2010 winter, another cohort of 37 healthy adults received vaccination in 2011/2012 winter, and a cohort of 20 healthy children (M 12, F 8; Age 11 ± 3) received vaccination in 2010/2011 winter. 2009/2010 vaccine contained A/Brisbane/59/2007 (H1N1)-like, A/Brisbane/10/2007 (H3N2)-like, and B/Brisbane/60/2008-like, while 2010/2011 and 2011/12 vaccines contained A/California/7/2009 (H1N1)-like, A/Perth/16/2009 (H3N2)-like, and B/Brisbane/60/2008-like. Blood apheresis was performed from healthy adults at 7 d after administration of influenza vaccines. Whole blood samples were used for phenotypic analysis of $CD4^+$ T cells by flow cytometry. PBMCs were isolated by centrifugation on a gradient (Ficoll-Paque, Stemcell), frozen and stored in liquid nitrogen. The study was approved by the Institutional Review Boards (IRBs) of Baylor Health Care System and Nationwide Children's Hospital. Informed consent was obtained from subjects, parents, or legal guardians.

Flow cytometry

Whole blood samples (200 μL) were incubated with the indicated antibodies and LIVE/DEAD fixable Aqua (Invitrogen) for 15 minutes at room temperature. mAbs: CXCR5 (1G10), CD3 (UCHT1), CD8 (SK1), CD4 (RPA-T4), CCR6 (11A9), CXCR3 (1C6/CXCR3), from BD. ICOS (C398.4A) from Biolegend. CD45RA (2H4) from Beckman Coulter. CD45

(HI30) mAbs were from Invitrogen. Stained cells were acquired on a BD LSRII. Phenotype of CD4+ T cells was assessed with FlowJo software (TreeStar).

For the assessment of Ki67 expression, enriched $CD4^+$ T cells (2×10^6) at day 7 post vaccination were stained with anti- Ki67 A488 (B56; BD pharmigen). The phenotype of $CD4^+$ Th subsets was also analyzed for with anti-PD1 PE (J105; eBioscience).

Antibody assays

Hemagglutination inhibition (HI) and virus neutralization (VN) antibody titers for each strain of influenza virus incorporated in the vaccines were determined at baseline and at day 28 post-vaccination.

Hemagglutination inhibition (HI) assay. HI assays were performed by standard methods. Briefly, collected sera were first treated with receptor-destroying enzyme (RDE) to remove non specific inhibitors of hemagglutination. Each influenza virus strain was mixed with twofold dilutions of the specific RDE-treated serum in PBS in V-bottomed 96-well plates. After 30 min of incubation at room temperature, chicken erythrocytes were added to the mixtures. The plates were kept at 4°C until a positive hemagglutination was developed in non-serum containing control wells. The HI titer was defined as the highest dilution of the serum able to inhibit hemagglutination.

Microneutralization assays. Briefly, Influenza virus containing 100 PFU was incubated with the specific RDE-treated serum for 1 h at room temperature in a 96-well plate containing an MDCK cell monolayer. After the incubation, the virus-serum samples were removed from the wells. The cells were incubated at 37°C for 2 days in minimal essential medium-bovine albumin.

The microneutralization titer was defined as the highest dilution of serum that neutralized 100 PFU of virus in MDCK cell cultures.

CD154 assay

PBMCs at day 7 post-vaccination were stimulated for 6 h with Fluzone® or killed Flu-virus (PR8) in the presence of Brefeldin A and monensin, and expression of intracytoplasmic cytokines together with CD154 was analyzed. After the stimulation period, cells were washed twice with PBS. Cells were subsequently incubated with anti- CD3 (UCHT1), anti- CD4 (S3.5), anti-CXCR5 (RF8B2), anti-ICOS (C398.4A), and Aqua live/Dead (Invitrogen). After permeabilization, cells were stained intracellularly with the following combination of antibodies: anti-IL-2 (MQ1-17H12), anti-IL-10 (JES3-9D7), anti-IL-21 (3A3-N2.1), anti-IFN-γ (4S.B3), and CD154 (24-31) for 30 min at room temperature. The stained cells were acquired with LSR Fortessa.

In vitro cell culture

Memory $CD4^+$ T cells enriched by using memory human $CD4^+$ T cell isolation kit (Miltenyi Biotec) were stained with anti-CD4 (RPA-T4), anti-CXCR5 (RF8B2), anti-CCR6 (11A9), anti-CXCR3 (1C6/CXCR3), anti-ICOS (C398.4A), anti-CD45RA (HI100), and an APC-conjugated mAb cocktail including anti-BDCA 3 (AD5-14H12), anti-CD16 APC (3G8), anti-CD19 APC (HIB19), anti-CD20 APC (2H7), anti-BDCA-2 (AC144), anti-CD56 (B159), anti-CD8 (SK1), anti-CD11c (S-HCL-3), anti-NKp46 (9E2). $CXCR5^+$ memory $CD4^+$ Th subsets were sorted from the dump-APC negative cells. For the isolation of B cell subsets, B cells were first positively

selected with CD19 MACS Microbeads (Miltenyi Biotech), then stained with anti IgD (IA6-2;), anti-CD20 (2H7), anti-CD3 (UCHT1), anti-CD27 (O323). Naïve B cells ($CD20^+IgD^+CD27^-$) and memory B cells ($CD20^+IgD^-CD27^+$) were sorted after excluding $CD3^+$ cell population.

Sorted $CD4^+$ $CXCR5^+$ Th populations (2×10^4 cells/well) were co-cultured with naïve B cells (2×10^4 cells/well) for 12 days and with memory B (2×10^4 cells/well) cells for 6 days, in RPMI 1640 medium (GIBCO) supplemented with 1% L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma), 1% sodium pyruvate (Sigma), 1% non-essential amino acids (Sigma), 50 μM β-mercaptoethanol (Sigma), 50 μg/ml gentamycin (GIBCO) and 10% heat-inactivated FCS (ATCC) in the presence of SEB (1 μg/ml, Sigma-Aldrich) in 96 well U bottomed plates. For the phenotype analysis of recovered B cells, recovered cells were stained with anti-CD3, anti-CD4, anti-CD38 (HB7) and anti-CD138 (MI15). The frequency of $CD38^{hi}CD138^-$ plasmablasts and $CD38^+CD138^+$ plasma cells within the $CD3^-CD4^-$ B cells were analyzed with FACS LSRII. We calculated the cell recovery per well, based on the frequency of the indicated cell population in the flow data and the recovered viable cell numbers per well. In blocking experiments, endogenous IL-21 and IL-10 were neutralized by the addition of IL-21R/Fc (20 μg/ml; R&D Systems), anti-IL-10 blocking mAb (20 μg/ml, developed at the Institute) plus anti-IL-10R mAb (20 μg/ml; R&D Systems). In some experiment sorted $CD4^+$ Th subsets were co-cultured with memory B cells loaded with Fluzone, and the phenotype of recovered B cells and the secreted influenza-specific IgG were analyzed at day 6 of the culture.

Fluzone specific IgG ELISA

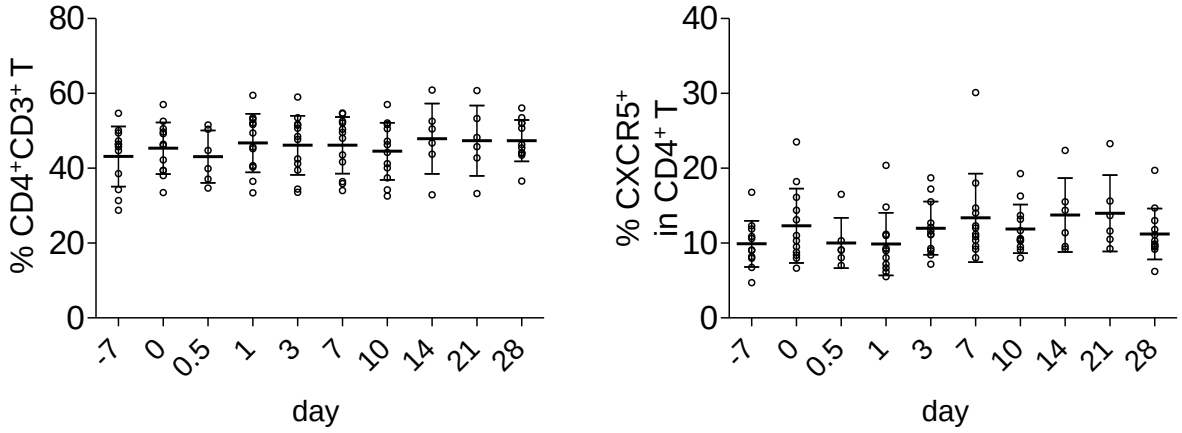
Flat-bottomed microtiter plates were coated with Fluzone diluted 1/10 in carbonate buffer (pH 9.5). After blocking with PBS containing 10% FBS for 1 h, the plates were incubated with

diluted supernatants at room temperature for 2 h. After washing, plates were incubated at room temperature for 1 h with alkaline phosphatase-conjugated goat anti-human, IgG at a final dilution 1/2500 (SouthernBiotech, Inc.), the plates were incubated with p-nitrophenyl phosphate (Sigma) after extensive wash. The reaction was stopped with 2 N H_2SO_4. The optical density was read by SpectraMax plate reader (Molecular Devices).

Assessment of influenza specific $CD4^+$ T cell proliferation and cytokines secretion

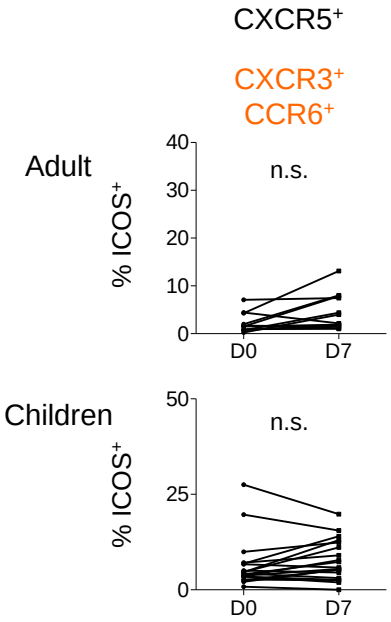
Sorted $CD4^+$ Th subsets (5 x 10^4 cells/well) were co-cultured with memory B cells (5 x 10^4 cells/well) loaded with Fluzone. Culture supernatants were harvested at day 2 and cytokines were measured by Luminex. At day 6, the cultured cells were stained with anti-CD3, and anti-CD4 and the frequency of $CD4^+CD3^+$ T cells in the culture were measured using FACS LSRII.

Supplementary Figure 1: Total $CD4^+$ and $CXCR5^+CD4^+$ T cells did not change after influenza vaccination in blood.



Percentage of total $CD4^+$ T cells within lymphocytes and $CXCR5^+$ cells within $CD4^+$ T cells are shown. Fresh blood samples were obtained from a healthy adult cohort (n=12) before and after vaccination with trivalent seasonal influenza vaccine (Fluzone® 2009/2010). No significance was observed in One-way ANOVA test.

Supplementary Figure 2: ICOS expression by CXCR3$^+$CCR6$^+$ cells within CXCR5$^+$ CD4$^+$ T cells did not change following influenza vaccination.



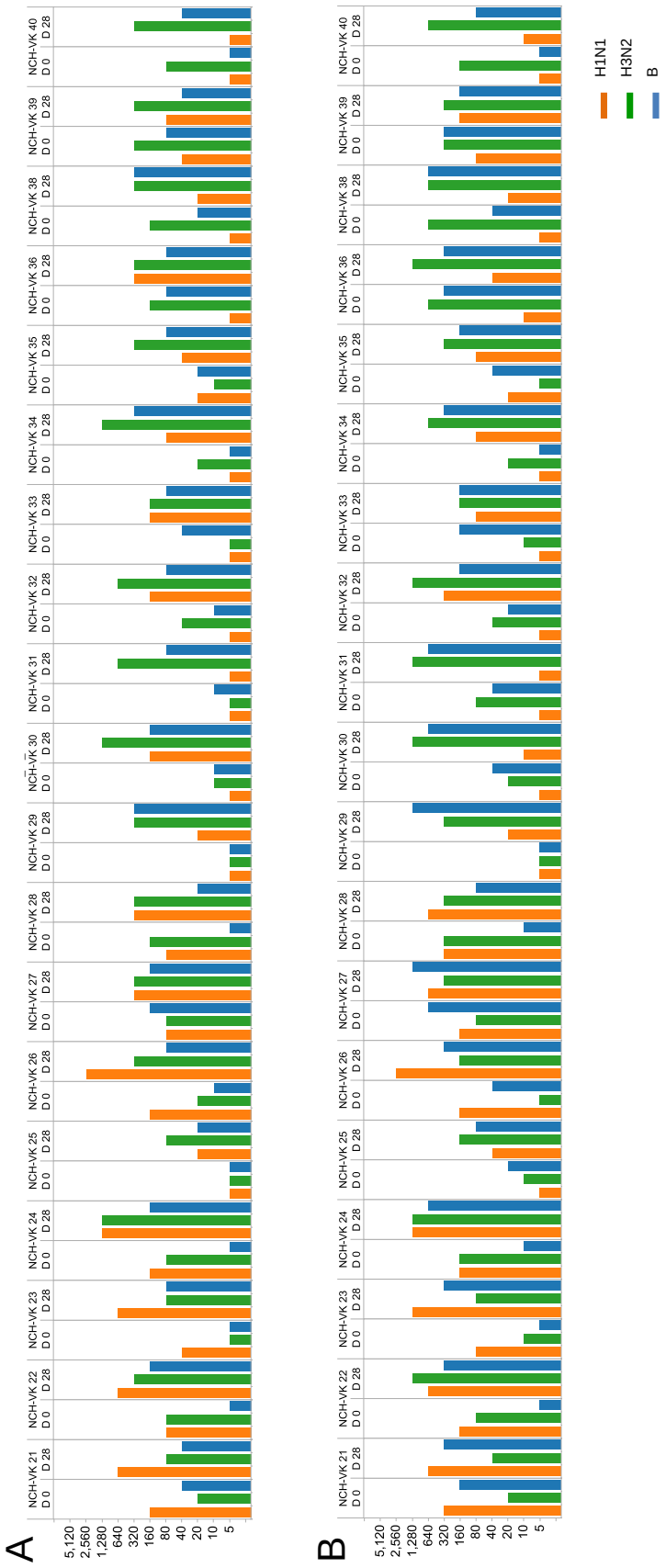
Percentage of ICOS$^+$ cells in CXCR3$^+$CCR6$^+$ CXCR5$^+$ CD4$^+$ T cells before and 7 d after influenza vaccination in the two cohorts. Paired t-test.

Supplementary Figure 3: HI and VN titers in first adult cohort

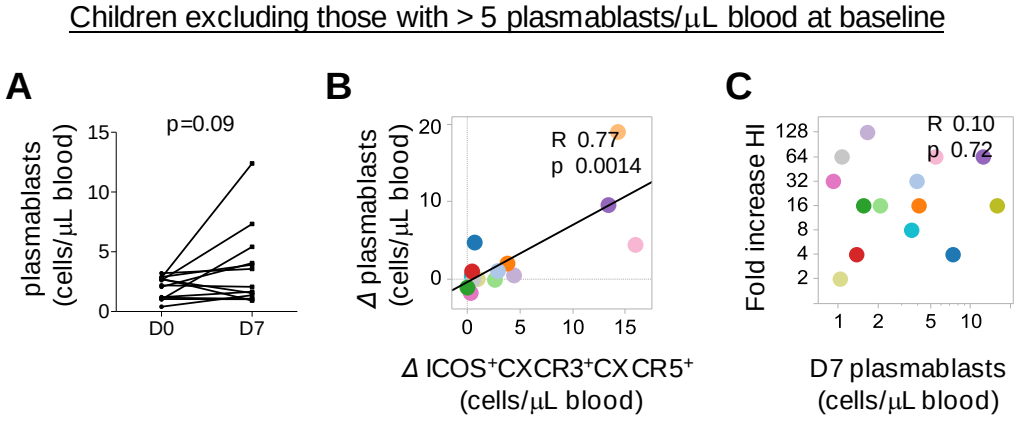
Hemagglutinin inhibition (HI, shown in panel **A**) and virus neutralization (VN, shown in panel **B**) antibody titers against each influenza virus strains incorporated in the vaccines were determined at baseline and at day 28 post-vaccination in the adult cohort.

Supplementary Figure 4: HI and VN titers in children cohort.

Hemagglutinin inhibition (HI, shown in panel **A**) and virus neutralization (VN, shown in panel **B**) antibody titers against each influenza virus strains incorporated in the vaccines were determined at baseline and at day 28 post-vaccination in the adult cohort.



Supplementary Figure 5: Positive correlation between the increase of ICOS$^+$ CXCR3$^+$CXCR5$^+$ CD4$^+$ T cells and the increase of plasmablasts in the children cohort, when the four donors who showed > 5 plasmablasts/μL blood at baseline were removed from the analysis.

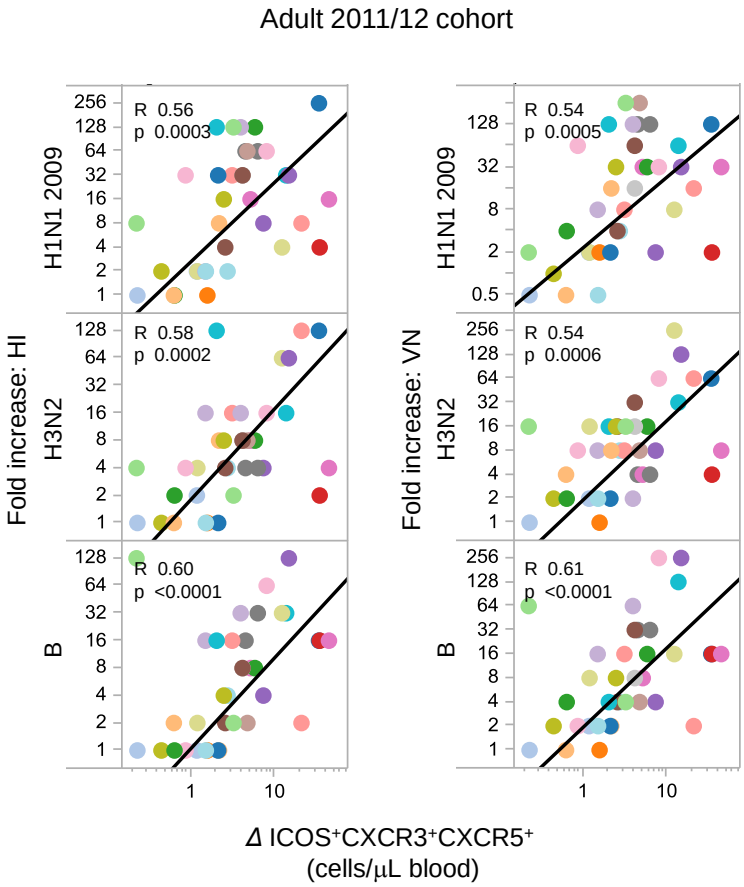


A. Absolute number of plasmablasts in blood before and 7 d after influenza vaccination in the children cohort excluding the samples from the 4 donors. Paired t-test.

B. Correlation between the increase in the absolute number of ICOS$^+$CXCR3$^+$CXCR5$^+$ CD4$^+$ T cells in blood and the increase in the absolute number of CD38$^+$CD27$^+$ plasmablasts in the children cohort excluding the samples from the 4 donors. R: Pearson R; p: p-value.

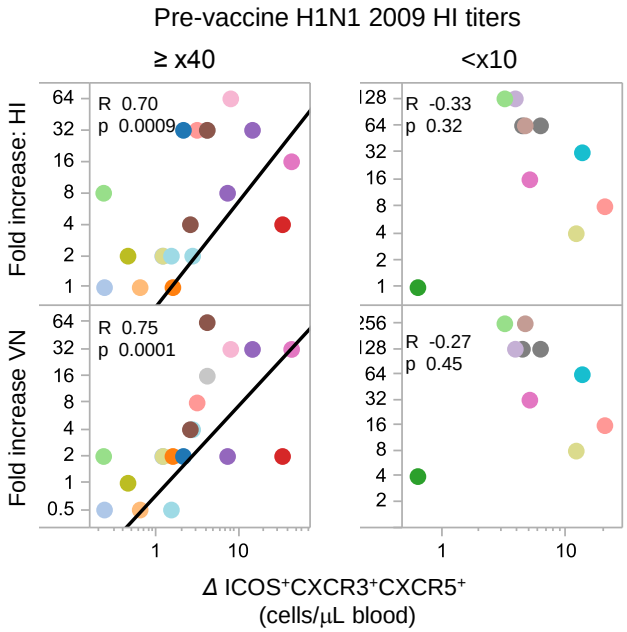
C. Correlation between the increase of global HI antibody titers and absolute number of plasmablasts at day 7 post-vaccination in the children cohort excluding the samples from the 4 donors.

Supplementary Figure 6: Positive correlation between the increase of ICOS$^+$ CXCR3$^+$CXCR5$^+$ CD4$^+$ T cells and the increased antibody titers against each viral strain in the vaccine.



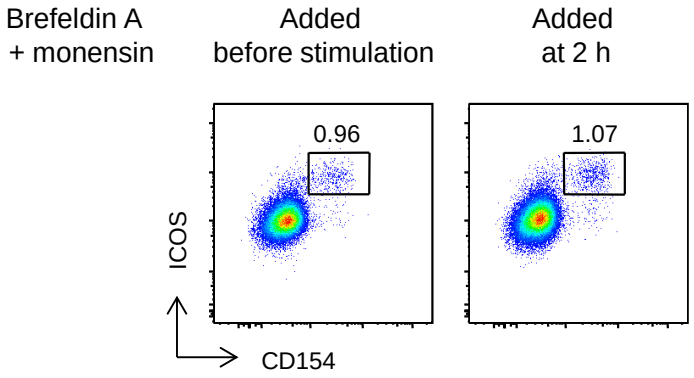
Correlation is shown between the increase of antibody (HI and VN) titers against each strain and the increase of ICOS$^+$CXCR3$^+$CXCR5$^+$ CD4$^+$ T cells in the adult 2011/12 validation cohort. R: Pearson R; p: p-value.

Supplementary Figure 7: Positive correlation between the increase of ICOS$^+$ CXCR3$^+$CXCR5$^+$ CD4$^+$ T cells and the increased antibody titers against H1N1 2009 in the vaccine.



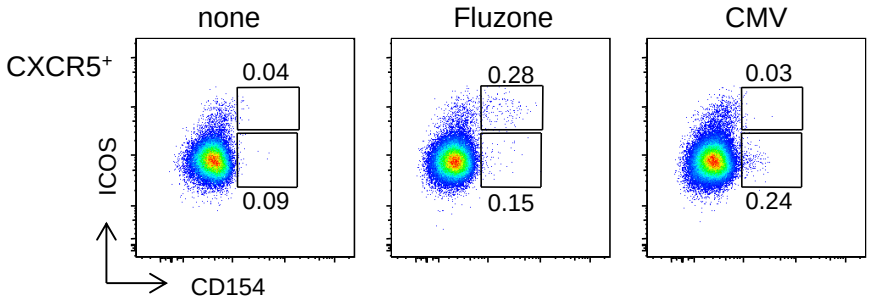
Correlation is shown between the increase of HI and VN titers against H1N1 2009 and the increase of ICOS$^+$CXCR3$^+$CXCR5$^+$ CD4$^+$ T cells, in subjects who did not carry pre-existing antibodies (right), and in subjects who showed pre-existing specific antibodies (HI or VN titer ≥ x40, left). R: Pearson R; p: p-value.

Supplementary Figure 8: CD154 expression by ICOS$^+$CXCR5$^+$ CD4$^+$ T cells was independent of soluble factors secreted during the stimulation.



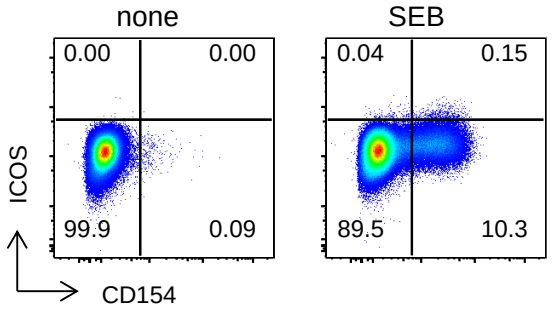
Brefeldin A and monensin were added to the PBMC culture with Fluzone either prior to the culture or at 2 h of the stimulation with Fluzone. CD154 expression on ICOS$^+$CXCR5$^+$ CD4$^+$ T cells was analyzed at 6 h of the stimulation. One representative of the four experiments is shown.

Supplementary Figure 9: ICOS^{+}CXCR5^{+} CD4^{+} T cells did not express CD154 upon stimulation with CMV peptides.



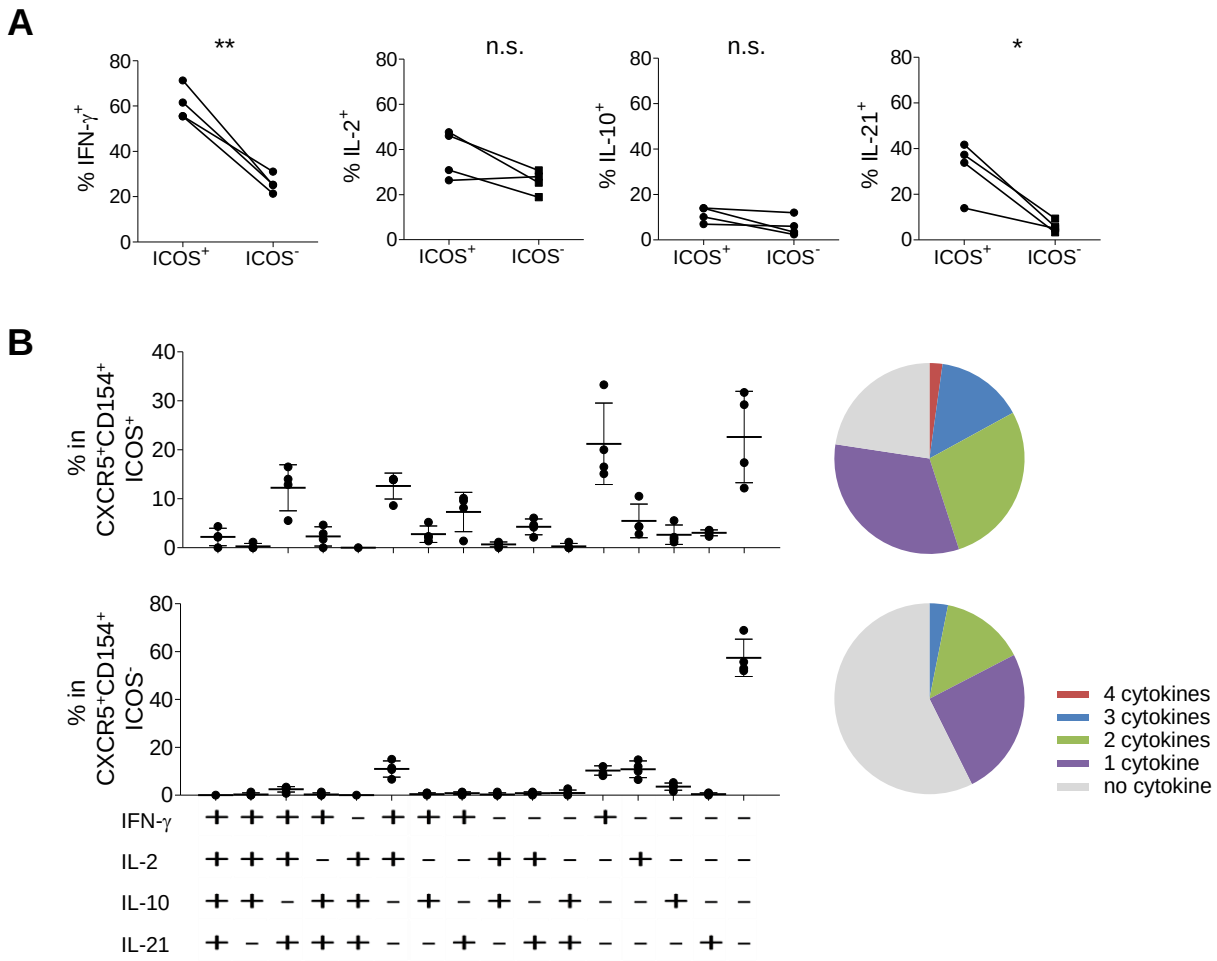
PBMCs from 7 d post-vaccination were stimulated with 15-19mer overlapping CMV peptides for 6 h in the presence of Brefeldin A and monensin. The majority of CD154^{+} cells were from ICOS^{neg} cells among CXCR5^{+} CD4^{+} T cells.

Supplementary Figure 10: ICOS^{neg}CD4^{+} T cells did not express ICOS within 6 h activation.



ICOS^{neg}CD4^{+} T cells were sorted from PBMCs obtained from healthy subjects. Sorted cells were cultured with SEB-pulsed or control autologous monocytes (ratio 1:1) for 6 h in the presence of Brefeldin A and monensin. ICOS expression on CD154^{+} CD4^{+} T cells was analyzed by flow cytometry. One representative of three independent experiments is shown.

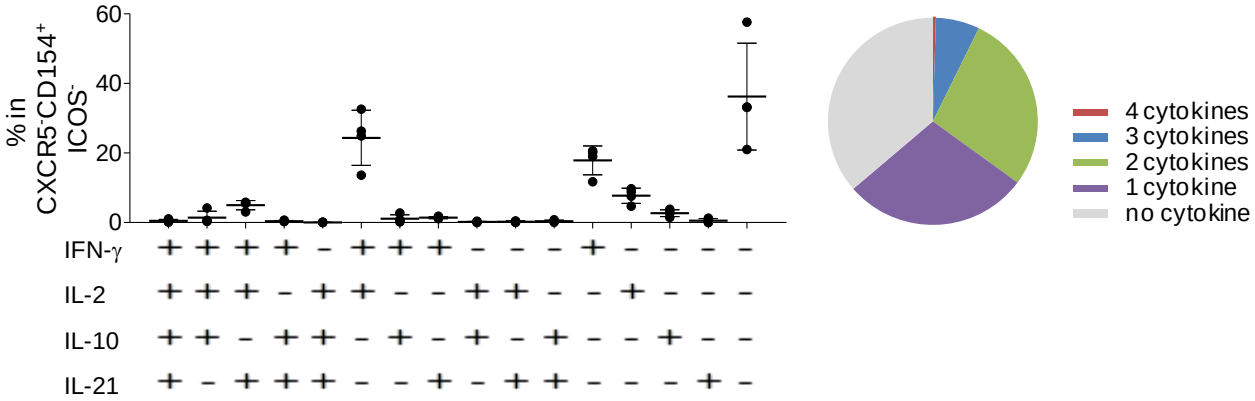
Supplementary Figure 11: Induced ICOS^{+}CXCR3^{+}CXCR5^{+} CD4^{+} T cells express multiple cytokines upon stimulation with heat-killed PR8 virus.



A. PBMCs obtained at day 7 post-vaccination were stimulated with heat-killed PR8 influenza virus for 6 h. Frequency of cytokine-expressing cells among CD154^{+} ICOS^{+}CXCR5^{+} and ICOS^{neg}CXCR5^{+} CD4^{+} T cells induced by PR8 stimulation. n=4. Paired t-test. p-values ** <0.01, * <0.05.

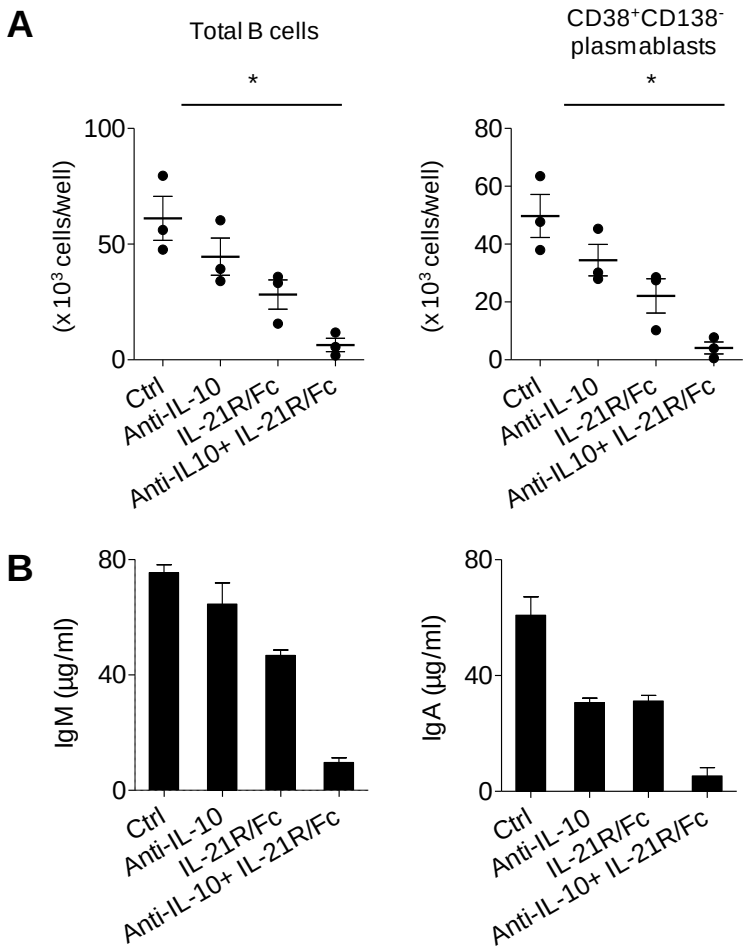
B. Cytokine expression pattern of $CD154^{+}$ $ICOS^{+}$ $CXCR5^{+}$ and $CD154^{+}$ $ICOS^{neg}CXCR5^{+}$ $CD4^{+}$ T cells induced by PR8 stimulation. N=4. The numbers of expressed cytokines are shown on the right in pie charts.

Supplementary Figure 12: Cytokine profiles of ICOS^{neg}CXCR5^{neg} CD4^{+} T cells responding to Fluzone.



PBMCs obtained at day 7 post-vaccination were stimulated with Fluzone virus for 6 h. Cytokine expression pattern of CD154^{+} ICOS^{neg} CXCR5^{neg} CD4^{+} T cells. N=4. The numbers of expressed cytokines are shown on the right in pie charts.

Supplementary Figure 13: ICOS$^+$CXCR3$^+$CXCR5$^+$ CD4$^+$ T cells help memory B cells in a manner dependent on IL-10 and IL-21.



Anti-IL-10 mAb and/or IL-21R-Fc chimera protein were added to the cultures of memory B cells and ICOS$^+$CXCR3$^+$CXCR5$^+$ CD4$^+$ T cells. **A.** The number of B cells and plasmablasts recovered from the cultured wells was determined. N=3. One way ANOVA. **B.** Produced Igs were measured by ELISA at day 6. IgG was not measured due to the cross-reactivity with the Fc portion of the IL-21R-Fc chimera protein.

Supplementary Table 1: Absolute cell number of ICOS$^+$CXCR3$^+$ CXCR5$^+$ CD4$^+$ T cells in blood at day 7 correlates with antibody responses. Correlation with the increase of HI titers is shown. Spearman R and p-value are shown in the table.

	Adult			Children	
	Spearman R	p-value		Spearman R	p-value
CD4	-0.1844	0.5661		0.3468	0.1458
ICOS+ CD4	0.1277	0.6925		0.4303	0.0659
CXCR5+	0.2553	0.4231		0.3521	0.1392
ICOS+ CXCR5+	0.5816	0.0473		0.345	0.1481
CXCR5+ CXCR3+	0.4539	0.1382		0.5363	0.0179
ICOS+ CXCR5+CXCR3+	0.5887	0.044		0.6657	0.0019
CXCR5+ CXCR3-CCR6-	0.2695	0.3969		-0.05929	0.8095
ICOS+ CXCR5+ CXCR3-CCR6-	0.5674	0.0543		0.07815	0.7505
CXCR5+ CXCR3-CCR6+	0.2553	0.4231		0.04402	0.858
ICOS+ CXCR5+CXCR3-CCR6+	0.2412	0.4502		-0.124	0.6131
CXCR5-	0.3263	0.3007		0.2381	0.3264
ICOS+ CXCR5-	-0.2766	0.3841		0.38	0.1085
CXCR5- CXCR3+CCR6-	-0.1135	0.7255		0.4186	0.0745
ICOS+ CXCR5- CXCR3+CCR6-	-0.3972	0.2011		0.5219	0.0219
CXCR5- CXCR3-CCR6-	-0.1348	0.6763		-0.03953	0.8724
ICOS+ CXCR5- CXCR3-CCR6-	-0.2979	0.347		0.2399	0.3226
CXCR5- CXCR3-CCR6+	0.5391	0.0705		0.01886	0.9389
ICOS+ CXCR5- CXCR3-CCR6+	0.1986	0.5361		0.02156	0.9302
CXCR5- CXCR3+CCR6+	0.3121	0.3234		0.2973	0.2163
ICOS+ CXCR5- CXCR3+CCR6+	-0.07802	0.8095		0.256	0.2901
CD8	0.532	0.075		0.04851	0.8437
PB+PC	0.6525	0.0214		0.02331	0.9268

Supplementary Table 2: Correlations between the increase of $ICOS^+CXCR3^+$ $CXCR5^+$ $CD4^+$ T cells and the increased antibody titers against each viral strain in the vaccines.

				Spearman R	p value
Adult (2009/2010)	$\Delta ICOS^+CXCR3^+CXCR5^+$ (cells/μL)	HI	H1N1	0.61	0.035
			H3N2	0.47	0.13
			B		
		VN	H1N1	0.32	0.31
			H3N2	0.65	0.021
			B	0.57	0.056
Adult (2011/2012)	$\Delta ICOS^+CXCR3^+CXCR5^+$ (cells/μL)	HI	H1N1	0.56	0.0003
			H3N2	0.58	0.002
			B	0.6	<0.0001
		VN	H1N1	0.54	0.0005
			H3N2	0.54	0.0006
			B	0.61	<0.0001
Children (2010/2011)	$\Delta ICOS^+CXCR3^+CXCR5^+$ (cells/μL)	HI	H1N1	0.12	0.63
			H3N2	0.59	0.0008
			B	0.64	0.0032
		VN	H1N1	0.12	0.63
			H3N2	0.54	0.017
			B	0.64	0.0028