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Supplementary Information for

Strong Influenza Induced T_{FH} Generation Requires CD4 Effectors Recognize Antigen Locally and Receive Signals from Continuing Infection

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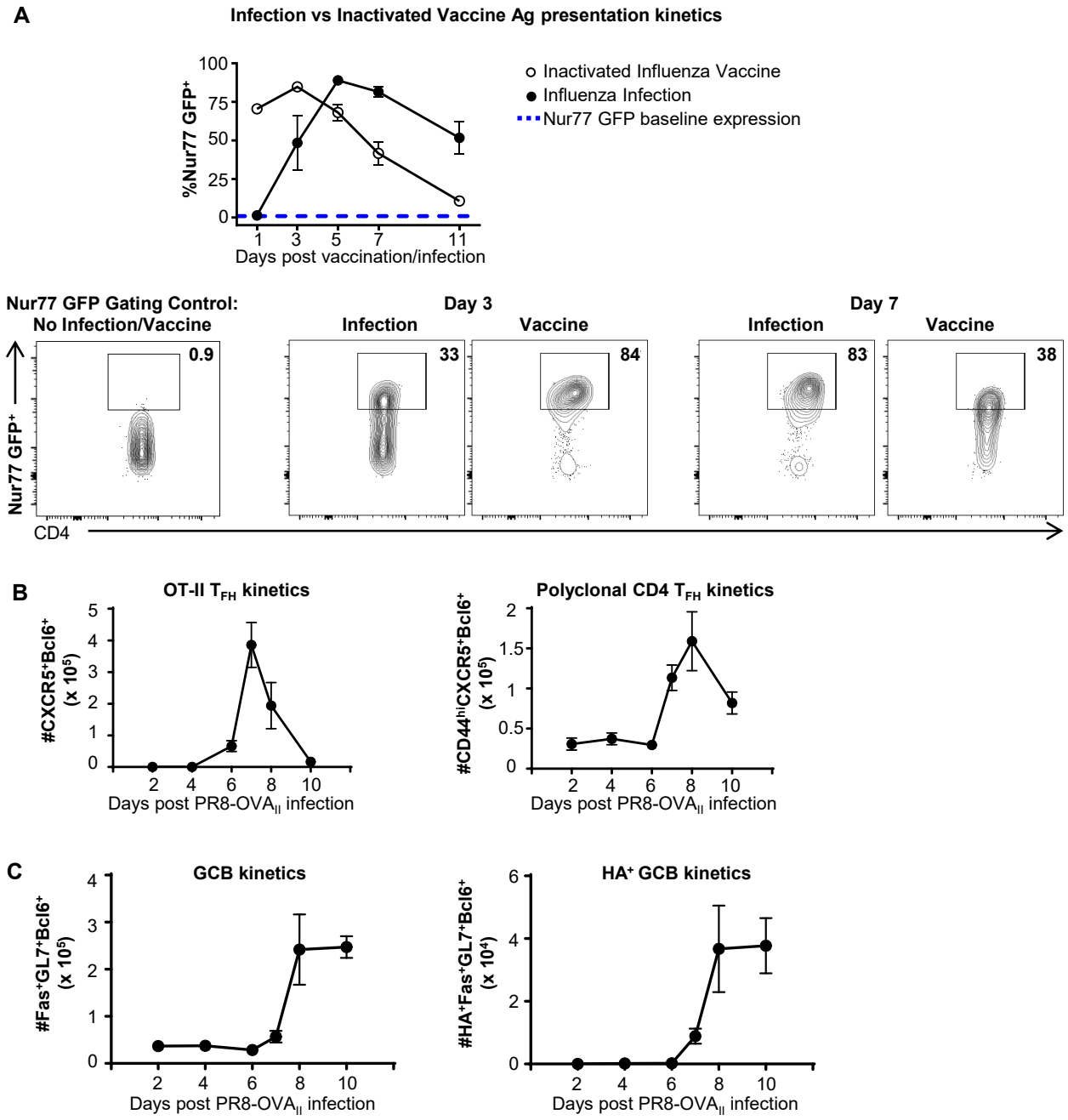
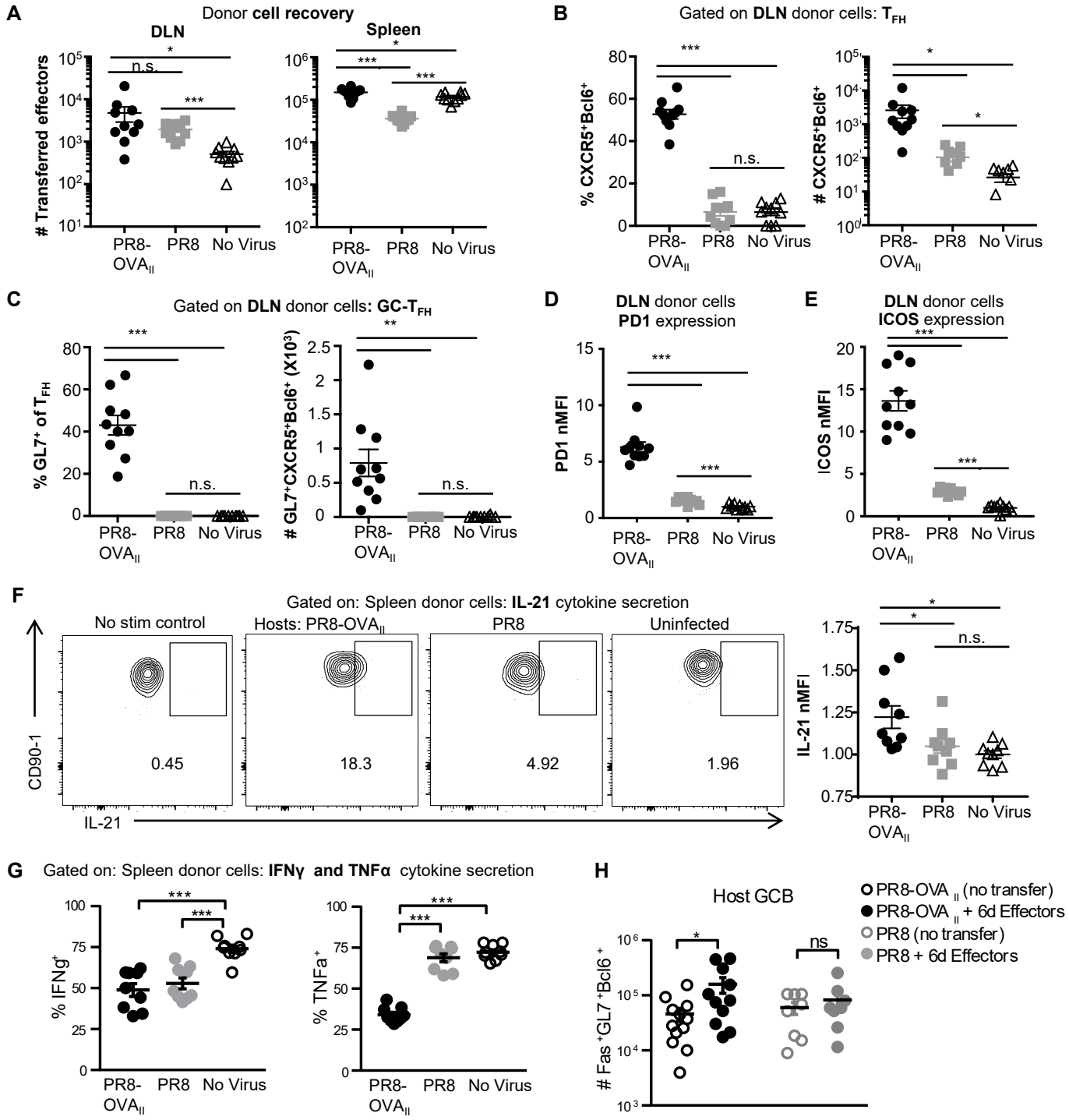
This PDF file includes:

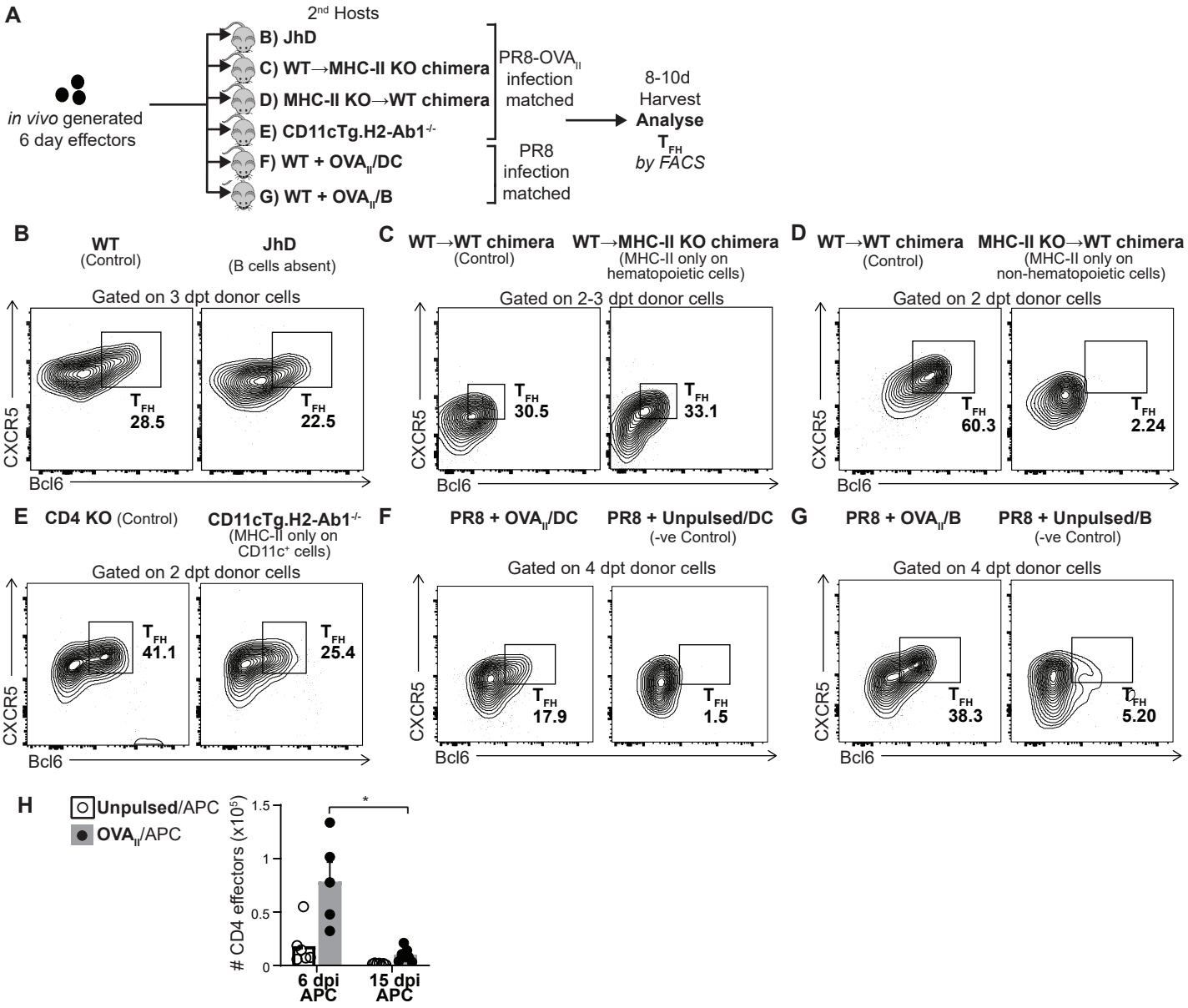
- **Supplementary Figures S1 to S6**
- **Supplemental Methods**



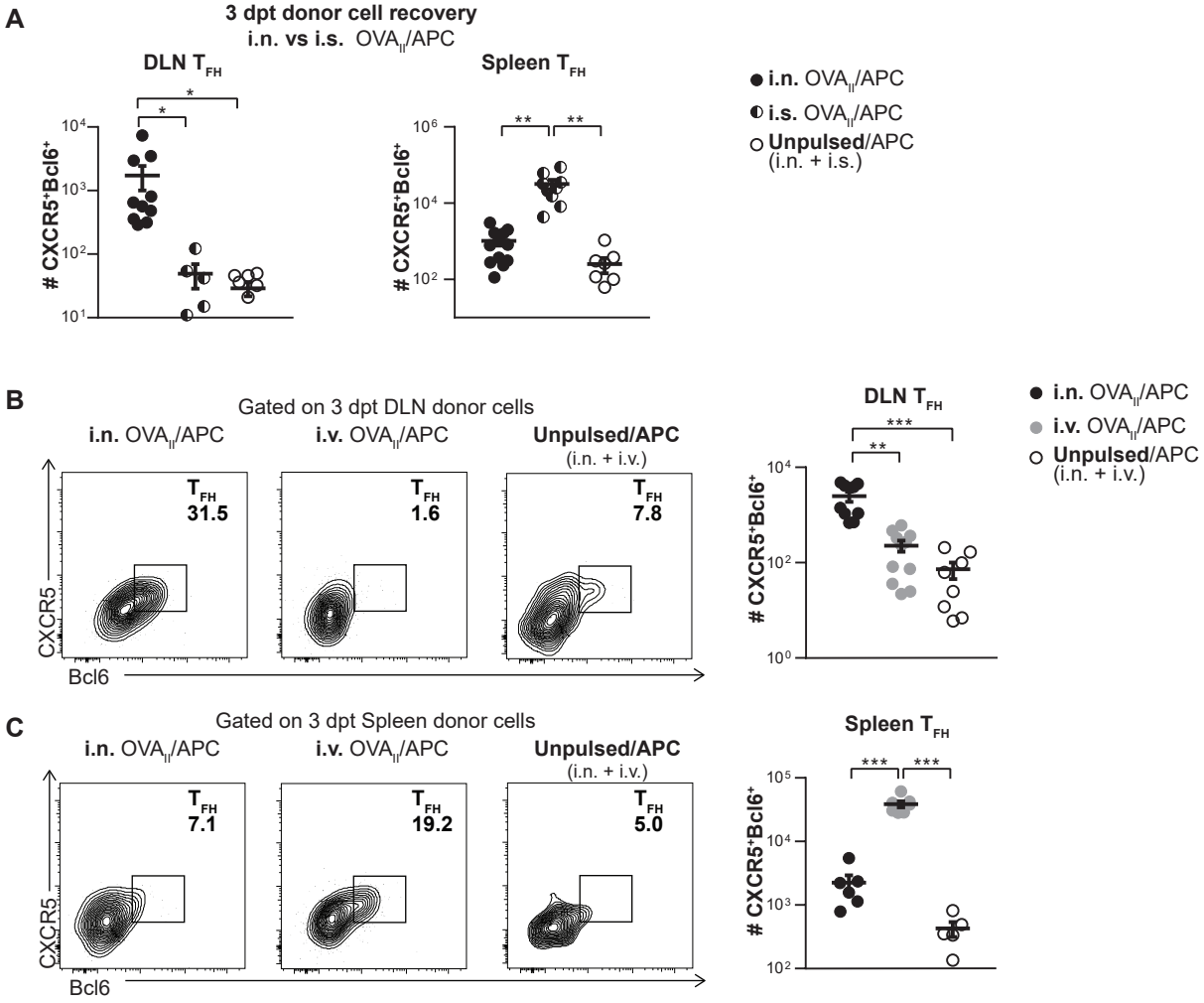
Supplementary Figure 1. Rapid decline of Ag with Immunization corresponds to the beginning of the GC phase during infection. (A) Mice were infected with PR8 influenza A virus or immunized with whole inactivated influenza. NP.Thy1.1.Nur77^{GFP} TCR Tg CD4 T cells, that recognize the nucleoprotein of influenza A viruses, were transferred into the mice 20 hours before sacrifice. Mice were sacrificed at 1, 3, 5, 7 and 11 days after infection/immunization and analyzed for Nur77^{GFP} expression by the transferred NP TCR Tg CD4 T cells. NP cells were also transferred into naive mice to serve as a negative control for determining Nur77^{GFP} gating. **(B-C)** Naïve OT-II.Thy1.1^{+} CD4 T cells were transferred into B6 mice and infected with PR8-OVA$_{II}$. Spleen T_{FH} (CXCR5^{+}Bcl6^{+}) (B) and total spleen GCB (CD19^{+}Fas^{+}GL7^{+}Bcl6^{+}) or HA^{+} GCB (C) generation was analyzed 2, 4, 6, 7, 8 and 10 dpi (B-C, n=7-8 pooled from 2 independent experiments).



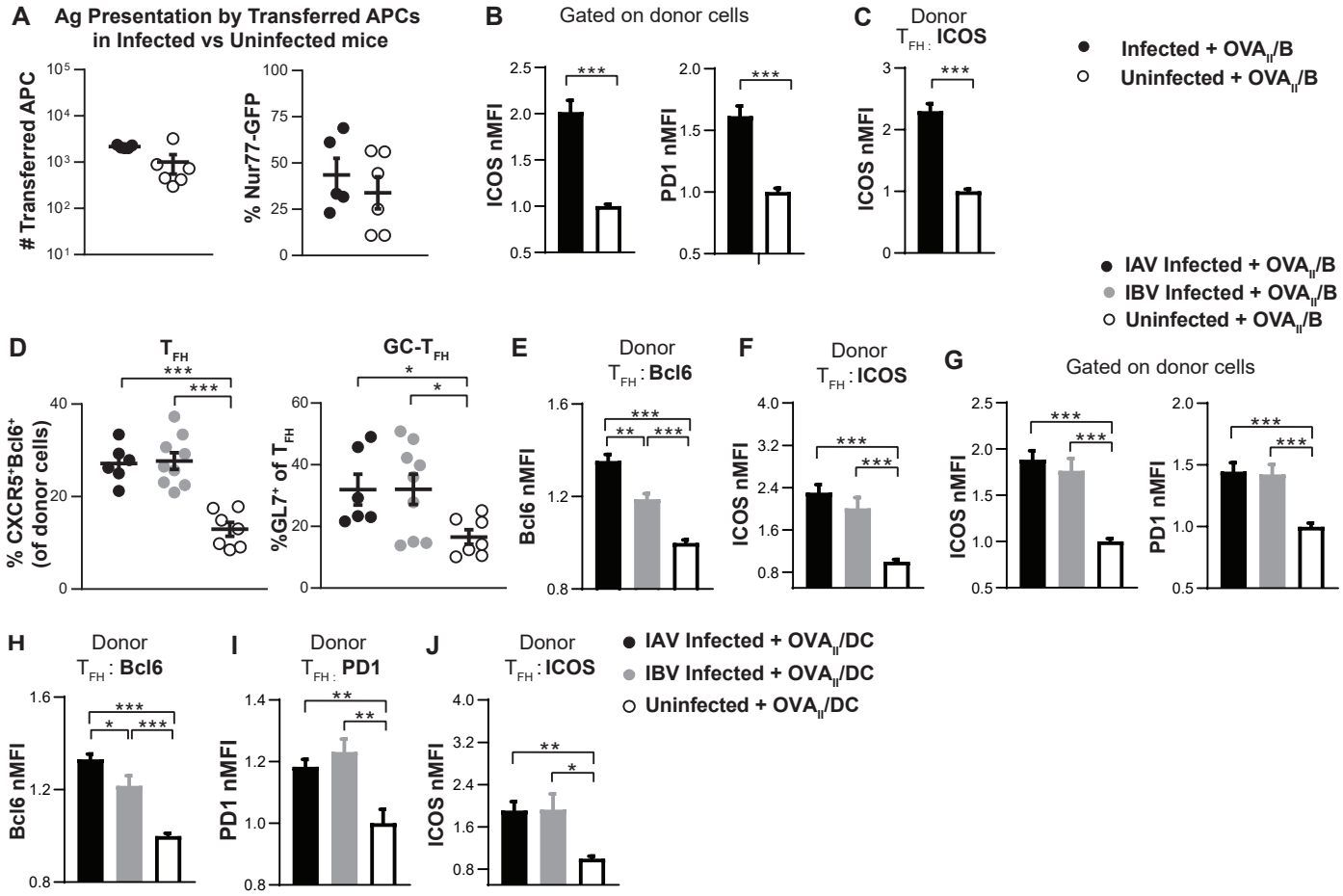
Supplementary Figure 2 (Related to Figure 1 and 2). Additional donor cell phenotype and function in the spleen and DLN. (A-G) Experiment performed as in Figure 1A. Spleen and DLN were harvested 8 dpi (2 dpt). **(A)** Numbers of total donor cells recovered in the DLN and spleen. **(B)** The percentage and number of DLN donor T_{FH} (CXCR5$^+$ Bcl6$^+$). **(C)** Percentage and number of DLN donor T_{FH} cells expressing GL7 (GC-T_{FH}). Normalized MFI of PD1 (**D**) or ICOS (**E**) expression by DLN donor cells. (A-E, n=10 per group pooled from 2 independent experiments). **(F)** (corresponding to Figure 2A) Representative FACS plots of IL-21 expression by spleen donor cells. Normalized MFI of IL-21 expression by spleen donor cells (n=9 per group pooled from 2 independent experiments). **(G)** Percentage of spleen donor cells expressing intracellular IFNγ and TNFα (n= 9 per group pooled, 2 independent experiments). **(H)** Experiment was performed as in Figure 2B. Number of total host GCB cells (CD19$^+$Fas$^+$GL7$^+$Bcl6$^+$) formed. (n=8-12 per group pooled, 2-3 independent experiments). Error bars represent s.e.m. Statistical significance determined by two-tailed, unpaired Student's t-test (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).



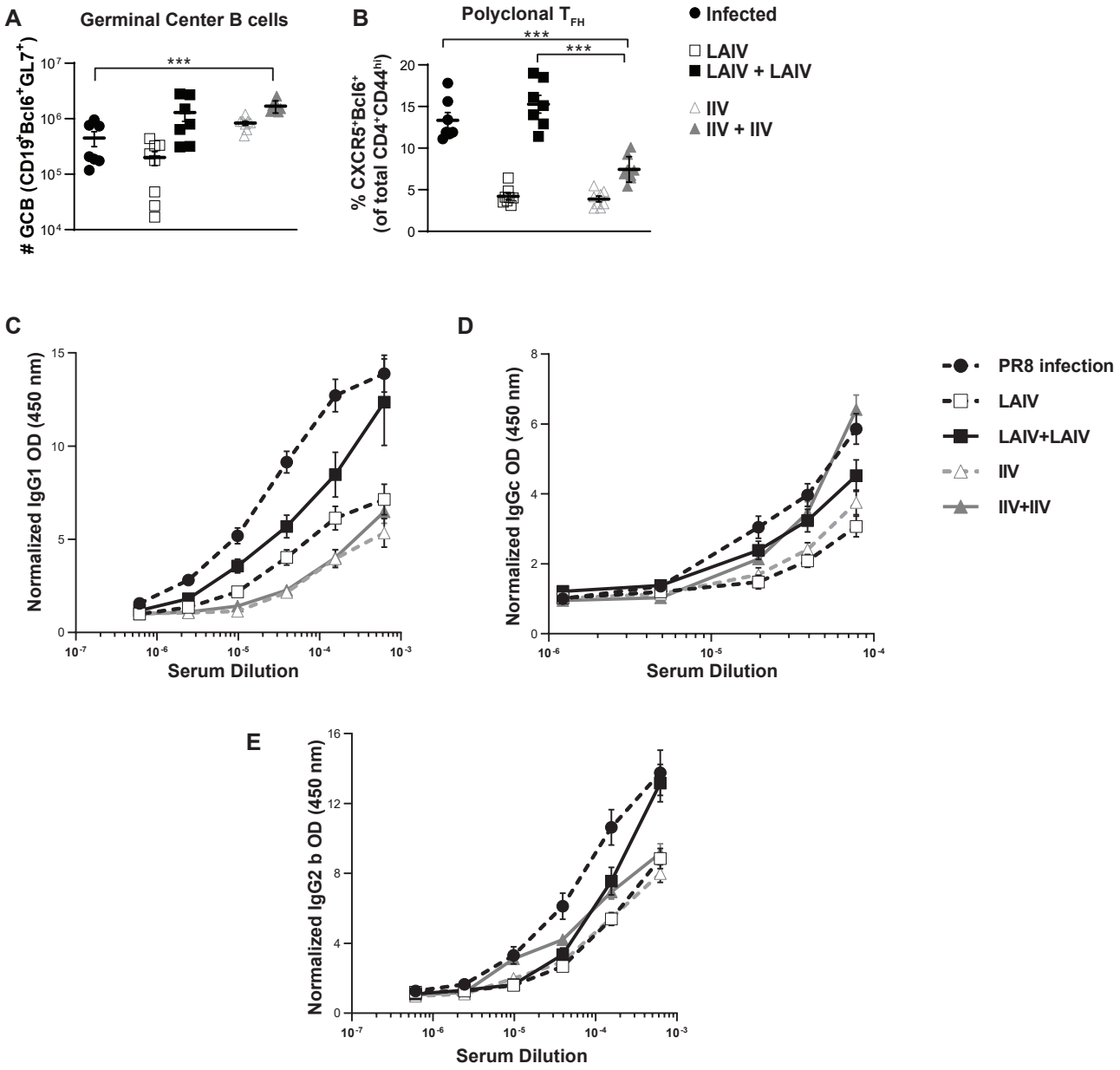
Supplementary Figure 3 (Related to Figure 3). Representative FACS plots showing T_{FH} generated with cognate Ag presented by multiple APC subsets during the effector phase. (A) Schematic for experimental setup in Figure 3A-F and Figure S3B-G: *In vivo* generated 6d OT-II.Thy1.1$^+$ or HNT.Thy1.1$^+$effectors were transferred into PR8 infection-matched hosts (B), PR8-OVA_{II} infection-matched hosts (C-E), or into PR8 infection-matched hosts together with OVA_{II}/APC (F-G). Representative FACS plots show the percentage of donor T_{FH} generated (gated on donor 6d OT-II.Thy1.1$^+$ cells) 2-4 dpt in the following models. **(B)** JhD mice where B cells are absent or into WT control mice (n=8 per group, 2 independent experiments). **(C)** WT→MHC-II KO (H2-Ab1$^{-/-}$) bone marrow chimera mice where MHC-II is restricted to the hematopoietic compartment or into WT→WT bone marrow chimera control mice (n=7-8 per group, 3 independent experiments). **(D)** MHC-II KO→WT bone marrow chimera mice where MHCII is restricted to the non-hematopoietic compartment or into WT→WT bone marrow chimera control mice (n=8-11 per group, 3 independent experiments). **(E)** CD11cTg.H2-Ab1$^{-/-}$ mice where MHC-II is restricted to CD11c$^+$ cells or into CD4 KO control mice (n=7-11 per group pooled from 2-3 independent experiments). **(F)** WT mice with cognate Ag supplied via OVA_{II} pulsed BMDC or via unpulsed BMDC controls (n=8-10 per group, 3 independent experiments). **(G)** WT mice with cognate Ag supplied via OVA_{II} pulsed B cells or via unpulsed B cell controls (n=5-6 per group, 2 independent experiments). **(H)** Experiment was done as in Fig. 3G: Numbers of total OT-II recovered in the spleen. (n=5-6 per group, 2 independent experiments). Error bars represent s.e.m. Statistical significance determined by two-tailed, unpaired Student's t-test (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).



Supplementary Figure 4 (Related to Figure 4). DLN vs Spleen T_{FH} generation when Ag presentation is supplied i.n. vs i.s. vs i.v. Experiment was done as in Figure 4A. Spleen and DLN were harvested 3-4 dpt and analyzed for T_{FH} generation from transferred donor cells. **(A)** Related to Main Figure 4E-F (i.n. vs i.s. APC transfer): Numbers of donor spleen and DLN T_{FH}. (DLN T_{FH}: n=5-10 per group pooled, 3 independent experiments, Spleen T_{FH}: n=9-12 per group pooled, 4 independent experiments) **(B)** Related to Main Figure 4J (i.n. vs i.v. APC transfer): Representative FACS plots for donor DLN T_{FH} and numbers of donor DLN T_{FH}. **(C)** Related to Main Figure 4K (i.n. vs i.v. APC transfer): Representative FACS plots for donor spleen T_{FH} and numbers of donor spleen T_{FH}. (B-C, n=5-11 per group pooled, 2-3 independent experiments). Error bars represent s.e.m. Statistical significance determined by two-tailed, unpaired Student's t-test (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).



Supplemental Figure 5 (Related to Figure 5). Reduction in T_{FH} associated marker expression in the absence of signals from infection during the effector phase. (A) CD45.1 OVA_{II}/B were transferred i.n. and i.v. into infection-matched hosts 6 dpi or into uninfected mice with *in vivo* generated 6d OT-II.Nur77^{GFP}.Thy1.1^{+} effectors. Numbers of transferred APC and Nur77^{GFP} expression was assessed 14-16 hr pt. (n=5-6 per group pooled from 2 independent experiments) **(B-C)** Related to Figure 5B-D. *In vivo* generated 6d OT-II.Nur77^{GFP}.Thy1.1^{+} effectors were transferred i.v. along with OVA_{II} peptide pulsed B cells both i.n. and i.v. into 2nd hosts that were either infection-matched with Influenza A Virus or that were uninfected. Spleens were harvested 3-4 dpt and donor cells were analyzed for **(B)** ICOS and PD1 expression **(C)** ICOS expression gated on the T_{FH} generated. B-C, n=16-17 per group pooled from 5 independent experiments. **(D-G)** Experiment was done as in (B-C) but 6d effectors were also transferred into a separate group of Influenza B Virus infection matched mice. **(D)** %T_{FH} generation from transferred donor cells. **(E-F)** Bcl6 expression (E) and ICOS expression (F) by T_{FH} generated from donors. **(G)** ICOS and PD1 expression by total transferred donor cells. **(H-J)** Related to Figure 5E. Experiment was done as in (B-G) except that OVA_{II} peptide pulsed DC were used as APC. Bcl6 expression (H), PD1 expression (I) and ICOS expression (J) by T_{FH} generated from donors. D-J, n=6-10 per group pooled from 2 independent experiments



Supplemental Figure 6 (Related to Figure 6). Two doses of LAIV induce superior polyclonal T_{FH} responses that correspond to superior IgG1 serum Ab titers. Experiment was done as in Figure 6A. **(A)** Total number germinal center B cells generated were analyzed at 9 dpi. **(B)** Polyclonal T_{FH} generated expressed as a % of total $CD4^+CD44^{hi}$ polyclonal effectors analyzed at 9 dpi. (A-B), n=7-8 per group pooled from 2 independent experiments. Statistical significance determined by two-tailed, unpaired Student's t-test. **(C-E)** Serum Ab titers were analyzed at 31-33 dpi by ELISA in two independent experiments, n=8 per group. OD values were normalized to the background in each ELISA experiment. Normalized OD values from each group were pooled to generate curves shown. (C) IgG1 ELISA (D) IgG2c ELISA (E) IgG2b ELISA. AUC (Area under the curve) analyses were done and one-way ANOVA used to determine statistical significance that is shown in Fig 6K-M. Error bars represent s.e.m. (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$)

Supplemental Materials and Methods

In vivo day 6 effector generation and transfer

Briefly, cells from lymph nodes and spleens of naïve OT-II or HNT transgenic mice were enriched for naïve cells by percoll gradients and CD4 T cells isolated by CD4 positive selection (Miltenyi Biotec) or using a CD4 naïve positive selection kit (Miltenyi Biotec). Naïve CD4 T cells were adoptively transferred into mice (1st hosts), which were then infected with IAV (PR8 or PR8-OVA_{II}). On 6 days post infection(dpi), the lung draining lymph nodes (DLN) and spleens were harvested and donor T cells were isolated using MACS (Miltenyi Biotec) based on their congenic marker (CD90.1). Immediately after isolation, the *in vivo* generated 1-2x10^6 6d CD4 effectors were adoptively transferred intravenously (i.v.) into host mice (2nd hosts).

Germinal Center B cell functional assay:

6d OT-II effectors were generated *in vivo* and transferred into either 2dpi PR8-OVA_{II} hosts or into 2dpi PR8 hosts. As a control there was a group of 2dpi PR8-OVA_{II} and 2dpi PR8 mice that received no 6d effectors. 2dpi hosts were chosen because it allowed us to readout T_{FH} induced GCB formation, 4 days after transfer of 6d effs at 6dpi, when endogenous GCB formation was still low (Figure S1C). 4 days after transfer (corresponding to 6dpi), spleens from the four groups of mice were harvested and analyzed for GCB formation. In the control groups of mice that received no transferred effectors, GCB were only beginning to be formed (baseline) in concert with the GCB formation kinetics in Figure S1C. Thus, any GCB formation induced over the control baseline was due to the transferred 6d effectors.

Bone marrow chimera mice generation

Host mice for bone marrow chimeras were lethally irradiated with 2 doses of 570 rads, 3 hours apart. Bone marrow was isolated from the femurs and tibia of donor mice. The bone marrow was T cell depleted (using CD90.2 magnetic beads from Miltenyi) and adoptively transferred into lethally irradiated host mice by tail-vein i.v. injections. Bone marrow was transferred at a 1:1 or 1:2 donor:host mice ratio. Mice were allowed to recover and reconstitute for at least 6 weeks prior to use during which they were treated with antibiotics (0.63mg/ml Sulfadiazine and 0.13mg/mL Trimethoprim) added to their drinking water. Reconstitution was confirmed by flow cytometry of peripheral blood before use and again in all tissues harvested when the mice were used in experiments.

In vitro APC culture and activation

BMDC (bone marrow derived dendritic cells) (6, 54) and activated B cell (6) generation was done as described previously. Briefly, bone marrow cells were flushed from femurs and tibia of mice and cultured *in vitro* with 10ng/mL GMCSF (Biolegend). After 7 days, cells were harvested and enriched for dendritic cells with CD11c positive selection (Miltenyi Biotec). Dendritic cells were then matured with 10ug/mL Poly I:C (InVivoGen) overnight before use. Activated B cells were

generated by isolating T depleted splenocytes using CD90.2 negative selection (Miltenyi Biotec) and culturing these *in vitro* for 2 days with 10ng/mL LPS and 10ng/mL dextran sulfate.

In vivo intrasplenic APC delivery

The animal was initially anaesthetized at 2.5%, then maintained at 1.5 – 1.75% isoflurane. Animal's hair was clipped from the hip to mid chest on the animal's left side. The area was sterilized and bupivacaine 1mg/kg was subcutaneously injected at the proposed incision site. Just below the last rib, using a pair of forceps, a 2 mm area of skin was held up and away from the body cavity and a 6-8mm incision was made by blunt dissection. PBS soaked cotton tipped applicators were used to lift the spleen out and hold in place. A 25µl Hamilton syringe with a 31-gauge Hamilton needle was used to inject the cells into the spleen. Sterile PBS was drawn into the syringe 3 times prior to the cells being drawn up. The syringe was held in a vertical position to the center of the spleen. The center of the top of spleen was penetrated by the needle at a depth of 2mm. The plunger was pushed slowly over a period of 10 seconds, and then the needle was left in the spleen for an additional 10 seconds. Using the cotton tipped applicators, the spleen was placed back into the abdominal cavity. Muscle and skin layers were sutured closed. Upon completion of the surgery, meloxicam SR 4.0 mg/kg was administered subcutaneously over the right flank.

Flow cytometry

Cells were harvested and passed through a 70uM nylon mesh, washed, and stained in FACS buffer [0.5% Bovine Serum Albumin, 0.01% sodium azide (Sigma-Aldrich) in PBS]. For $NP_{311-325}$ tetramer staining, cells were incubated with the tetramer for 1hr at 37ºC prior to surface staining. Cells were blocked with anti-FcR (2.4G2) and then stained with amine reactive viability dyes to exclude dead cells (Invitrogen) at 4ºC. Surface antigens were stained with fluorochrome conjugated antibodies at 4ºC. Antibodies used: anti- CD4 (GK1.5), CD19 (6D5), CD44 (IM7), CD90.1 (OX-7 and HIS51), CD95 (Fas, Jo2), CD185 (CXCR5, SPRCL5), CD278 (ICOS, C398.4A), CD279 (PD1, 29F.1A12), CD335 (NKp46, 29A1.4), GL-7, IgD (11-26c). HA reactivity was detected using HA conjugated to FITC. Following surface staining, cells were fixed with 2% paraformaldehyde (Sigma-Aldrich).

T cell cytokine production was measured by *ex vivo* stimulation with plate bound 0.5ug/mL anti-CD3 and 20ug/mL anti-CD28 or with 10ng/mL PMA and 500ng/mL Ionomycin for 4 hours at 37ºC, 5% CO_2 with brefeldin A (10ug/ml). Cells were harvested and stained for intracellular cytokines. For intracellular staining of cytokines, cells were first surface stained then fixed with 4% paraformaldehyde for 20 min, washed, and permeabilized with 0.1% saponin buffer (1% FBS, 0.1%NaN_3 and 0.1% saponin in PBS, (Sigma-Aldrich) for 15 mins. Subsequent staining for cytokines using the following antibodies: anti-IFNγ (XMG1.2), anti- TNFα (MP6-XT22). IL-21 was detected using IL-21RFc (R&D systems), washed and detected with fluorochrome conjugated secondary goat anti-human antibodies (Jackson ImmunoResearch). For Bcl-6 staining, cells were first surface stained then fixed and permeabilized using the FoxP3 fix/perm kit (eBioscience) overnight following manufacturer's protocol and stained with anti-Bcl-6 (K112-91) at 4ºC for 1hr. Antibodies were obtained from eBioscience, Biolegend, or BD Bioscience. Stained cells were acquired on an LSRII flow cytometer (BD) and analyzed using FlowJo analysis software.

ELISA

Plates (Nunc) coated overnight at room temperature with PR8 or with LAIV in carbonate buffer, were washed and blocked with PBS containing 1%BSA and 0.01% Tween. Serum samples serially diluted in PBS-0.01% Tween-1% BSA were incubated 2 h at room temperature. After washing, 100uL of HRP-conjugated Abs specific for mouse IgG1, IgG2c or IgG2b, (Southern Biotechnology Associates) were added at dilutions of 1:8000, 1:2000 and 1:8000 respectively, in PBS-0.01% Tween-1% BSA, and plates were incubated 1 h at room temperature. After washing, TMB was added, stopped with 1N Sulfuric Acid and the OD of the color reaction was measured at 450 nm. OD values from different experiments were normalized by dividing by background OD. The ODs were then pooled to generate the ELISA curves. AUC was determined and statistical significance analyze by one-way ANOVA.