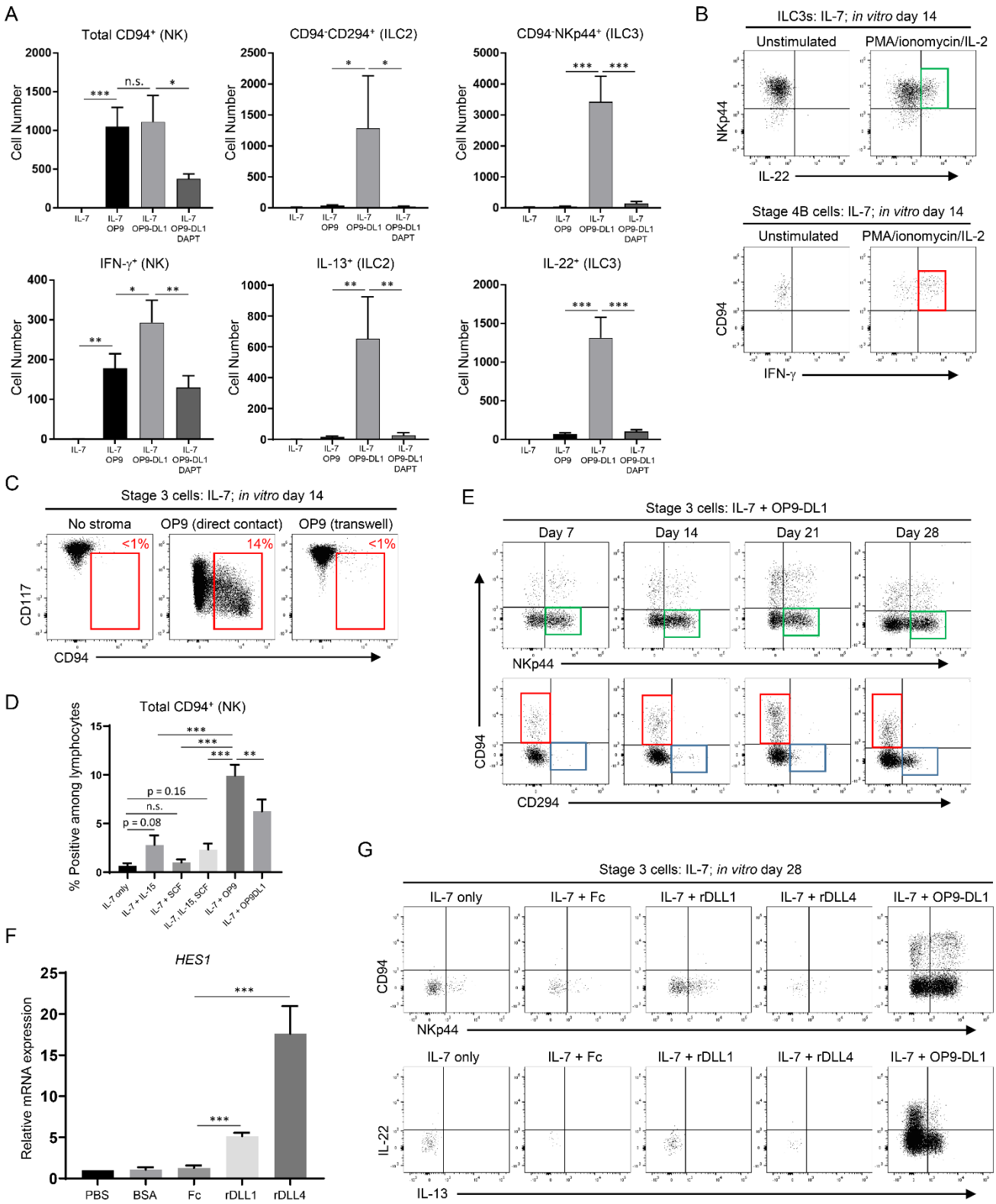
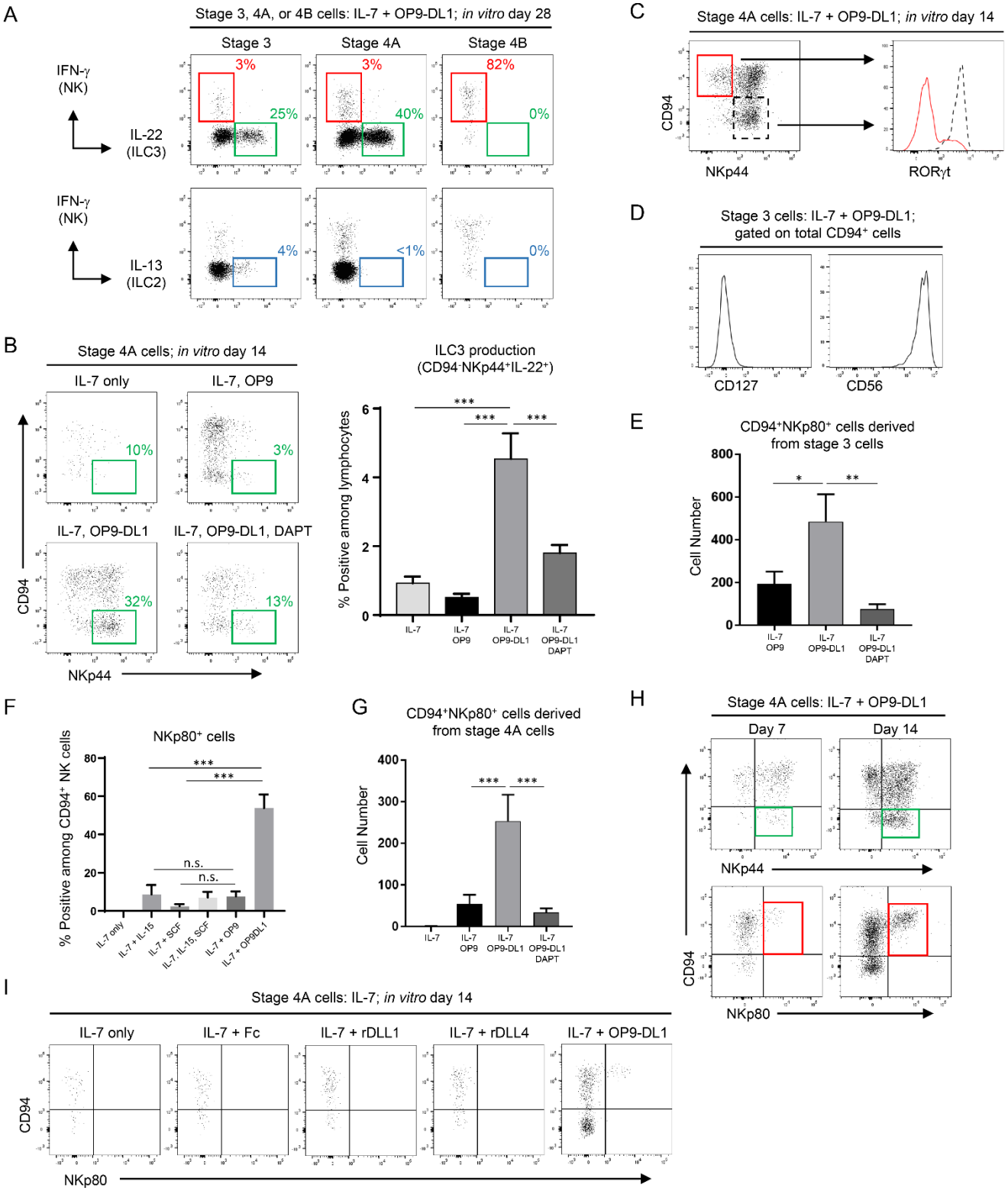
Supplemental Figure 1



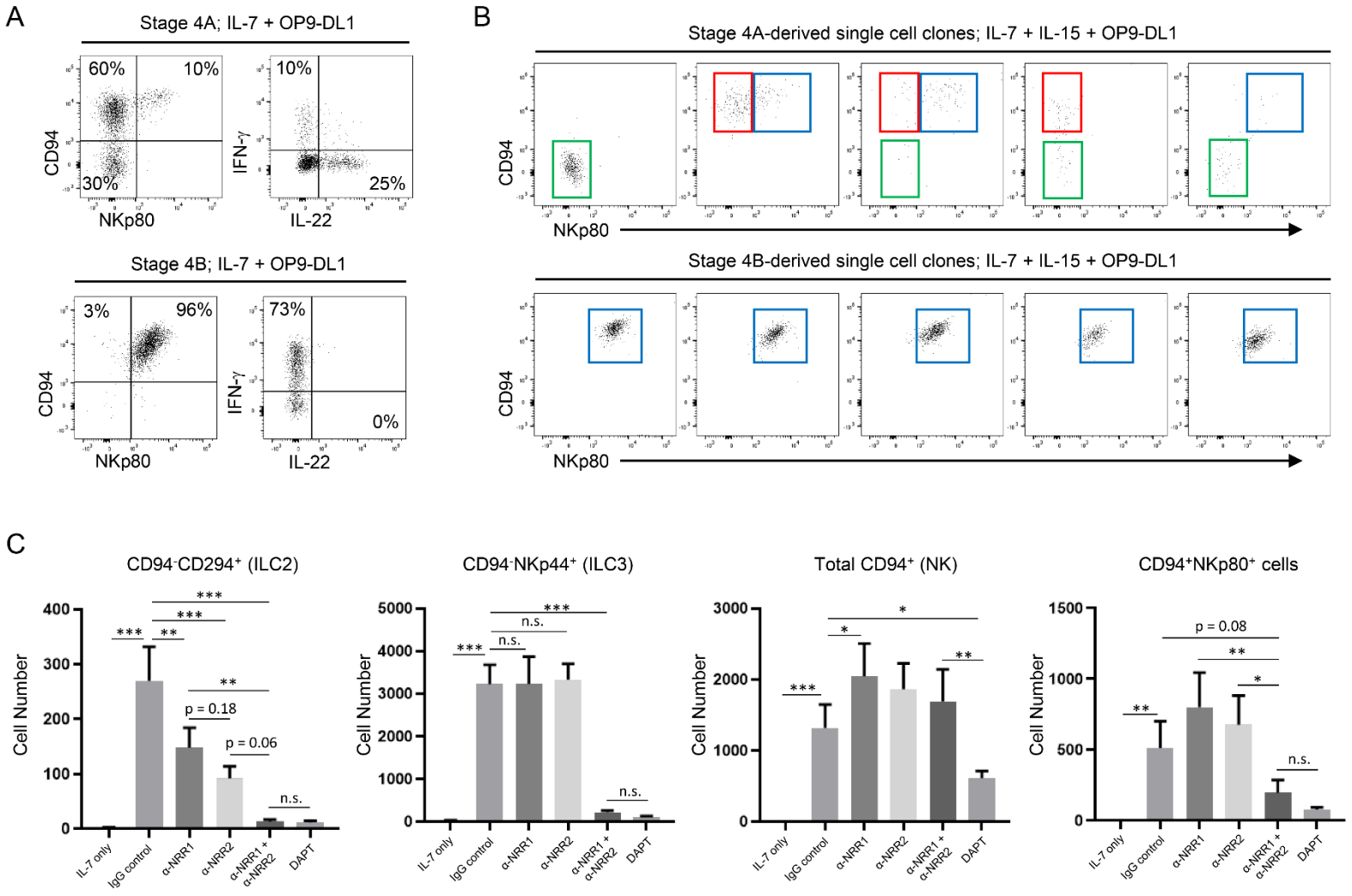
Supplemental Figure S1. Stromal cells and Notch are required for ILC differentiation. **(A)** Absolute numbers of NK cells (CD94$^+$; IFN-γ$^+$), ILC2s (CD94$^-$CD294$^+$; IL-13$^+$), and ILC3s (CD94$^-$NKp44$^+$; IL-22$^+$) generated *in vitro* following 28 day culture of freshly purified tonsil-derived stage 3 cells with recombinant human IL-7 (10 ng/ml for all experiments) alone, IL-7 + OP9 cells, or IL-7 + OP9-DL1 cells and treated with either vehicle control or DAPT (10 μM for all experiments). For analysis of cytokine production, ILCs were first stimulated with PMA, ionomycin, and IL-2 (10 ng/ml) for 4 hr prior to analysis. Dot plots in this and all successive figures are gated on live CD3$^-$CD14$^-$CD45$^+$ lymphocytes unless otherwise labeled. Data are represented as mean ± SEM, n = 16; 5 independent experiments. n.s. = not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **(B)** Representative (n = 5; 1 independent experiment) flow cytometry analyses of ILCs following 14 day culture of either tonsil-derived ILC3s (top) or stage 4B cells (bottom) with IL-7 in the absence of stroma. Cells were either not stimulated (left panels) or stimulated with PMA, ionomycin, and IL-2 (right panels) for 4 hr prior to analysis of cytokine production. **(C)** Representative (n = 3; 1 independent experiment) surface flow cytometry analyses of ILCs generated *in vitro* following 14 day culture of freshly purified tonsil-derived stage 3 cells with IL-7 alone or IL-7 + OP9 cells in direct contact or separated by transwell. Boxes and percentages refer to CD94$^+$ NK cell differentiation. **(D)** Quantification of CD94$^+$ cells generated *in vitro* following 28 day culture of freshly purified tonsil-derived stage 3 cells with IL-7 alone, IL-7 + IL-15 (10 ng/ml), IL-7 + SCF (10 ng/ml), IL-7 + IL-15 + SCF, IL-7 + OP9 cells, or IL-7 + OP9-DL1 cells. Data are represented as mean ± SEM, n = 7; 3 independent experiments. n.s. = not significant ($p > 0.05$); ** $p < 0.01$; *** $p < 0.001$. **(E)** Representative (n = 6; 2 independent experiments) surface flow cytometry analyses of ILC2s (CD94$^-$CD294$^+$; blue boxes), ILC3s (CD94$^-$NKp44$^+$; green boxes), and NK cells (CD94$^+$; red boxes) generated *in vitro* following culture of tonsil-derived stage 3 cells for 7, 14, 21, or 28 days. **(F)** Validation of Notch receptor activation by plate-bound Notch ligands. Shown are relative transcript expression amounts by quantitative real-time RT-PCR of *HES1* (Notch target gene) in THP1 cells incubated for 24 hr with PBS (vehicle control), bovine serum albumin (BSA, protein control), Fc control, recombinant human DLL1, or recombinant human DLL4 (each in triplicate; 1 independent experiment) as described in Methods. Gene expression was normalized to *18S* internal control and expressed relative to *HES1* expression in PBS condition set to 1.0 arbitrary units. *** $p < 0.001$. **(G)** Representative (n = 3; 1 independent experiment) flow cytometry analyses of ILCs generated *in vitro* following 28 day culture of freshly purified tonsil-derived stage 3 cells with IL-7 alone or IL-7 plus Fc control, recombinant human DLL1 (4 μg/ml), recombinant human DLL4 (4 μg/ml), or OP9-DL1 cells.

Supplemental Figure 2



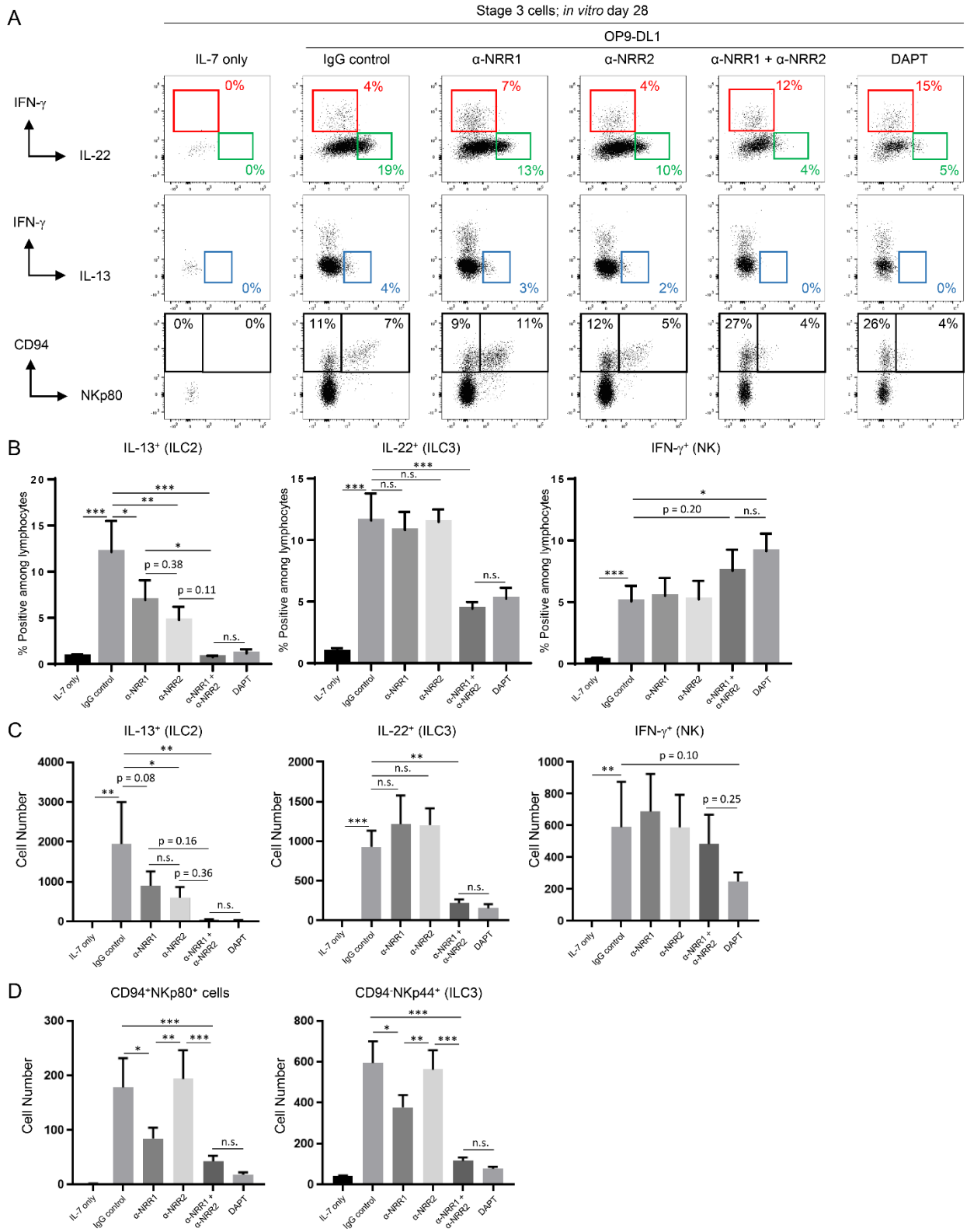
Supplemental Figure S2. Stage 4A cells, but not stage 4B cells, differentiate into ILC3s. **(A)** Representative (n = 15; 4 independent experiments) intracellular flow cytometry analyses of cytokine-producing NK cells (IFN-γ$^+$), ILC3s (IL-22$^+$), and ILC2s (IL-13$^+$) generated *in vitro* following 28 day culture of the indicated freshly purified tonsil-derived populations with OP9-DL1 stromal feeder cells and IL-7. Percentages shown represent each population relative to total *in vitro*-derived live CD45$^+$ lymphocytes. **(B)** Left panels, representative (n = 10; 3 independent experiments) surface flow cytometry analyses of ILCs generated *in vitro* following 14 day culture of freshly purified tonsil-derived stage 4A cells with IL-7 alone, IL-7 + OP9 cells, or IL-7 + OP9-DL1 cells + either vehicle control or DAPT. Green boxes quantify CD94$^-$NKp44$^+$ cells as percentages of total *in vitro*-derived live CD45$^+$ lymphocytes. Right, quantification of CD94$^-$NKp44$^+$IL-22$^+$ ILC3s generated *in vitro* following 14 day culture of freshly purified tonsil-derived stage 4A cells with IL-7 alone, IL-7 + OP9 cells, or IL-7 + OP9-DL1 cells + either vehicle control or DAPT. Cells were stimulated with PMA, ionomycin, and IL-2 for 4 hr prior to analysis of cytokine production. *** $p < 0.001$. **(C)** Representative (n = 8; 2 independent experiments) intracellular flow cytometry analysis of RORγt expression among CD94$^+$NKp44$^-$ (red histogram) or CD94$^-$NKp44$^+$ (ILC3s, dotted black histogram) cells generated *in vitro* following 14 day culture of stage 4A cells with IL-7 plus OP9-DL1 cells. **(D)** Representative (n = 7; 3 independent experiments) surface cytometry analyses of CD127 and CD56 among total CD94$^+$ cells generated following 28 day culture of freshly purified tonsil-derived stage 3 cells with IL-7 + OP9-DL1 cells. **(E)** Absolute numbers of CD94$^+$NKp80$^+$ cells generated *in vitro* following 28 day culture of freshly purified tonsil-derived stage 3 cells with IL-7 + OP9 cells, or IL-7 + OP9-DL1 cells and treated with either vehicle control or DAPT. Data are represented as mean ± SEM, n = 16; 5 independent experiments. * $p < 0.05$; ** $p < 0.01$. **(F)** Quantification of NKp80$^+$ cells among CD94$^+$ cells generated *in vitro* following 28 day culture of freshly purified tonsil-derived stage 3 cells with IL-7 alone, IL-7 + IL-15, IL-7 + SCF, IL-7 + IL-15 + SCF, IL-7 + OP9 cells, or IL-7 + OP9-DL1 cells. Data are represented as mean ± SEM, n = 7; 3 independent experiments. n.s. = not significant ($p > 0.05$); *** $p < 0.001$. **(G)** Absolute numbers of CD94$^+$NKp80$^+$ cells generated *in vitro* following 14 day culture of freshly purified tonsil-derived stage 4A cells with IL-7 alone, IL-7 + OP9 cells, or IL-7 + OP9-DL1 cells and treated with either vehicle control or DAPT. Data are represented as mean ± SEM, n = 14; 4 independent experiments. *** $p < 0.001$. **(H)** Representative (n = 6; 2 independent experiments) surface flow cytometry analyses of ILC3s (CD94$^-$NKp44$^+$; green boxes) and CD94$^+$NKp80$^+$ NK cells (red boxes) generated *in vitro* following culture of tonsil-derived stage 4A cells for 7 or 14 days. **(I)** Representative (n = 3; 1 independent experiment) flow cytometry analyses of ILCs generated *in vitro* following 14 day culture of freshly purified tonsil-derived stage 4A cells with IL-7 alone or IL-7 plus Fc control, recombinant human DLL1 (4 μg/ml), recombinant human DLL4 (4 μg/ml), or OP9-DL1 cells.

Supplemental Figure 3



Supplemental Figure S3. Stage 4B cells are NK lineage-committed compared to stage 3 and 4A cells. **(A)** Representative (n = 11; 3 independent experiments) surface and intracellular flow cytometry analyses of ILCs generated *in vitro* following 14 day culture of the indicated freshly purified tonsil-derived populations with IL-7 + OP9-DL1 cells. Percentages shown represent each population relative to total *in vitro*-derived live $CD45^+$ lymphocytes. **(B)** Representative surface flow cytometry analyses of ILCs generated *in vitro* following 21 day culture of either stage 4A (top row) or stage 4B (bottom row) cells individually sorted into round-bottom wells containing OP9-DL1 stromal feeder cells and IL-7 and IL-15 (10 ng/ml each). Plots show ILC3/stage 3-like (green boxes), stage 4A-like (red), and stage 4B-like (blue) cells among culture-derived live $CD45^+$ lymphocytes. Data shown here are representative of 54 and 53 clones evaluated from tonsillar FACS-purified stage 4A and stage 4B cells, respectively (obtained from a total of 6 tonsils; 3 independent experiments). Cloning efficiency was approximately 10% for stage 4A clones and 25% for stage 4B clones. **(C)** Absolute numbers of $CD94^-CD294^+$ (ILC2s), $CD94^-NKp44^+$ (ILC3s), total $CD94^+$ (NK), and $CD94^+NKp80^+$ cells generated *in vitro* following 28 day culture of freshly purified tonsil-derived stage 3 cells with IL-7 alone or IL-7 + OP9-DL1 cells with the addition of IgG control antibody, anti-NRR1 and/or anti-NRR2 (5 μg/ml each), or DAPT. Data are represented as mean ± SEM, n = 13; 4 independent experiments. n.s. = not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Supplemental Figure 4



Supplemental Figure S4. NOTCH1 and NOTCH2 regulate ILC differentiation from stage 3 and 4A cells. **(A)** Representative (n = 12; 4 independent experiments) intracellular flow cytometry analyses of ILCs generated *in vitro* following 28 day culture of freshly purified tonsil-derived stage 3 cells with IL-7 alone or IL-7 + OP9-DL1 cells with the addition of IgG control antibody, anti-NRR1 and/or anti-NRR2 (5 μg/ml each), or DAPT. Percentages shown represent each population relative to total *in vitro*-derived live CD45$^+$ lymphocytes. **(B)** Quantification of IL-13$^+$, IL-22$^+$, and IFN-γ$^+$ cells generated *in vitro* following 28 day culture of freshly purified tonsil-derived stage 3 cells in the conditions described in (A). Data are represented as mean ± SEM. n.s. = not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **(C)** Absolute numbers of IL-13$^+$, IL-22$^+$, and IFN-γ$^+$ cells generated *in vitro* following 28 day culture of freshly purified tonsil-derived stage 3 cells in the conditions described in (A). Data are represented as mean ± SEM. n.s. = not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **(D)** Absolute numbers of CD94$^+$NKp80$^+$ NK cells and ILC3s (CD94$^-$NKp44$^+$) generated *in vitro* following 14 day culture of freshly purified tonsil-derived stage 4A cells with IL-7 alone or IL-7 + OP9-DL1 in the presence of IgG control, anti-NRR1 and/or anti-NRR2, or DAPT. Data are represented as mean ± SEM, n = 18; 6 independent experiments. n.s. = not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.