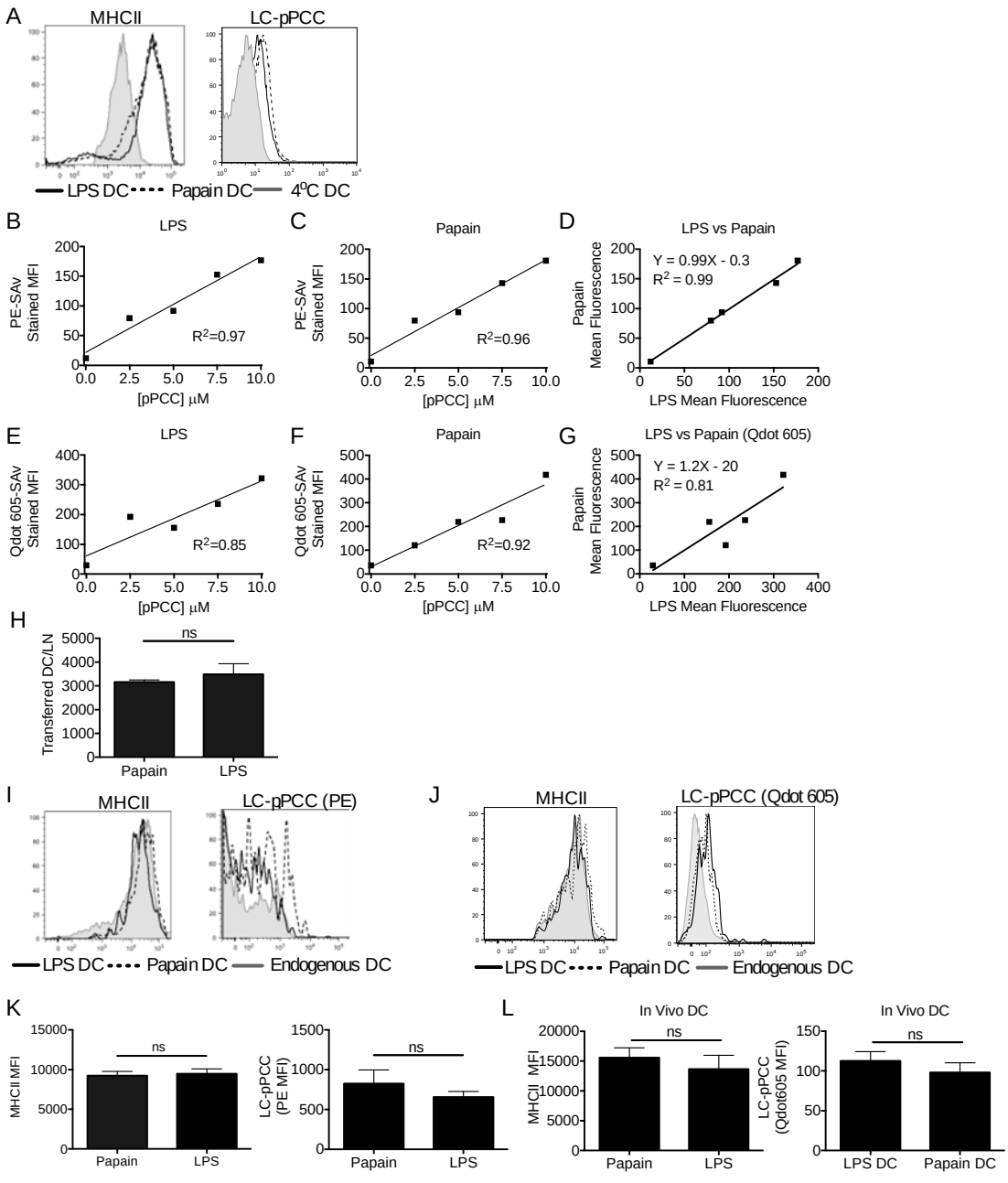

Figure S1: Antigen presentation by in vitro activated DC following in vitro activation and ex vivo examination. Related to Figure 1.

(A) CD11c^{+} splenic DC were isolated and incubated at 37^{o}C for 4 hr with papain or LPS in the presence of 10.0μM long chain biotin-pPCC (LC-pPCC). Following in vitro culture activation status was ascertained via flow cytometry for MHCII expression and PCC peptide presentation was determined following staining with Streptavidin(SAv)-PE. In vitro cultured DC were compared to DC maintained at 4^{o}C for the period of stimulation. (B-G) DC were

cultured as in (A) at a range of pPCC concentrations as indicated and stained with either (B,C,D) SAv-PE or (E,F,G) SAv-Qdot 605 in the presence of (B,E) LPS or (C,F) papain and mean fluorescence intensity (MFI) vs. [pPCC] plotted. MFIs of LPS or papain treated DC stained with either (D) SAv-PE or (G) SAv-Qdot 605 were compared. $CD11c^+$ splenic DC were isolated and incubated as in (A). DC were then adoptively transferred into the rear footpads of $CD45.2^-$ congenic hosts and recovered from the dLN at 24 hr post-transfer without digestion. Following recovery (H) DC numbers were determined and (I&J) activation status was ascertained via flow cytometry for MHCII expression and PCC peptide presentation was determined following staining with (I&K) Streptavidin-PE or with (J&L) Streptavidin-Qdot605. *In vivo* recovered DC were compared to endogenous DC. Data are representative of three experiments (n=3-4 animals). Error bars represent mean +/- s.e.m. ns = non-significant as determined by Student's t test (two-tailed).

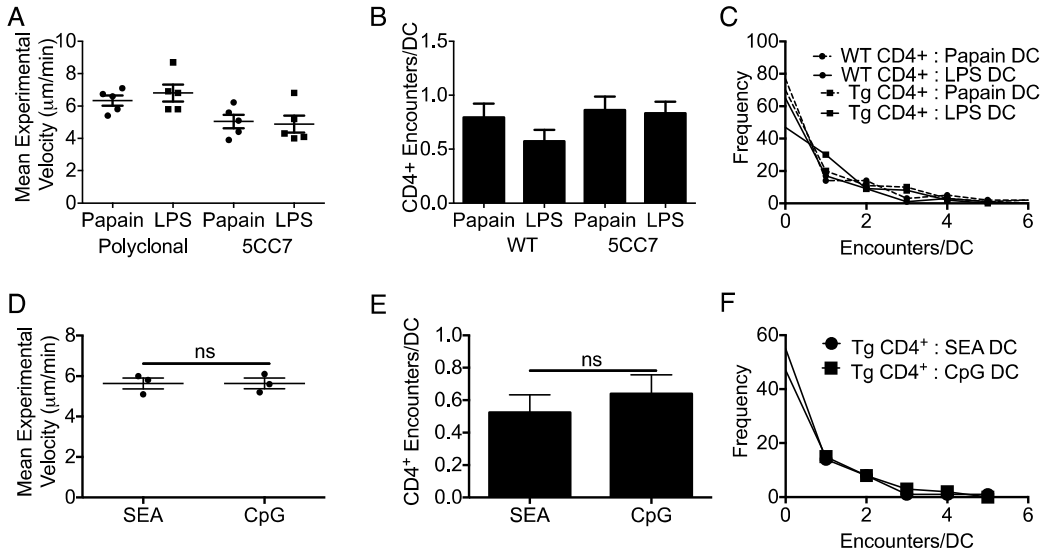

Figure S2: Different adjuvant treatments of DC alter contact duration, but not initial contact frequency with $CD4^+$ T cells. Related to figure 1.

$CD11c^+$ splenic DC were isolated and incubated at 37°C for four hr with adjuvants as indicated in the presence of 0.1μM pPCC and adoptively transferred into the rear footpad of $CD45.1^+$ B10.A animals. Eighteen hrs. post transfer 5CC7 $CD4^+$ T cells labeled with CMTPX and B10.A polyclonal $CD4^+$ T cells labeled with CMFDA were co-transferred by i.v. injection and imaged immediately afterwards for 2hr by 2-P IVM. (A&D)

Individual CD4$^+$ T cells were tracked and mean experimental velocities were determined. (B&E) Frequency distribution of the number of CD4$^+$ T cell contacts per DC. (C&F) Mean experimental number of contacts made by CD4$^+$ T cells per DC. (A-C) Data are pooled from five individual experiments. (D-F) Data are pooled from three individual experiments. Error bars represent mean +/- s.e.m.

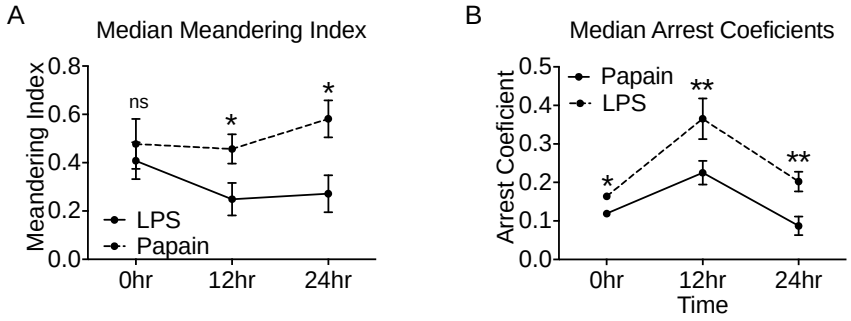

Figure S3: Different adjuvant treatments of DCs alter the motility of CD4$^+$ T cells in lymph nodes during activation. Related to Figure 2.

CD11c$^+$ splenic DC were isolated and incubated at 37^{o}C for four hr with adjuvants as indicated in the presence of 0.1μM pPCC and adoptively transferred into the rear footpad of CD45.1$^+$ B10.A animals. Eighteen hrs. post transfer 5CC7 CD4$^+$ T cells labeled with CMTPX and B10.A polyclonal CD4$^+$ T cells labeled with CMFDA were co-transferred by i.v. injection and imaged at times indicated by 2-P IVM. Individual CD4$^+$ T cells were tracked and meandering indices and arrest coefficients calculated, median experimental values were then determined. (A) Median experimental meandering indices and (B) median experimental arrest coefficients Data are pooled from 3-5 individual experiments. Error bars represent mean +/- s.e.m. ns = non-significant *P<0.05, **P<0.01 and ***P<0.001, as determined by Student's t test (two-tailed).

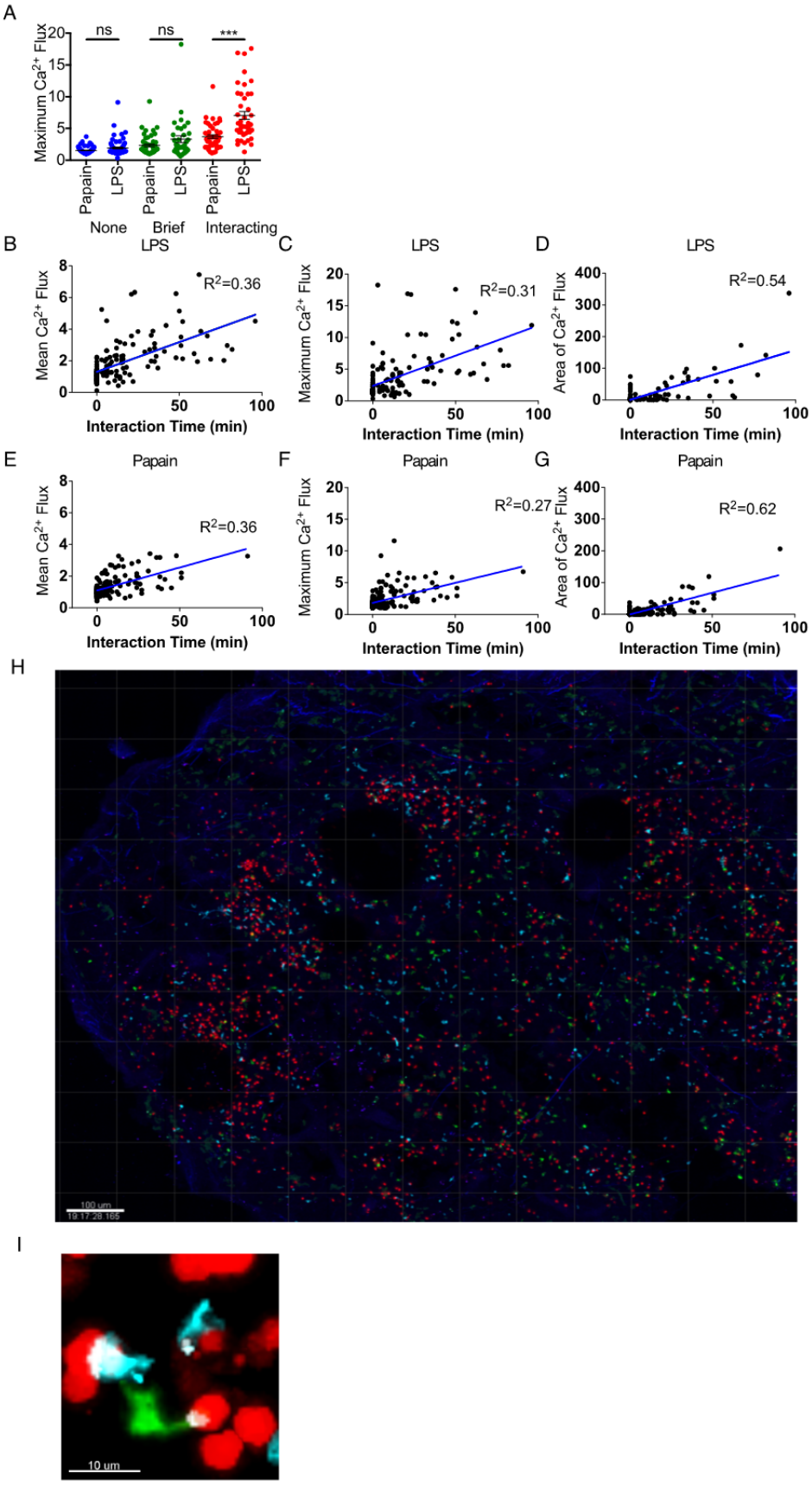
A
Maximum Ca2+ Flux
ns
ns
***
Papain
LPS
Papain
LPS
Papain
LPS
None
Brief
Interacting
B
LPS
Mean Ca2+ Flux
R2=0.36
Interaction Time (min)
C
LPS
Maximum Ca2+ Flux
R2=0.31
Interaction Time (min)
D
LPS
Area of Ca2+ Flux
R2=0.54
Interaction Time (min)
E
Papain
Mean Ca2+ Flux
R2=0.36
Interaction Time (min)
F
Papain
Maximum Ca2+ Flux
R2=0.27
Interaction Time (min)
G
Papain
Area of Ca2+ Flux
R2=0.62
Interaction Time (min)
H
100 um
I
10 um

Figure S4: Strength of signaling correlates with the length of cellular interactions. Related to Figure 4.

$CD11c^+$ splenic DC were isolated and incubated in the presence of 0.1μM pPCC at 37°C for 4hr with either LPS or papain. DC were adoptively transferred into the rear foot pads of naïve B10 animals. Eighteen hrs. post-transfer 5CC7 $CD4^+$ T cell labeled with CMTPX and Fluor-4 were adoptively transferred by i.v. injection and imaged immediately afterwards for 2hr by 2-P IVM. (A) Maximum Ca^{2+} flux for individually tracked cells was calculated for the duration of brief interactions (green) and interactions (red), while mean calcium flux for full track lengths of non-interacting cells (blue) is shown for five pooled experiments. (B & E) Mean Ca^{2+} flux, (C & F) maximum Ca^{2+} and (D & G) area under the Ca^{2+} trace for individually tracked CD4+ cells vs. interaction times for CD4+ T cells interacting with (B-D) LPS- or (C-F) papain-treated DC. Linear regression analysis was conducted and R^2 values determined (Data are the sum of 5 pooled experiments). Error bars represent mean +/- s.e.m. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ as determined by 1 way ANOVA with Tukeys post testing. $CD11c^+$ DC were isolated and incubated with either papain or LPS in the presence of 0.1μM pPCC. Papain- and LPS-treated DC were labeled with either CTB or CMFDA and co-transferred via s.c. injection into the foot pads of naïve B10 animals. Eighteen hrs. post DC transfer 5CC7 $CD4^+$ T cells labeled with CMTPX were adoptively transferred and imaged by 2-P IVM for 2hr immediately after transfer. (H) Representative micrograph of a lymph node section shows papain cultured DC in teal, LPS cultured DC in green, 5CC7 $CD4^+$ T cells in red and second harmonic collagen signals in blue. (I) Cellular bodies and interaction interfaces were digitally quantified; interaction interfaces shown in white. Same color scheme as in (H).

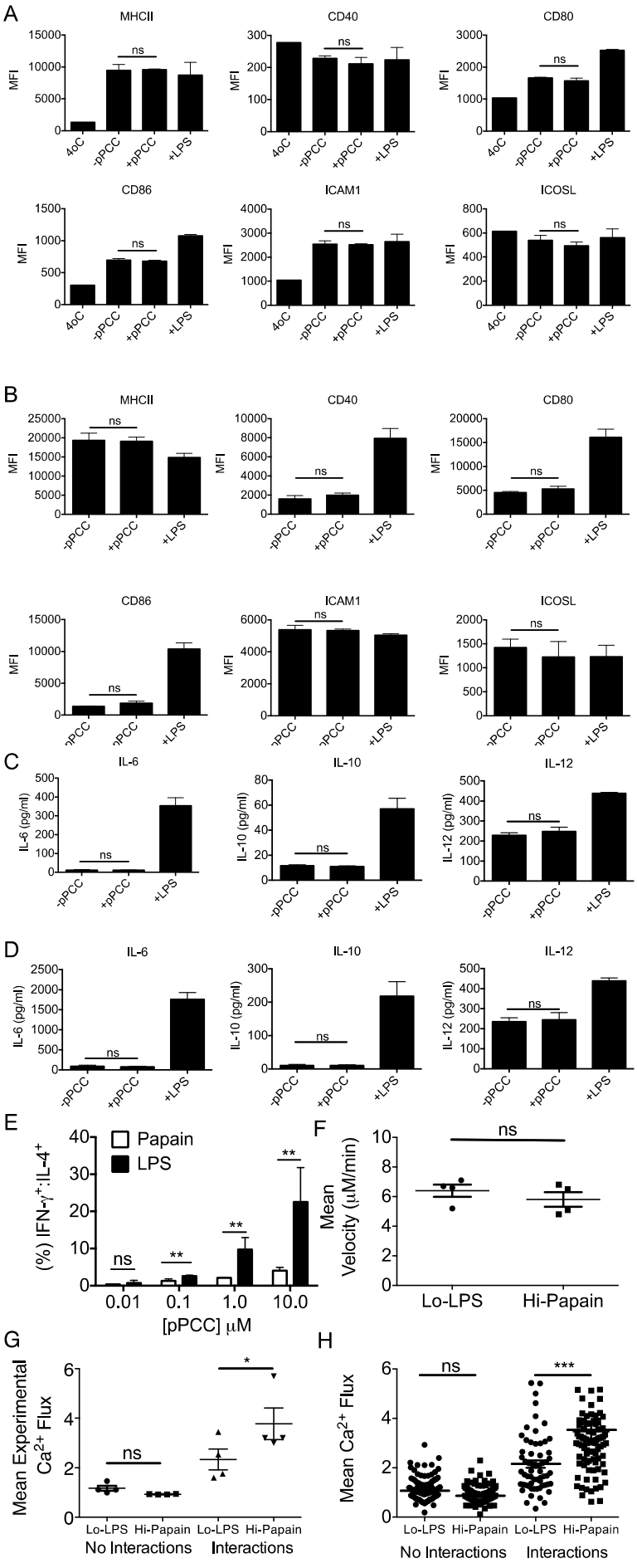
A
MHCII
CD40
CD80
CD86
ICAM1
ICOSL
MFI
ns
4oC
-pPCC
+pPCC
+LPS
B
MHCII
CD40
CD80
CD86
ICAM1
ICOSL
MFI
ns
-pPCC
+pPCC
+LPS
C
IL-6
IL-10
IL-12
IL-6 (pg/ml)
IL-10 (pg/ml)
IL-12 (pg/ml)
ns
-pPCC
+pPCC
+LPS
D
IL-6
IL-10
IL-12
IL-6 (pg/ml)
IL-10 (pg/ml)
IL-12 (pg/ml)
ns
-pPCC
+pPCC
+LPS
E
Papain
LPS
(%) IFN-γ+:IL-4+
ns
**
**
**
0.01
0.1
1.0
10.0
[pPCC] μM
F
ns
Mean Velocity (μM/min)
Lo-LPS
Hi-Papain
G
Mean Experimental Ca2+ Flux
ns
*
Lo-LPS
Hi-Papain
Lo-LPS
Hi-Papain
No Interactions
Interactions
H
Mean Ca2+ Flux
ns
***
Lo-LPS
Hi-Papain
Lo-LPS
Hi-Papain
No Interactions
Interactions

Figure S5: pPCC loading does not alter DC maturation state, but antigen-dependent signal strength dominates over qualitative effects of adjuvants on DC function. Related to Figure 5.

$CD11c^+$ splenic DC were isolated and incubated at 37°C for (A&C) 4hr or (B&D) 24hr in medium alone (-pPCC) the presence of 10μM pPCC (+pPCC) or with 10μM pPCC + LPS. (A&B) DC activation markers were assessed by flow cytometry. (C&D) DC cytokine production was assessed by ELISA. Error bars represent mean +/- s.e.m. ns = non-significant as determined by Student's t test (two-tailed) (n=3). $CD11c^+$ DC were exposed to LPS and 0.01μM pPCC (Lo-LPS) or papain and 10.0μM pPCC (Hi-Papain) and adoptively transferred into the rear footpad. Eighteen hrs. post transfer 5CC7 $CD4^+$ T cells labeled with CMTPX and Fluor-4 were adoptively transferred and imaged immediately afterwards for 1hr by 2P IVM. (E) Data as in Figure 5D in the main text, but displayed in linear format to emphasize the change in response with ligand dose. (F) Comparison of mean velocities of $CD4^+$ T cells migration speeds for 5CC7 in lymph nodes with Lo-LPS- or Hi Papain-treated DC for experimental data displayed in Figure 5 E-H. (G) Mean experimental Ca^{2+} flux. (H) Mean Ca^{2+} flux per interaction. (G&H) Data are representative of four individual experiments. (H) Data are representative of four pooled experiments, error bars indicate mean +/- SEM. *P<0.05, **P<0.01 and ***P<0.001 as determined by (F) Student's t test (two-tailed) or (G&H) 1 way ANOVA with Tukeys post testing

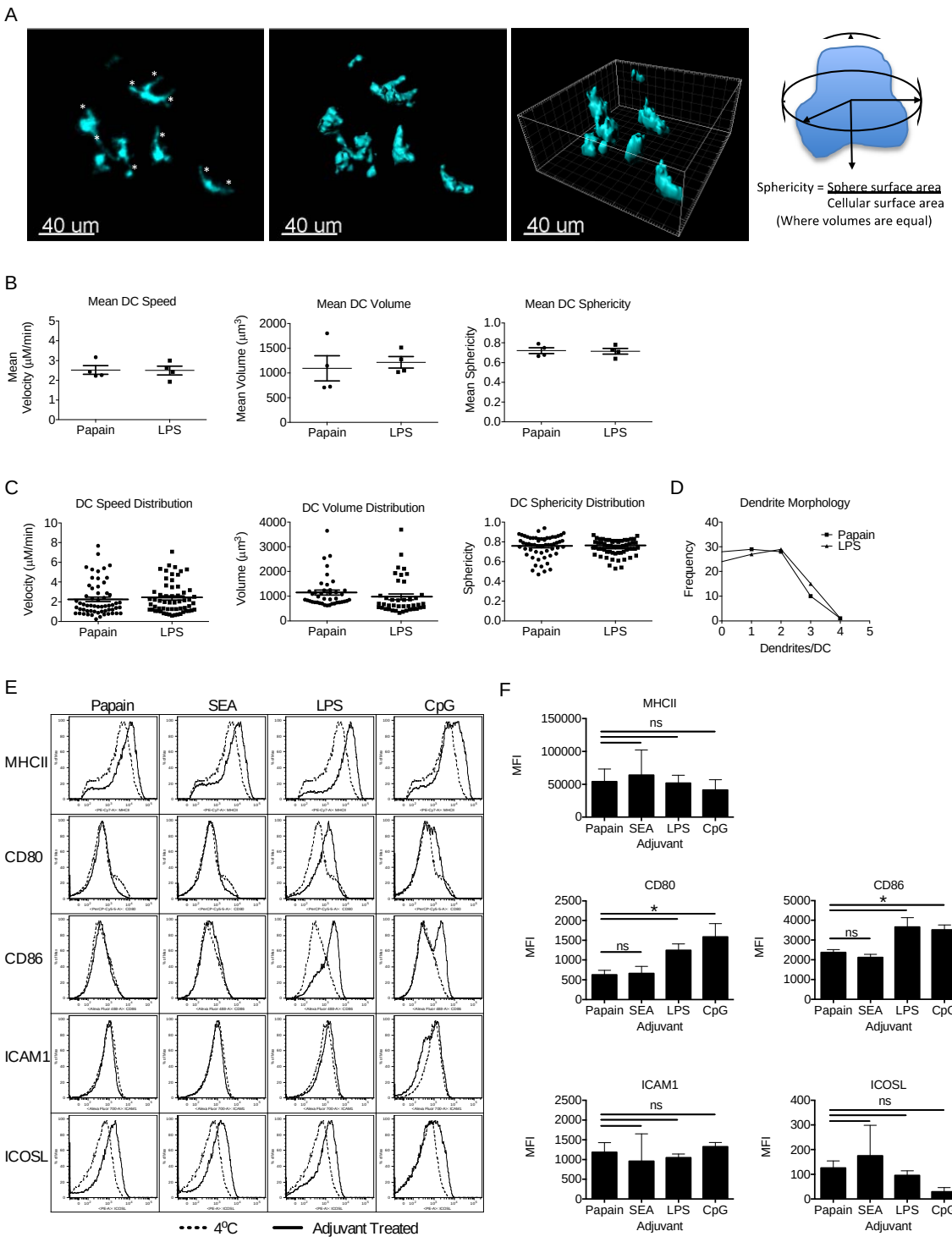

Figure S6: Physical characteristics and phenotypes of DC exposed to Th1 or Th2 cell promoting adjuvants. Related to Figure 6.

$CD11c^+$ splenic DC were isolated and incubated with 0.1μM pPCC at 37°C for 4hr with either papain or LPS. DC were labeled with CTB and adoptively transferred via s.c. injection into right rear foot pads of naïve B10 animals. Eighteen hrs. post transfer DC were imaged for 2hr by 2-P IVM. (A) Velocity, volume, and sphericity were calculated following construction of isosurface areas in Imaris Imageworks. (B) Mean velocity, volume, and sphericity from four individual experiments. (C) Representative distributions of an individual experiment. (D) Number of dendritic extensions from the main cell body were determined from static images captured at 18hr post transfer (*=dendrite, as indicated in (A)). $CD11c^+$ splenic DC were isolated and incubated at 37°C for 4 hr. with papain, SEA, LPS, or CpG. (E) DC were analyzed by flow cytometry for MHCII, CD80, CD86, ICAM1, and ICOSL surface expression. In vitro cultured DC (solid lines) were compared to DC maintained at 4°C for the period of stimulation (dashed lines). (F) DC were adoptively transferred via s.c. injection into right rear foot pads of $CD45.1^+$ B10 animals. Twenty-four hrs. post-transfer of DC, LN were processed to a single cell suspension (no digestion) and DC were analyzed by flow cytometry for MHCII, CD80, CD86, ICAM1, and ICOSL surface expression. Ex vivo transferred DC were compared to ex vivo endogenous DC. (A,C) data are representative of four individual experiments. (B) Mean values from four individual experiments. (D) Data are pooled from four individual experiments (E) Results are representative of two individual experiments. (F) Results are representative of two individual experiments (n=4 animals). Error bars indicate mean +/- SEM. *P<0.05, **P<0.01 and ***P<0.001 as determined by 1 way ANOVA with Tukeys post testing.

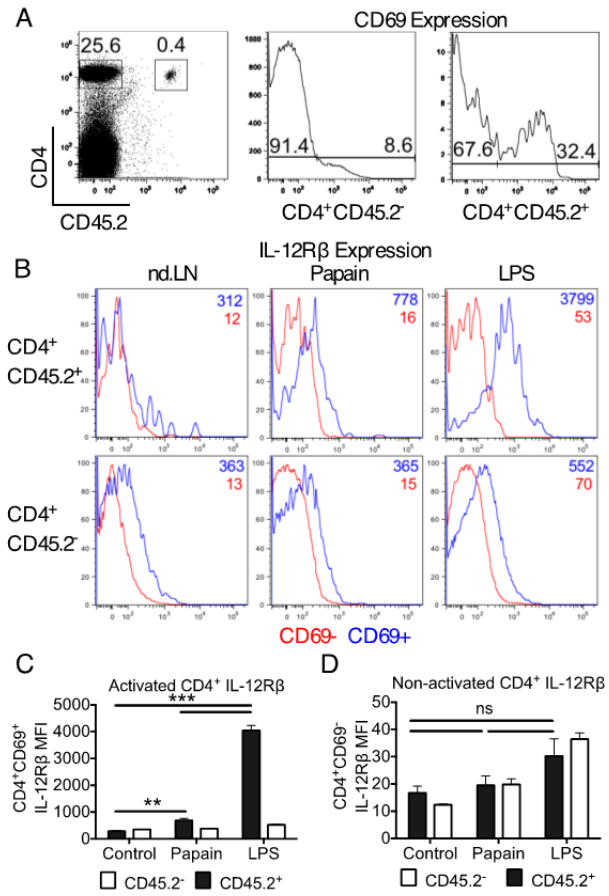

Figure S7: Signal strength determines the ability of CD4$^+$ T cells to respond to polarizing cytokine signaling *in vivo*. Related to Figure 7.

5CC7 CD4$^+$ T cells were activated in vivo with papain or LPS treated DC. At 24hr post transfer lymph nodes were processed to a single cell suspension and (A) activated 5CC7 CD4$^+$ T cells were identified as CD4$^+$ CD45.2$^+$ CD69$^+$. (B) Representative FACS plots of ex vivo IL-12Rβ2 expression of CD69$^+$ (blue) vs. CD69$^-$ (red) cells. Comparison of MFI for (C) activated (CD69$^+$) and (D) non-activated (CD69$^-$) cells. Data in (A-D) are representative of two individual experiments (n=4 animals), means are plotted +/- s.e.m. *P<0.05, **P<0.01 and ***P<0.001 as determined by 1 way ANOVA with Tukeys post testing.