Online supplemental material

Supplemental Figure 1. *A,* Panel shows DEC-205 mean fluorescence intensity ±SD on $CD8^+$ and $CD8^-$ DCs, germinal center ($CD19^+$ Fas^+ $GL\text{-}7^+$) and naive ($CD19^+$ Fas^- $GL\text{-}7^-$) B cells isolated from spleen and B cells cultured with LPS and IL-4 for 3 days from WT (n = 3) and DEC-205 deficient (n = 2) mice. *B.* Panel shows DEC-205 expression (mean fluorescence intensity ±SD) on B cells from WT (n = 3) and DEC-205 deficient (n = 2) mice that were cultured for 1, 2 or 3 days with LPS (25 μg/ml), IL-4 (5 ng/ml) or anti-CD40 (IC10 clone, 5 μg/ml), as indicated. *A* and *B*, show representative data of one experiment that was repeated 3 times with similar results.

Supplemental Figure 2. Antigen presentation after DEC205-mediated endocytosis *in vitro*. *A-B,* APCs from CD11c-hDEC mice were isolated from spleen of naive or mBSA-CFA immunized mice, or cultured from bone marrow *A,* Panels show the percentage of divided OTI (upper panel) and OTII (lower panel) T cells as in Fig. 2B but each symbol indicates independent experiments and represents the average of duplicate measurements where APCs were targeted with 1,000 ng/ml αhumanDEC-OVA. *B*, Activation and proliferation of OTI T cells (upper panels) and OTII (lower panels) T cells in response to APCs that were targeted with 300 ng/ml of αhDEC-OVA (left) or pulsed with 1 μM peptide (right). The Y-axis shows the percentage of divided T cells. The X-axis shows the number of APCs plated in each 96-well. *C*, APCs were isolated from spleen or cultured from bone marrow or spleen of WT mice. Panels show the percentage of divided OTI (upper panel) and OTII (lower panel) T cells as in Fig. 2E but each symbol indicates independent experiments and represents the average of duplicate measurements where

APCs were targeted with 1,000 ng/ml αmDEC-OVA. *A* and *C*, data was analyzed by ANOVA and Tukey's test was used to compare groups: ∗ $p < 0.05$; **$p < 0.01$; ***$p<0.001$. *B,* represents pooled data from 2 independent experiments.

Supplemental Figure 3. Activated monocytes differ from conventional DCs in that they are GM-CSF dependent and do not express Flk2 but express M-CSF receptor. WT and GMCSF-receptor β KO (GMR KO) mice were immunized with mBSA-CFA and i.p. challenged with mBSA 24 hours before analysis. *A*, Representative flow cytometry profile of splenocytes (after exclusion of T, B and NK cells, granulocytes and pDCs) showing CD11c and CD11b expression (upper panels). Sorting scheme for activated monocytes ($CD11c^+$ iMono) and $CD8^-$ DCs, after further gating on $CD11b^+$ $CD8^-$ cells (lower panels). *B*, Absolute number of different cell populations in the spleen, where N stands for naive mice and I for immunized mice. Graph shows mean ± SE, of two independent experiments with three mice/group each. *C*, Representative histograms show CD86, MHCI and MHCII expression on different cell populations. *D*. Representative dot-plots show expression of CD11b, CD11c, DCIR2, DEC-205, CD135 (Flk2) and CD115 (M-CSF receptor) on monocytes, $CD8^+$ DCs, $CD8^-$ DCs and activated monocytes ($CD11c^+$, iMono).

Supplemental Figure 4. CD86, MHCI and MHCII expression. *A,* CD86; *B,* MHCI; *C*, MHCII expression on the indicated APCs. The mean fluorescence intensity is represented in the Y-axis. Data represents the mean± SE data from 3 independent experiments.

Supplemental Figure 5. Antigen presentation after pinocytosis. *A*, Histograms show OVA staining on the indicated APC populations. Filled grey histograms represent background fluorescence (APCs not incubated with OVA antigen). $CD11c^+$ enriched splenocytes, GM-DCs and FL-DCs were incubated with OVA at 100 μg/ml (black lines) or anti-DEC-OVA at 10 μg/ml (blue line) at 4°C in PBS 2% FCS 0.05% azide for 20 minutes. Excess antigen was washed off and cells were incubated with Rabbit anti-OVA and subsequently with anti-Rabbit-Cy5 and antibodies to identify APCs as described in methods. *B*, Panels show the percentage of divided, CFSE low OTI (upper panel) and OTII (lower panel) T cells on the Y-axis, after incubation with the indicated APCs that were previously pulsed with OVA at 10 μg/ml for two hours at 37°C (as in Fig. 4) or for 20 minutes at 4°C. In addition, control APCs that were not pulsed with antigen were also incubated with T cells. *C,* Panels show the percentage of divided, CFSE low OTI (upper panels) and OTII (lower panels) T cells as in Fig. 4B, but each symbol indicates independent experiments and represents the average of duplicate measurements where APCs were incubated with 30 μg/ml OVA. Data was analyzed by ANOVA and Tukey's test was used to compare groups: $* p < 0.05$; $**p < 0.01$; $***p<0.001$. In *C*, FL-$CD8^-$ DCs were significantly different than all cell types, except i$CD8^-$ DCs.

Supplemental Figure 6. OVA-beads phagocytosis. APCs that had captured a single OVA-bead were sorted as in Fig. 5. Cells were then stained at 4°C in PBS 1% BSA 0.05% azide with CD11c-, CD11b-, CD8- and MHCII-biotin, followed by steptavidin pacific blue and analyzed on Image Stream 100 (Amnis). In focus, single cells were gated

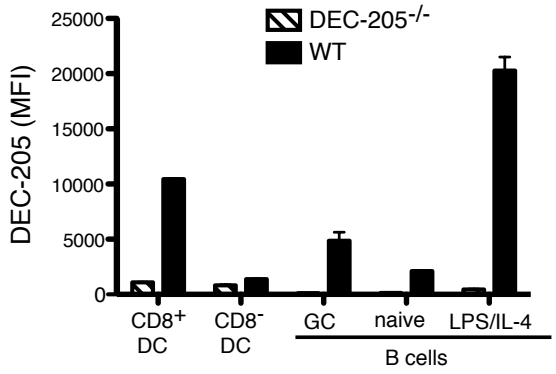
and the pacific blue membrane staining was used to create a mask to enable analysis of the beads internalization according to manufacturer's instructions (Amnis). Histograms show distribution of internalization scores for the different APC populations, where positive scores indicate internalization. The percentage of cells with internalized beads is shown in each histogram. Representative images of cells with positive internalization scores are shown on the right. We also show representative images from LPS-GM DCs that had a negative internalization score (lower images).

Supplemental Figure 7. Comparison of tip-DCs elicited by *Listeria monocytogenes* infection and activated monocytes elicited by mBSA-CFA immunization. WT mice were immunized with mBSA-CFA and i.p. challenged with mBSA 24 hours before analysis. Alternatively, WT mice were infected with 3,000 *Listeria monocytogenes* i.v. and analyzed 48 hrs later . *A*, Representative flow cytometry profile of splenocytes (after exclusion of T, B and NK cells, granulocytes and pDCs) showing CD11c and CD11b expression (upper panels). Sorting scheme (lower panels), after further gating on $CD11b^+$ $CD8^-$ cells, for $CD8^-$ DCs, activated monocytes ($CD11c^+$ monocytes from immunized mice; iMono CFA), or tip-DCs (monocytes from *Listeria* infected mice; iMono *Listeria*). *B*, Representative dot-plots show expression of CD11b, CD11c, CD115 (M-CSF receptor) and MHCII on $CD8^+$ DCs, $CD8^-$ DCs, monocytes and $CD11c^+$ monocytes. *C*, Representative histograms show CD86, MHCI and MHCII expression on the indicated different cell populations. *D*, DCs ($CD8^+$ and $CD8^-$) were isolated from spleen of naive mice and iMono were isolated from mBSA-CFA immunized mice ($CD11c^+$ monocytes) or *Listeria* infected mice. Enriched populations were incubated with OVA-beads before

cell sorting. Activation and proliferation of OTI (top panel) and OTII (lower panel) T cells after incubation with the indicated number of APCs in the X-axis, that were sorted to contain a single OVA-bead or more than two beads, as indicated. The Y-axis shows percentage of divided T cells. *E*, as in *D*, but cells that did not capture any beads were sorted. $5x10^3$ APCs were pulsed with the indicated concentrations of peptides in the X-axis and subsequently co-cultured with OTI (top panel) or OTII (lower panel) T cells. The Y-axis shows percentage of divided T cells.

Fig.S1

A


DEC-205 (MFI)
25000
20000
15000
10000
5000
0
DEC-205-/-
WT
CD8+ DC
CD8- DC
GC
naive
LPS/IL-4
B cells

B

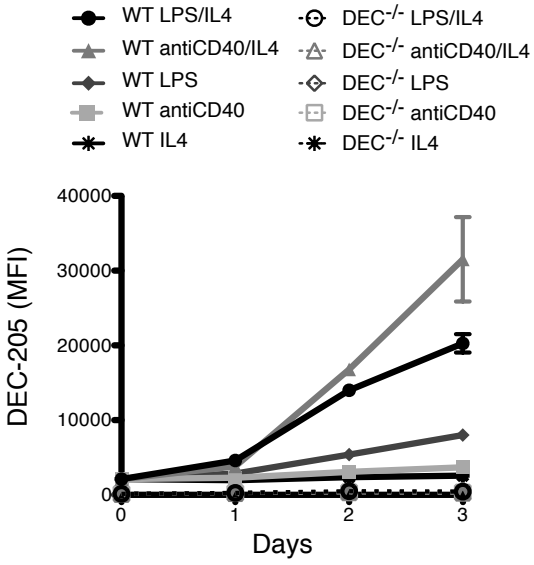

WT LPS/IL4
WT antiCD40/IL4
WT LPS
WT antiCD40
WT IL4
DEC-/- LPS/IL4
DEC-/- antiCD40/IL4
DEC-/- LPS
DEC-/- antiCD40
DEC-/- IL4
DEC-205 (MFI)
40000
30000
20000
10000
0
0
1
2
3
Days

Fig.S2

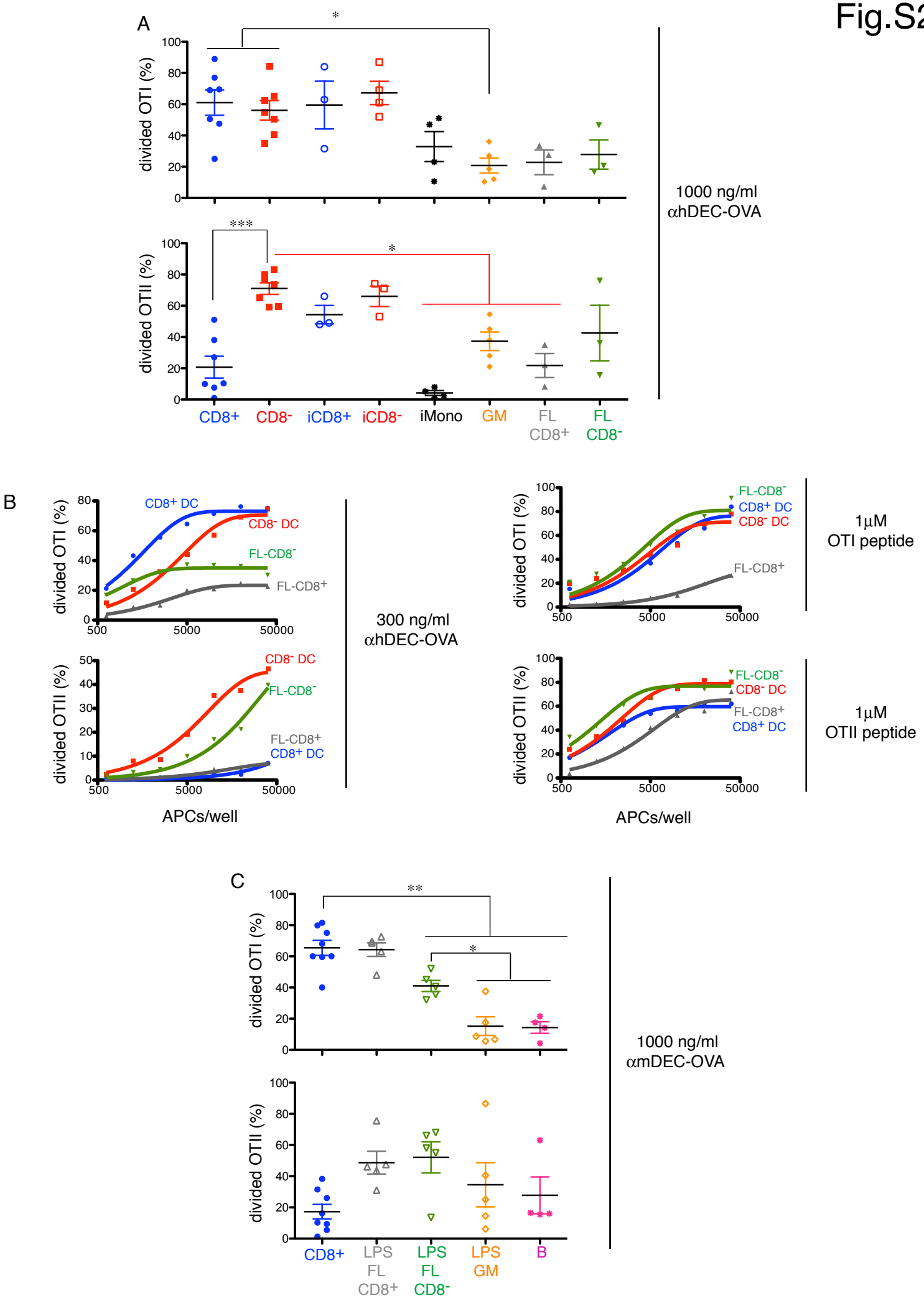

A
divided OTI (%)
divided OTII (%)
*
***
*
1000 ng/ml
αhDEC-OVA
CD8+
CD8-
iCD8+
iCD8-
iMono
GM
FL CD8+
FL CD8-
B
divided OTI (%)
divided OTII (%)
CD8+ DC
CD8- DC
FL-CD8-
FL-CD8+
CD8+ DC
300 ng/ml
αhDEC-OVA
1μM
OTI peptide
1μM
OTII peptide
APCs/well
C
divided OTI (%)
divided OTII (%)
**
*
1000 ng/ml
αmDEC-OVA
CD8+
LPS FL CD8+
LPS FL CD8-
LPS GM
B

Fig. S3

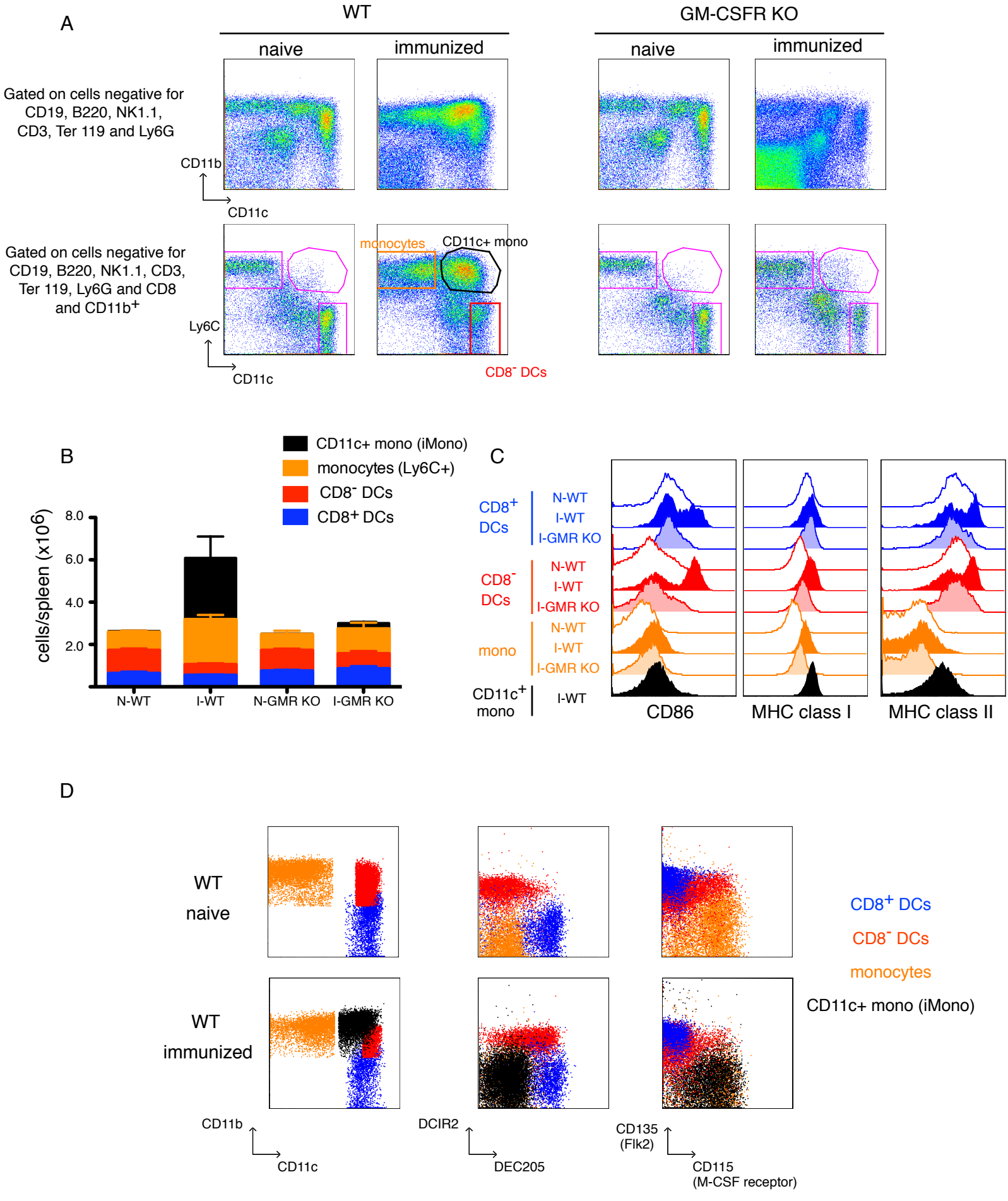

A
WT
GM-CSFR KO
naive
immunized
naive
immunized
Gated on cells negative for CD19, B220, NK1.1, CD3, Ter 119 and Ly6G
CD11b
CD11c
Gated on cells negative for CD19, B220, NK1.1, CD3, Ter 119, Ly6G and CD8 and CD11b+
monocytes
CD11c+ mono
Ly6C
CD11c
CD8- DCs
B
CD11c+ mono (iMono)
monocytes (Ly6C+)
CD8- DCs
CD8+ DCs
cells/spleen (x10^6)
8.0
6.0
4.0
2.0
N-WT
I-WT
N-GMR KO
I-GMR KO
C
CD8+ DCs
N-WT
I-WT
I-GMR KO
CD8- DCs
N-WT
I-WT
I-GMR KO
mono
N-WT
I-WT
I-GMR KO
CD11c+ mono
I-WT
CD86
MHC class I
MHC class II
D
WT naive
WT immunized
CD11b
CD11c
DCIR2
DEC205
CD135 (Flk2)
CD115 (M-CSF receptor)
CD8+ DCs
CD8- DCs
monocytes
CD11c+ mono (iMono)

Fig. S4

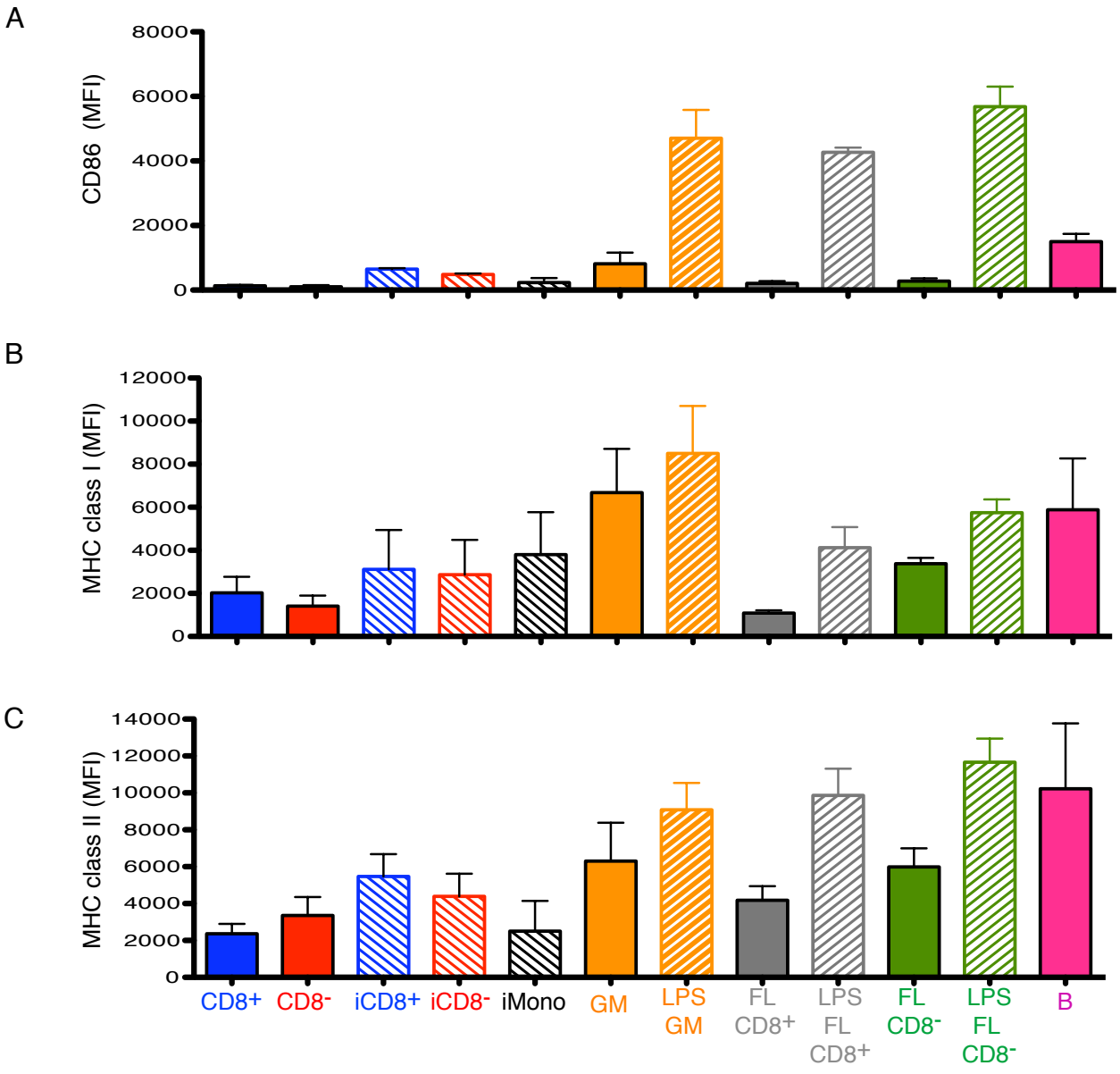

A
CD86 (MFI)
8000
6000
4000
2000
0
B
MHC class I (MFI)
12000
10000
8000
6000
4000
2000
0
C
MHC class II (MFI)
14000
12000
10000
8000
6000
4000
2000
0
CD8+
CD8-
iCD8+
iCD8-
iMono
GM
LPS GM
FL CD8+
LPS FL CD8+
FL CD8-
LPS FL CD8-
B

Fig. S5

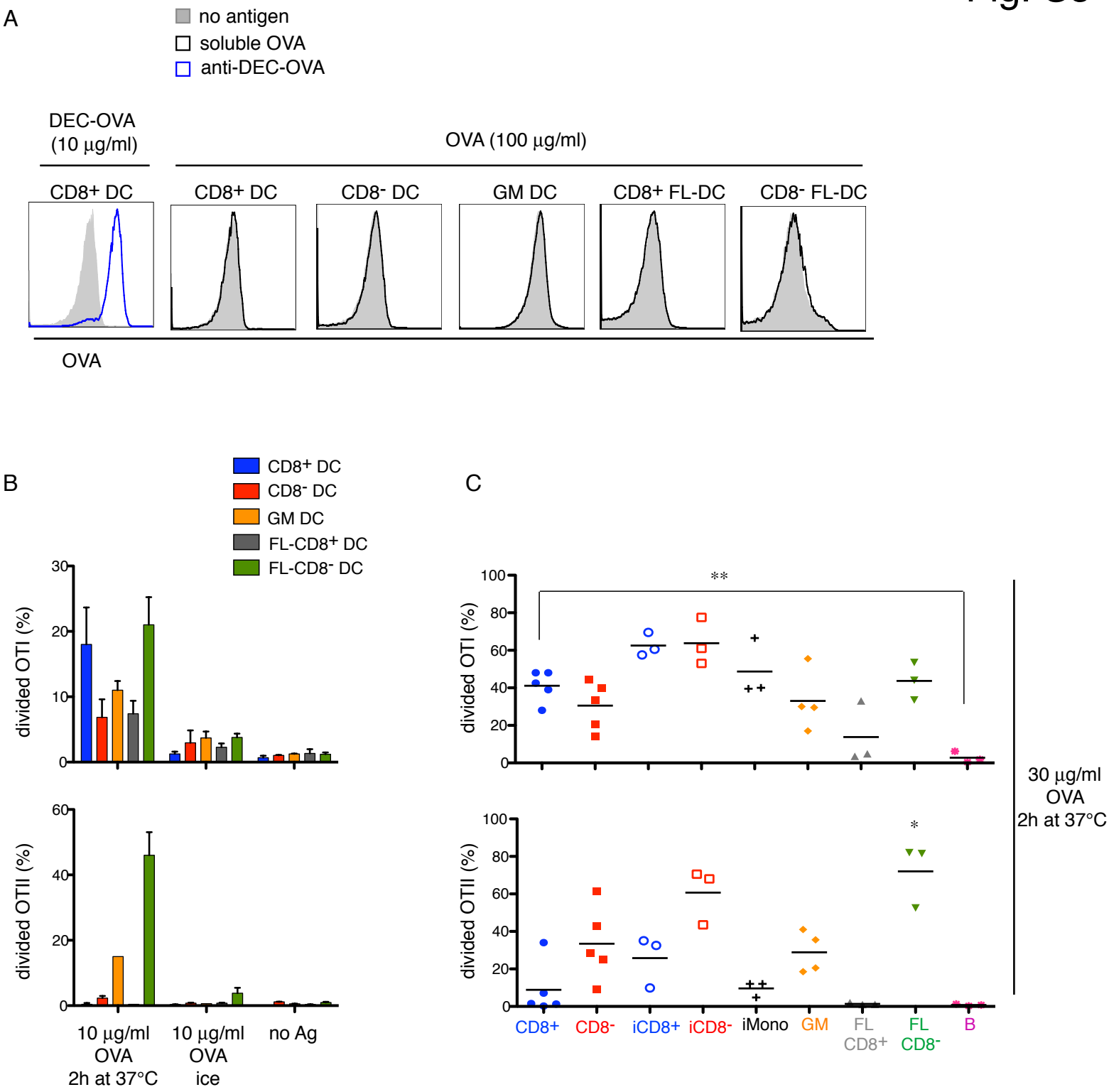

A
no antigen
soluble OVA
anti-DEC-OVA
DEC-OVA
(10 μg/ml)
OVA (100 μg/ml)
CD8+ DC
CD8+ DC
CD8- DC
GM DC
CD8+ FL-DC
CD8- FL-DC
OVA
B
CD8+ DC
CD8- DC
GM DC
FL-CD8+ DC
FL-CD8- DC
divided OTI (%)
divided OTII (%)
10 μg/ml OVA 2h at 37°C
10 μg/ml OVA ice
no Ag
C
divided OTI (%)
divided OTII (%)
**
*
CD8+
CD8-
iCD8+
iCD8-
iMono
GM
FL CD8+
FL CD8-
B
30 μg/ml OVA 2h at 37°C

Fig. S6

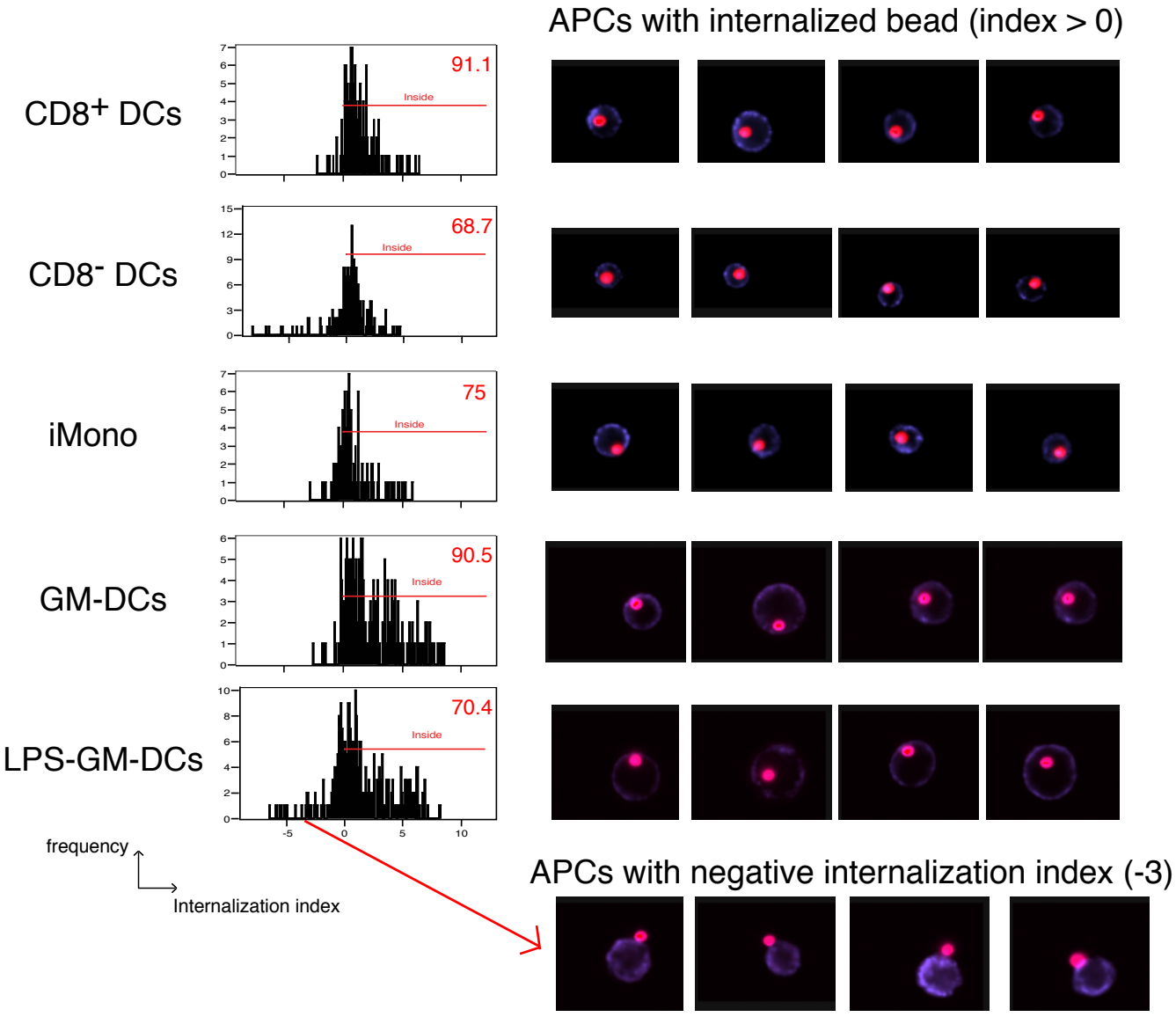
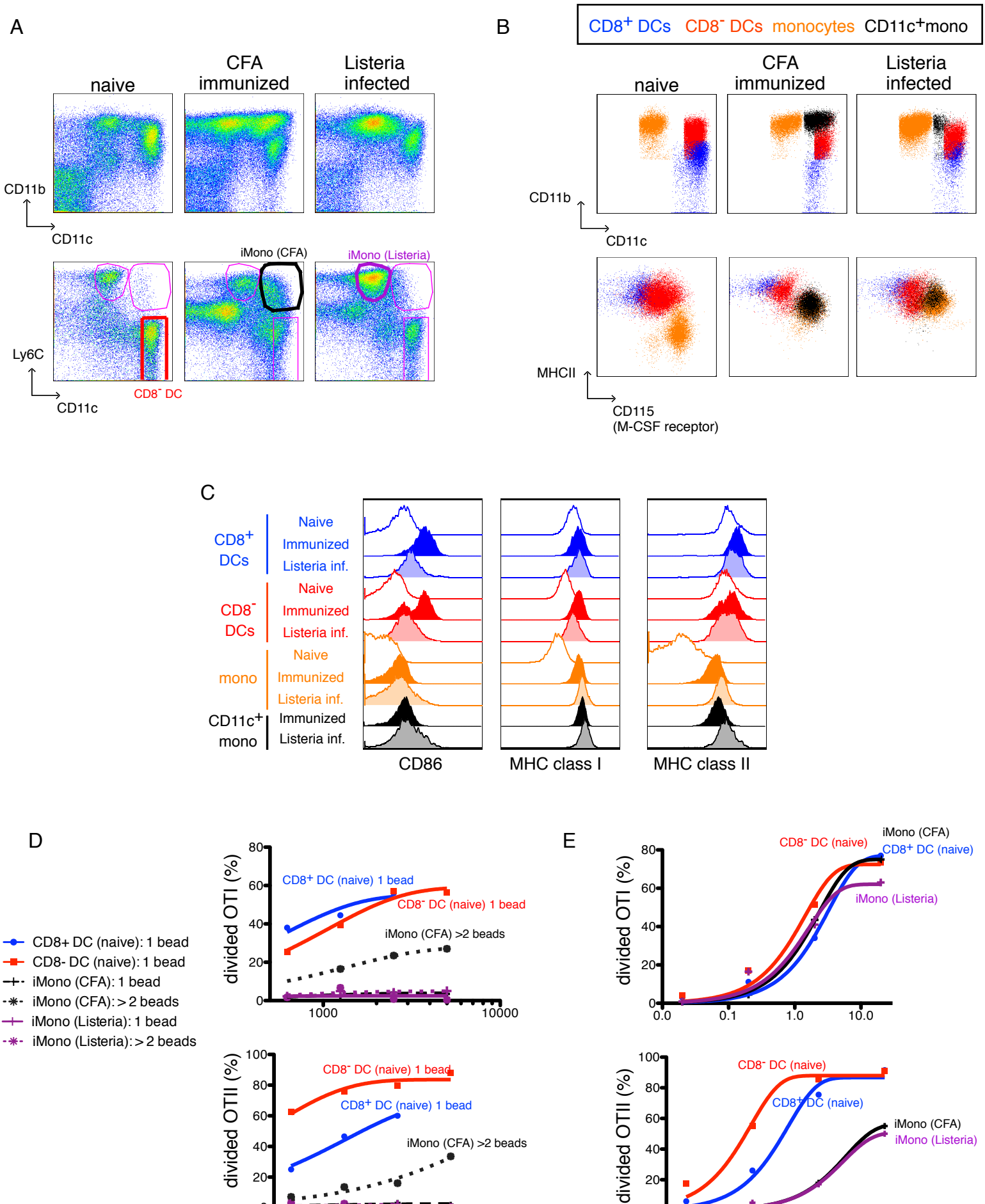

APCs with internalized bead (index > 0)
CD8+ DCs
91.1
Inside
CD8- DCs
68.7
Inside
iMono
75
Inside
GM-DCs
90.5
Inside
LPS-GM-DCs
70.4
Inside
frequency
Internalization index
APCs with negative internalization index (-3)

Fig. S7


A
naive
CFA immunized
Listeria infected
CD11b
CD11c
iMono (CFA)
iMono (Listeria)
Ly6C
CD11c
CD8⁻ DC
B
CD8⁺ DCs
CD8⁻ DCs
monocytes
CD11c⁺mono
naive
CFA immunized
Listeria infected
CD11b
CD11c
MHCII
CD115 (M-CSF receptor)
C
CD8⁺ DCs
Naive
Immunized
Listeria inf.
CD8⁻ DCs
Naive
Immunized
Listeria inf.
mono
Naive
Immunized
Listeria inf.
CD11c⁺ mono
Immunized
Listeria inf.
CD86
MHC class I
MHC class II
D
CD8+ DC (naive): 1 bead
CD8- DC (naive): 1 bead
iMono (CFA): 1 bead
iMono (CFA): > 2 beads
iMono (Listeria): 1 bead
iMono (Listeria): > 2 beads
divided OTI (%)
CD8⁺ DC (naive) 1 bead
CD8⁻ DC (naive) 1 bead
iMono (CFA) >2 beads
divided OTII (%)
CD8⁻ DC (naive) 1 bead
CD8⁺ DC (naive) 1 bead
iMono (CFA) >2 beads
APCs/ well
E
divided OTI (%)
CD8⁻ DC (naive)
iMono (CFA)
CD8⁺ DC (naive)
iMono (Listeria)
divided OTII (%)
CD8⁻ DC (naive)
CD8⁺ DC (naive)
iMono (CFA)
iMono (Listeria)
peptide (μM)