Supplementary Materials

SUPPLEMENTARY METHODS

Plasmid DNA constructs and adenoviral vectors

The MyD88 amino terminus consisting of the death and intermediate domains was amplified with Platinum® *Pfx* DNA polymerase (Invitrogen) using a *Sal* I flanked 5' primer (5MyD88LS), 5'-ACATCAAGTCGACATGGCTGCAGGAGG-3' and *Sal* I flanked 3' primer (3MyD88LS), 5'-ACTCATAGTCGACCAGGGACAAGG-3' from a human dendritic cell (DC) cDNA library. The PCR fragment was subcloned into a pCR4Blunt-TOPO vector (Invitrogen) and sequenced at Lone Star Labs Inc. Upon sequence verification, the 521-bp fragment was excised by *Sal* I digestion, and subcloned into a myristoylated pBJ5-M-F_v'F_{vls}-E backbone digested with *Xho* I or *Sal* I to generate pBJ5-M-MyD88-F_v'F_{vls}-E and pBJ5-M-F_v'F_{vls}-MyD88-E respectively. To generate the inducible MyD88.CD40 composite vector, the MyD88 fragment was excised as described above and subcloned into *Xho* I or *Sal* I cut pBJ5-M-CD40-F_v'F_{vls}-E and pBJ5-M-F_v'F_{vls}-CD40-E. This subcloning generated pBJ5-M-MyD88-CD40-F_v'F_{vls}-E, pBJ5-M-CD40-F_v'F_{vls}-MyD88-E, pBJ5-M-MyD88-F_v'F_{vls}-CD40-E and pBJ5-M-F_v'F_{vls}-CD40-MyD88-E. Conversely, the human CD40 cytoplasmic domain was obtained by *Xho* I and *Sal* I digestion of pCR4Blunt-TOPO-hCD40. The CD40 fragment was subcloned into *Sal* I cut pBJ5-M-F_v'F_{vls}-MyD88-E to yield pBJ5-M-CD40-MyD88-F_v'F_{vls}-E and pBJ5-M-F_v'F_{vls}-MyD88-CD40-E.

Thus, several combinations of MyD88 and CD40 signaling domains were generated in different orientations up or downstream of the FKBP dimerizer drug binding domains and tested for functionality in NF-κB SEAP reporter assays. The constitutive CD4/TLR4 construct was a kind gift from Dr. Sankar Ghosh, Yale University, New Haven, Connecticut.

To generate Ad5-based adenoviral vectors expressing iMyD88, iMyD88/CD40 and cTLR4, recombinant plasmids encoding the chimeric constructs were digested with *Xho* I and *Sal* I to generate inserts that were then subcloned into a similarly digested pShuttle vector. The pShuttle gene cassettes were used in an Adeno-X expression system deleted for E1, E3 and (BD Biosciences) as previously described {Hanks *et al*, Nature Medicine, 2005} to generate replication incompetent adenoviruses. Generation, purification, large-scale expansion and titration of recombinant adenoviruses serotype 5 (Ad5), expressing iMyD88/CD40, iMyD88 and iCD40, were performed in the Vector Development Core (Baylor College of Medicine). The expression of iMyD88/CD40 (iMC) and iMyD88 (iM) in mouse DCs was determined by western blot and flow cytometry using anti-HA-tag monoclonal antibodies (mAb). Ad5- encoding luciferase was from BCM's Vector Development Laboratory.

NF-κB SEAP reporter assay

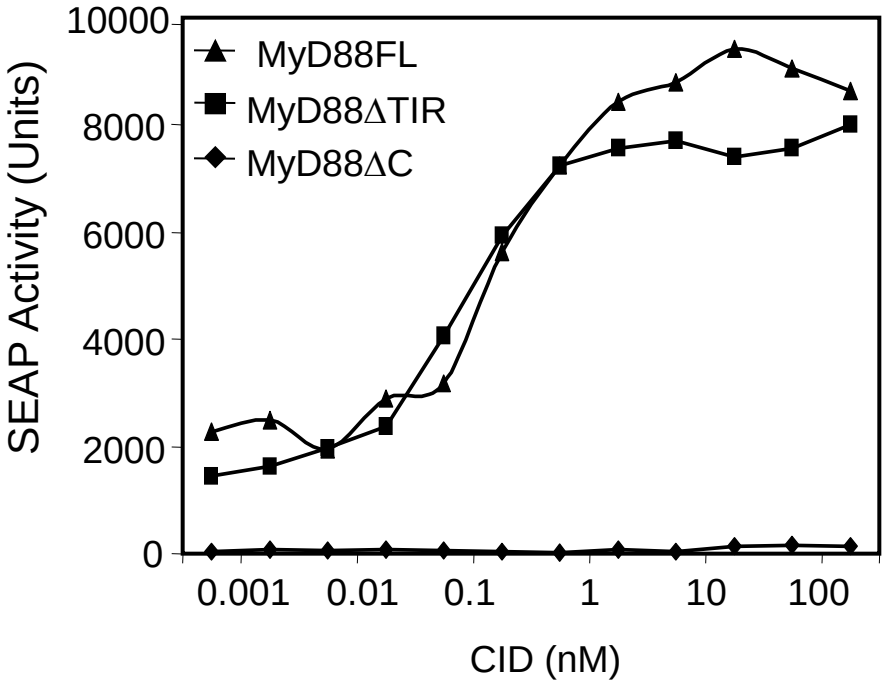
HEK 293 cells were transfected with 0.3-1μg of indicated recombinant plasmids and 1μg of NF-κB SEAP reporter using FuGENE-6 transfection reagent (Roche). The next day, cells were harvested and plated in the presence of increasing amounts of CID (AP20187/AP1903). 24 hours later, cell supernatants were harvested and SEAP activity was analyzed as previously described {Hanks *et al*, Nature Medicine, 2005}.

Flow Cytometry

For analysis by flow cytometry, cells were harvested by flushing with cold PBS. BMDCs were incubated with F_c blocking reagent (anti-mouse CD16/CD32 monoclonal antibody; BD Pharmingen) to prevent non-specific antibody binding. Cells were stained with FITC-labeled anti-mouse CD11c, PE-labeled anti-mouse MHC class II I-A^b, biotin conjugated anti-mouse CD40, CD80, CD86 and PE-conjugated Streptavidin (BD Pharmingen). CCR7 expression on BMDCs was tested using PE-conjugated anti-mouse CD197 monoclonal antibody (eBioscience). Human monocyte-derived DCs were stained with FITC/APC-conjugated anti-human CD40, FITC-conjugated anti-human CD86, FITC-conjugated anti-HLA-DR, APC-conjugated CD83 and PerCP.Cy5.5-conjugated CD80 (BD Biosciences, San Jose, CA).

Supplementary Figure 1. (Spencer)

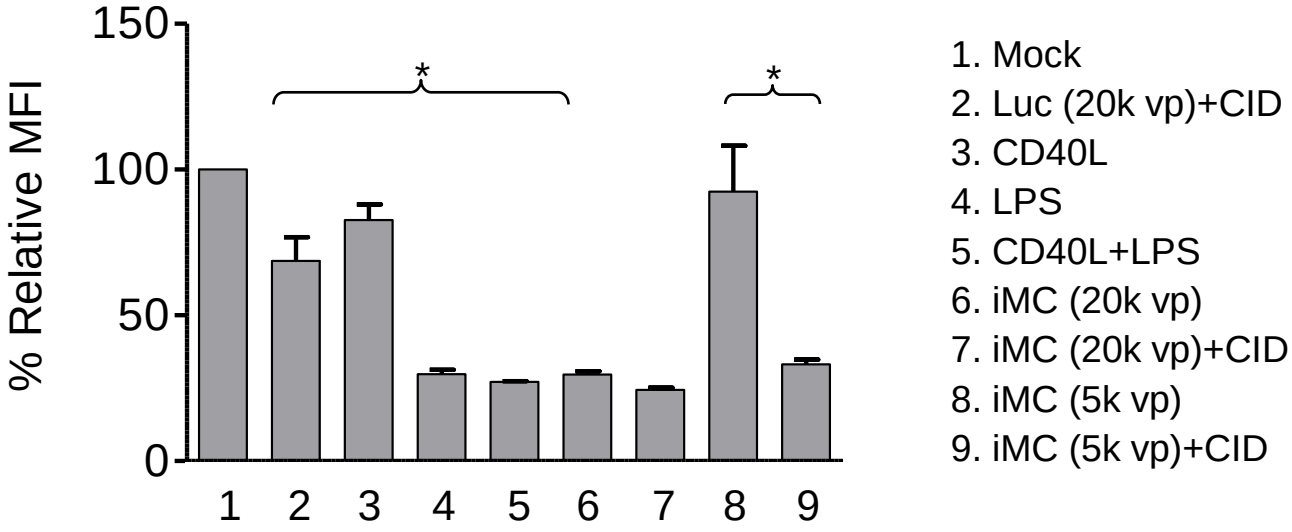
The intermediate domain of MyD88 is required for CID-dependent, iMyD88-mediated NF-κB activation.



293 cells were transiently transfected with constructs expressing full length iMyD88 (MyD88FL), iMyD88 lacking the C-terminal TIR domain (MyD88ΔTIR) or iMyD88 lacking the C-terminal TIR and intermediate domains (MyD88ΔC) along with an NF-κB-responsive SEAP reporter plasmid. 24 hours following transfection, cells were treated with CID overnight and cell supernatants were analyzed for SEAP activity.

Supplementary Figure 2. (Spencer)

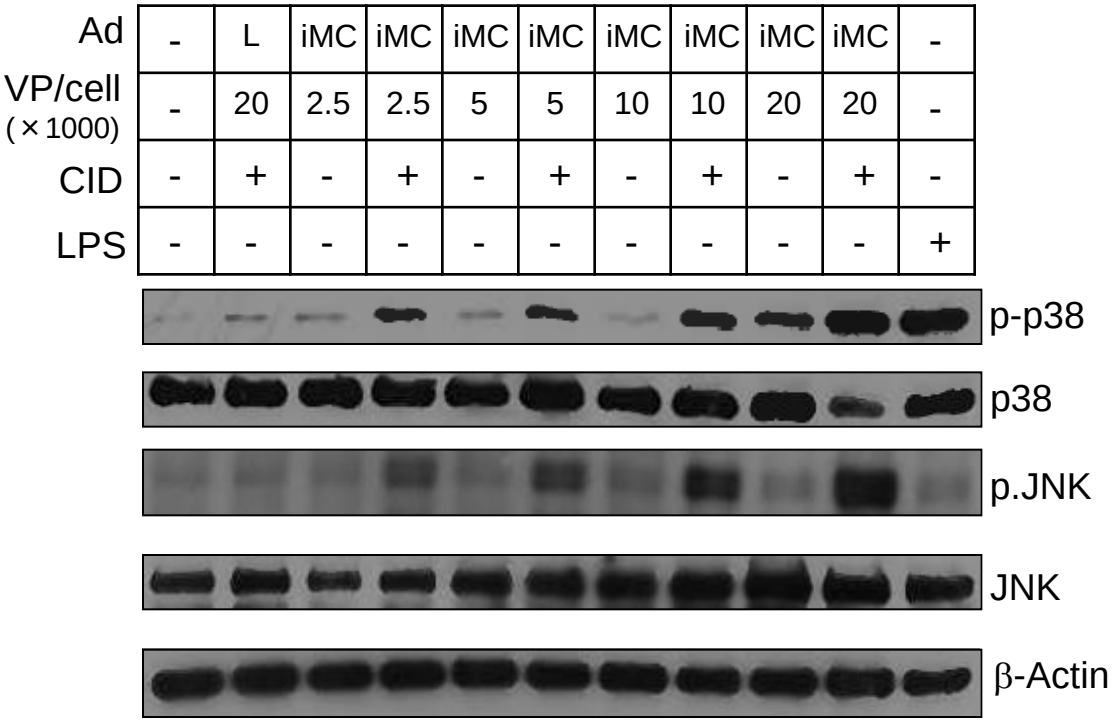
iMyD88/CD40 downregulates phagocytic capacity of BMDCs in a CID-dependent manner.



BMDCs were Ad-treated as described in **Fig. 2b** and activated with 100 nM CID or LPS (250 ng/ml) and CD40L (2 μg/ml). 24 hours later, cells were incubated with FITC-dextran at 37° C, for one hour. FITC-dextran uptake by $CD11c^+$ cells was analyzed by flow cytometry and data was normalized to mock treatment. Uptake at 0° C was negligible and served as the negative control. Data are mean ± SEM, $*P < 0.05$ (two-tailed), representative of at least two independent experiments

Supplementary Figure 3. (Spencer)

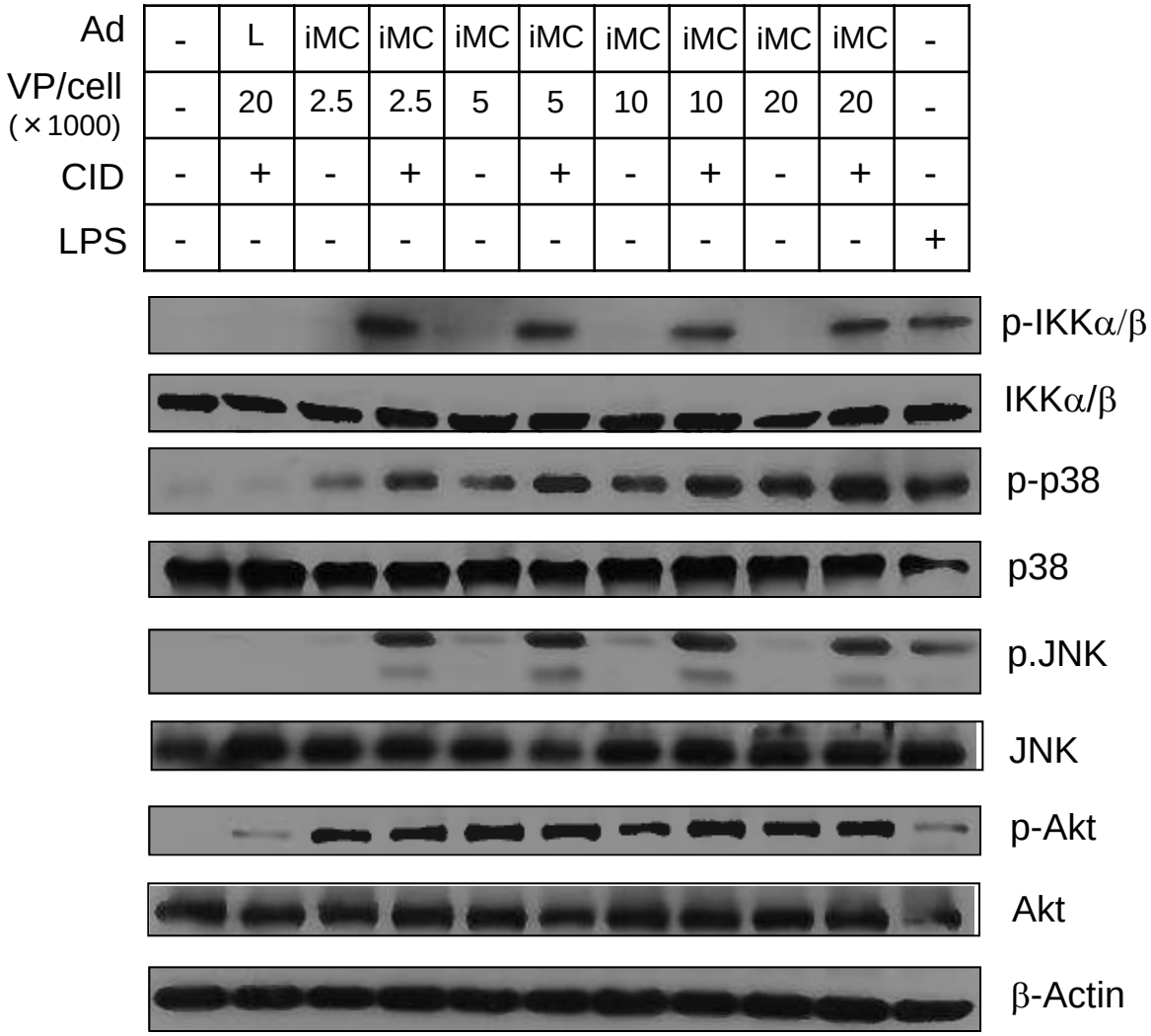
Differential CID-dependent activation of downstream signaling molecules by iMyD88/CD40 in BMDCs.



BMDCs were transduced with increasing amounts of Ad-iMyD88/CD40 (2500, 5000, 10,000 and 20,000 vp/cell). 20,000 vp/cell of Ad-Luciferase served as a control. 48 hours following virus treatment, cells were stimulated with CID (100 nM) or LPS (10 ng/ml) for 30 minutes, where indicated. Cell lysates were harvested and analyzed using antibodies as described in **Fig. 2c**.

Supplementary Figure 4. (Spencer)

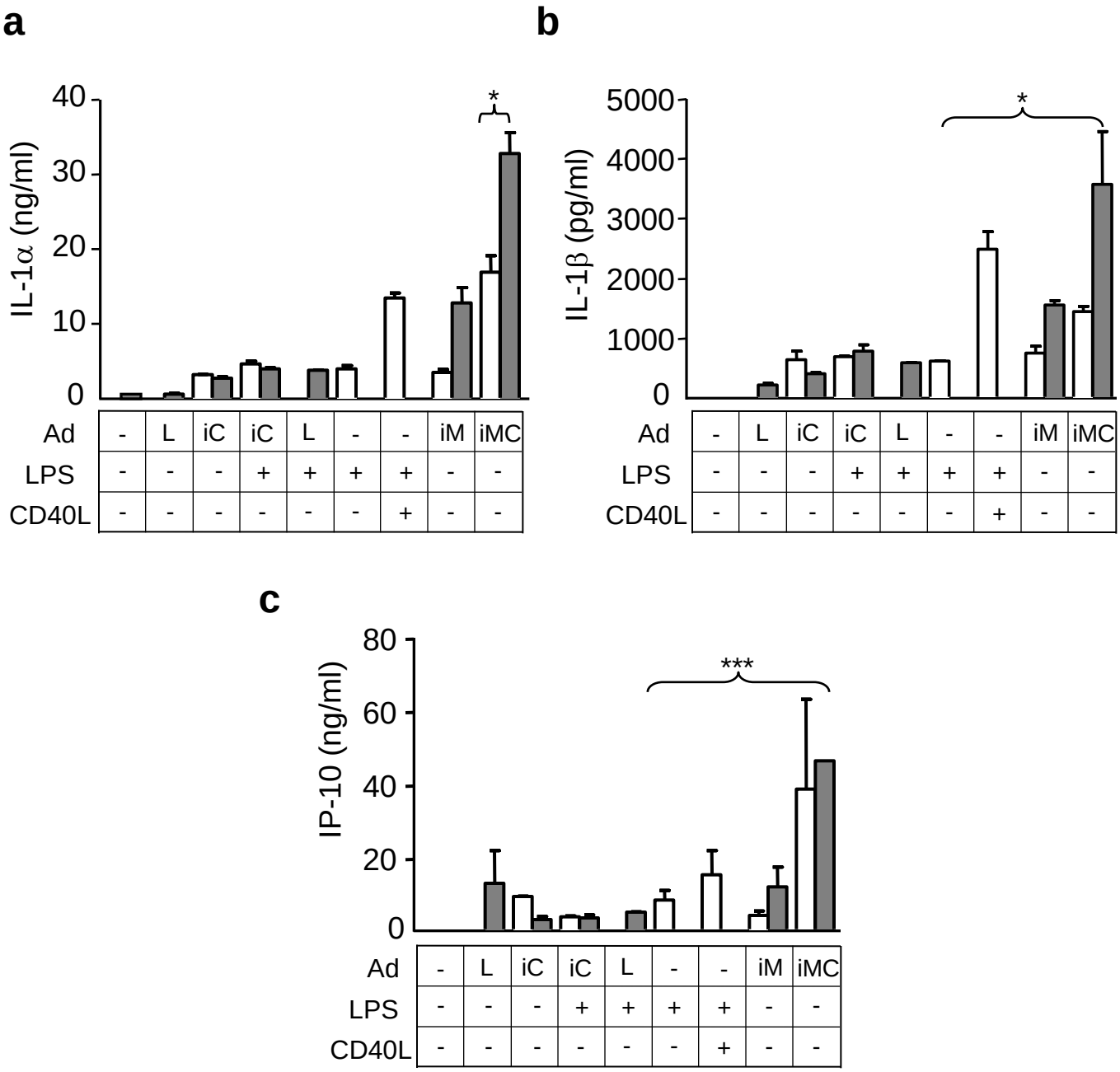
Differential CID-dependent activation of downstream signaling molecules by iMyD88/CD40 in THP-1 cells.



THP-1 cells were transduced with increasing amounts of Ad-iMyD88/CD40 (2500, 5000, 10,000 and 20,000 vp/cell). 20,000 vp/cell of Ad-Luciferase served as control. 48 hours following virus treatment, cells were stimulated with CID (100 nM) or LPS (10 ng/ml) for 30 minutes, as indicated. Cell lysates were harvested and analyzed using antibodies as described in **Fig. 2c**.

Supplementary Figure 5. (Spencer)

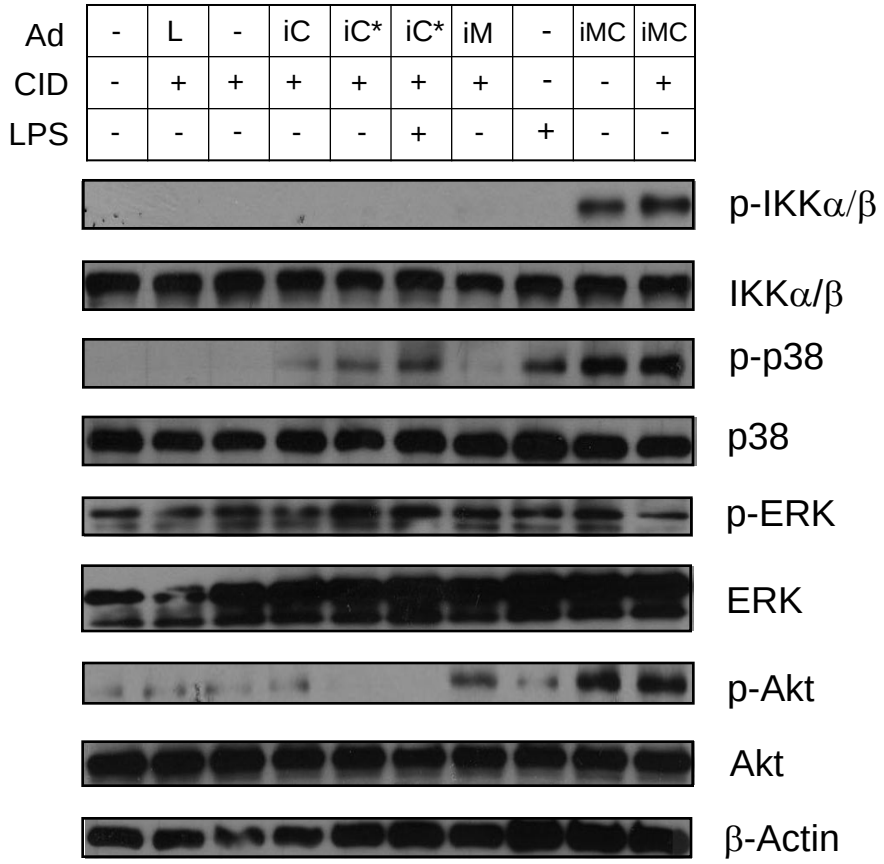
iMyD88/CD40 induces pro-inflammatory cytokines, IL-1α/β and chemokine, IP-10, by BMDCs upon CID treatment.



BMDCs were treated as described in **Fig. 2b**. Supernatants were harvested and analyzed in duplicate in a LINCOPLEX assay, **P* < 0.05 (two-tailed for IL-1α, one-tailed for IL-1β), ****P* < 0.0005 (two-tailed), representative of three independent experiments.

Supplementary Figure 6. (Spencer)

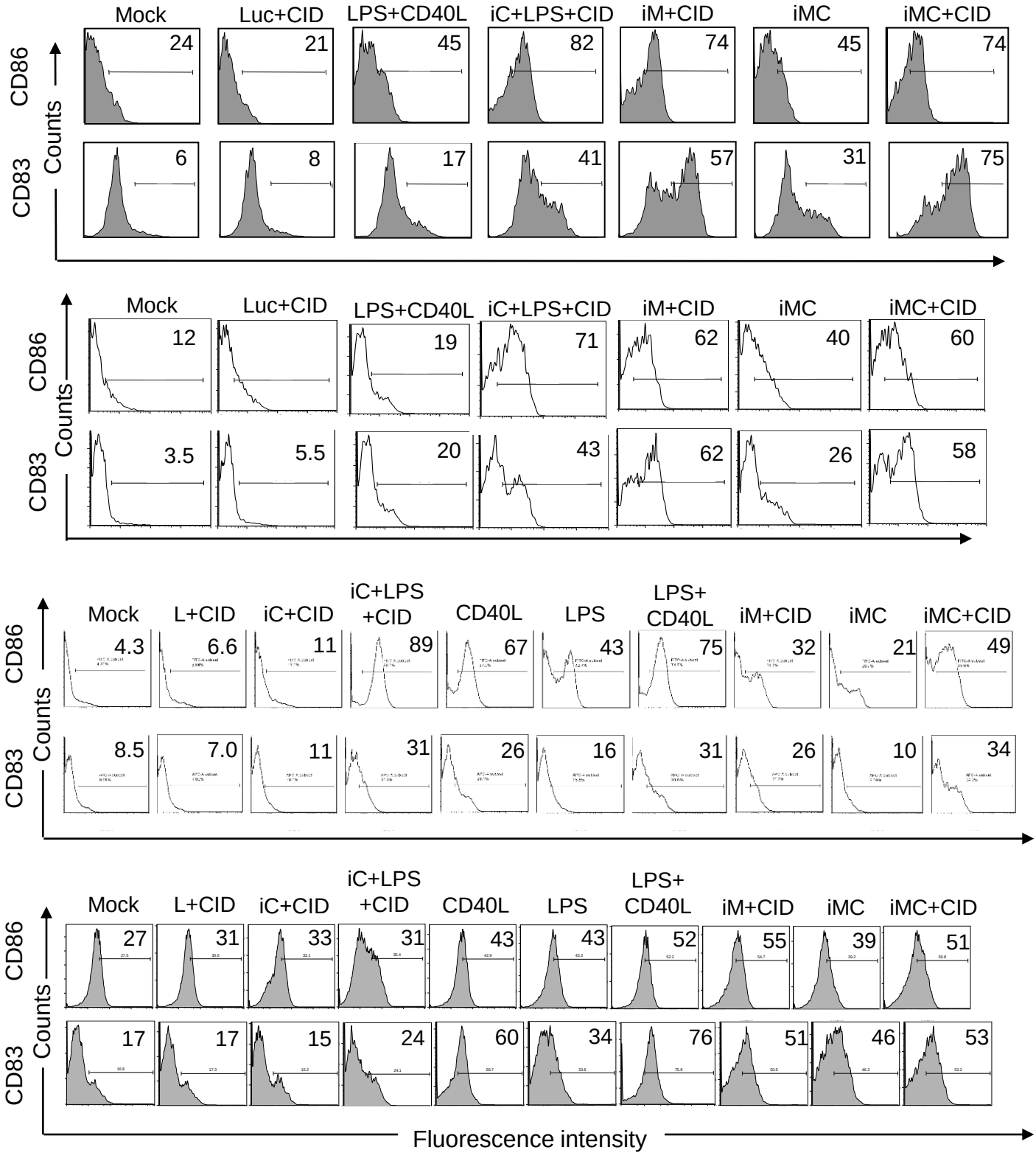
iMyD88/CD40 activates pro-T_H1 signaling molecules in a CID-dependent manner in MoDCs.



MoDCs were transduced with 100 MOI of various adenoviruses listed in **Fig. 2d**. 48 hours following virus treatment, cells were stimulated with CID (100 nM) or LPS (10 ng/ml) where indicated for 30 minutes. Cell lysates were harvested and analyzed using antibodies as described in **Fig. 2c**.

Supplementary Figure 7. (Spencer)

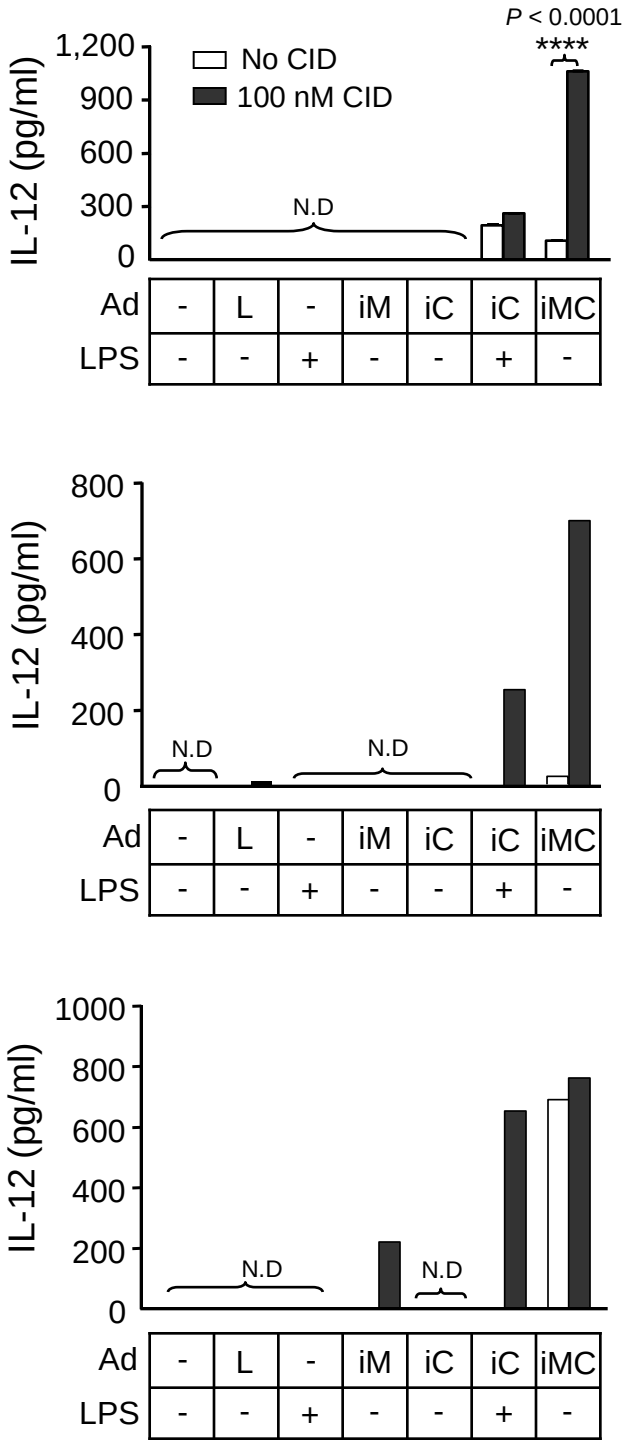
iMyD88/CD40 upregulates costimulatory molecules on the surface of human MoDCs.



MoDCs transduced with Ad (10,000 vp/cell) and treated with CID or enhanced CD40L (1 µg/ml) + LPS (1 µg/ml) for 48 hrs, were analyzed by flow cytomtery for CD83 and CD86 expression. Representative data from four different donors

Supplementary Figure 8. (Spencer)

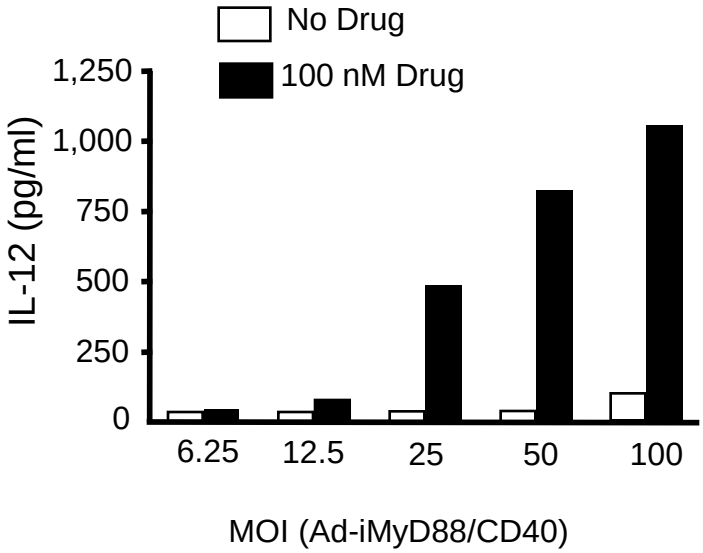
iMyD88/CD40 induces bioactive IL-12p70 production by human MoDCs.



MoDCs were Ad-transduced and activated with CID, LPS, and/or CD40L. 48 hrs later, supernatants were analyzed in duplicate by ELISA. Results from three representative donors shown.

Supplementary Figure 9. (Spencer)

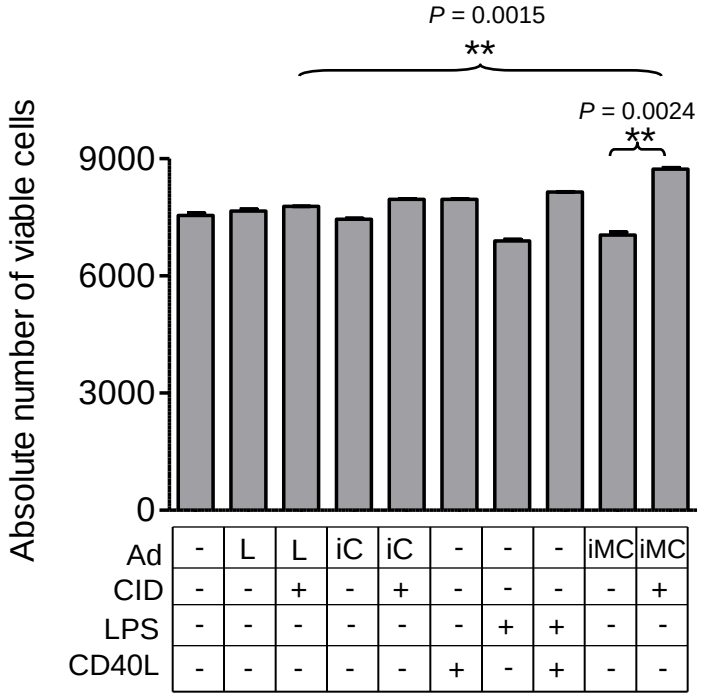
The magnitude of CID-dependent IL-12 production escalates with increasing iMyD88/CD40 transgene expression.



Immature human MoDCs were transduced with increasing MOI of Ad-iMyD88/CD40 and stimulated with 100 nM CID for 48 hrs. Supernatants were assayed in duplicate using ELISA. Data representative of three different donors.

Supplementary Figure 10. (Spencer)

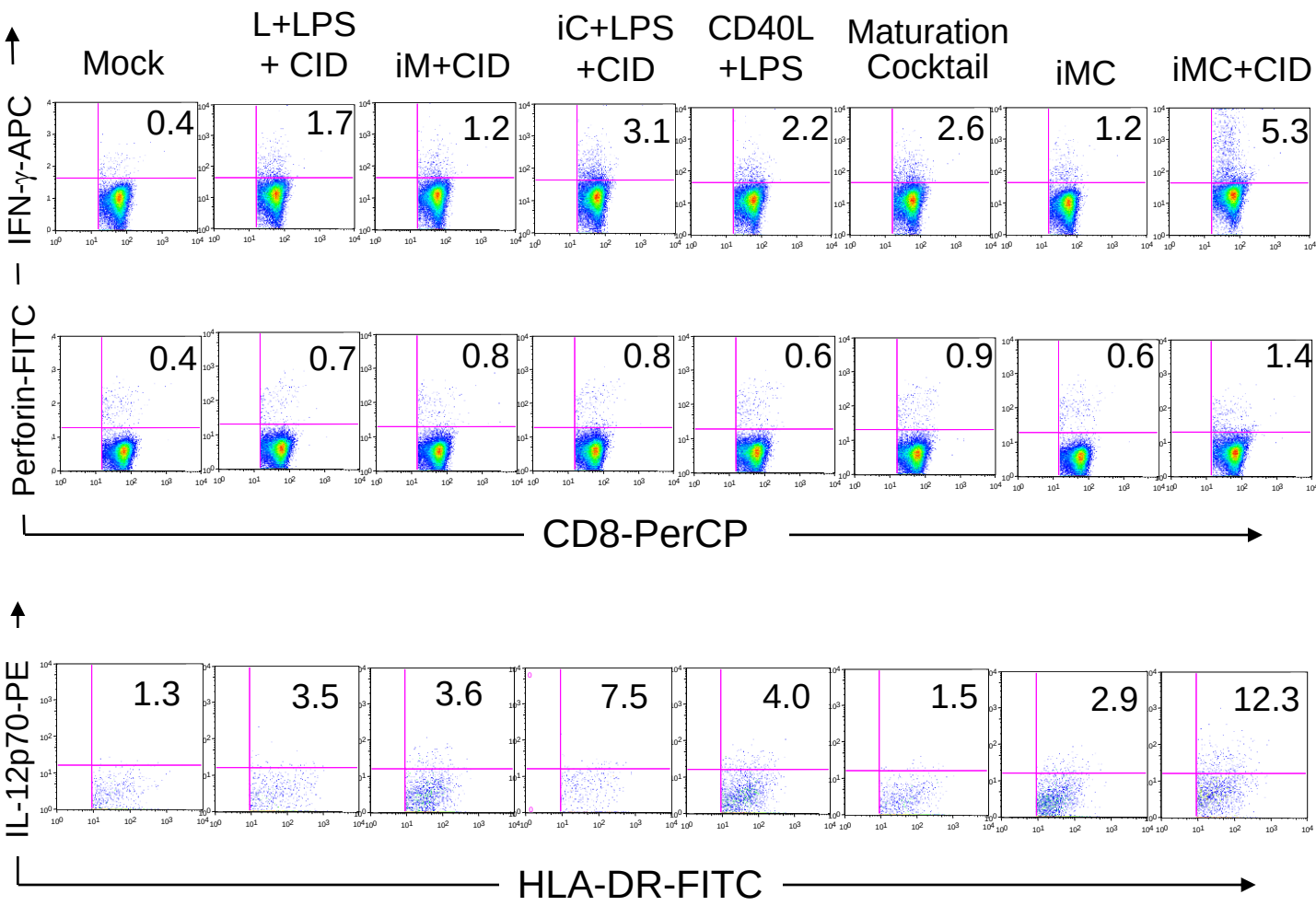
iMyD88/CD40 improves the *in vitro* survival of human MoDCs in a CID-dependent manner.



MoDCs were transduced with Ad as described in **Fig 3**, activated for one day, washed and plated in serum-free human DC media. Data indicate absolute number of viable cells on Day 9 as analyzed in duplicate by flow cytometry. Data are mean ± SEM, *P* values (two-tailed), representative results out of three (different donors) shown.

Supplementary Figure 11. (Spencer)

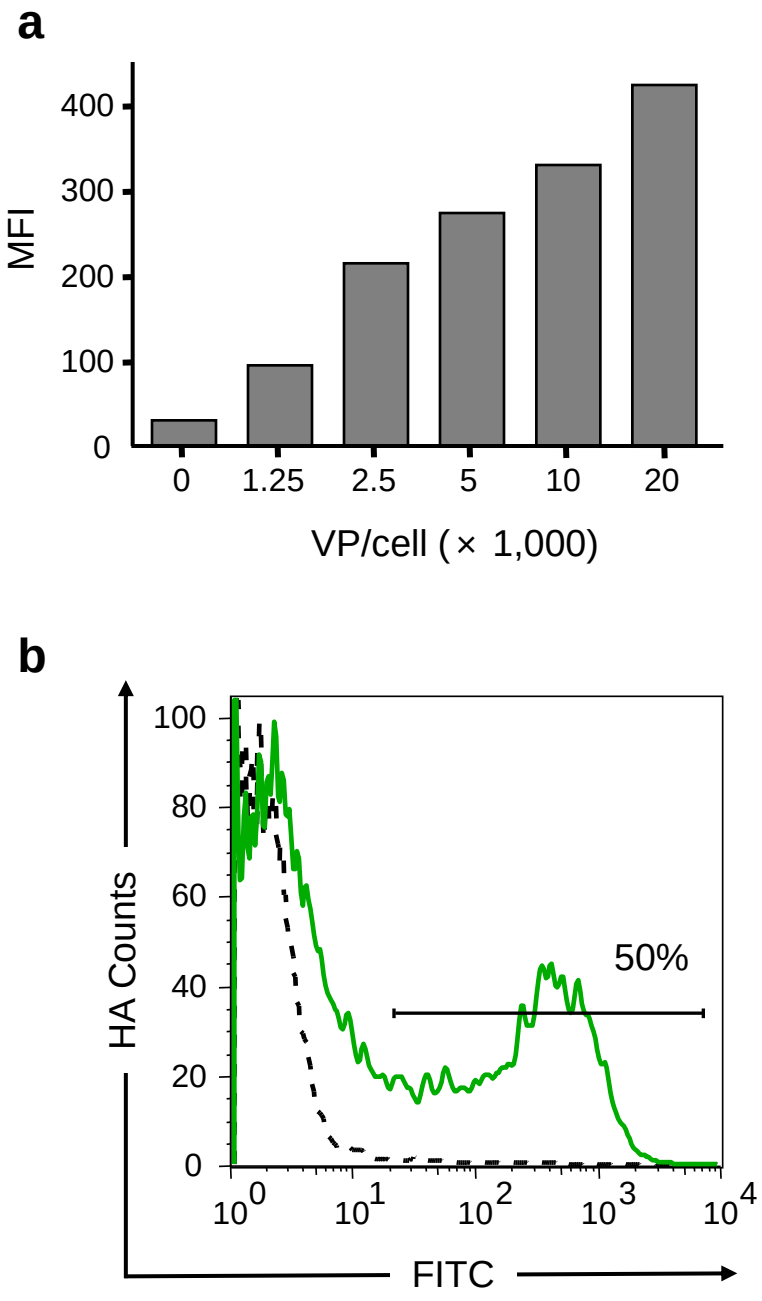
iMyD88/CD40-DCs express high levels of intracellular IL-12p70 and induce a higher frequency of antigen-specific IFN-γ/Perforin-expressing CD8⁺ T cells, following CID addition.



MoDCs derived from HLA-A2⁺ donors were pulsed with HLA-A2-restricted MAGE-$3_{271–279}$ peptide, transduced with Ad (10,000 vp/cell) and/or activated with 100 nM CID, 250 ng/ml LPS and 1 μg/ml enhanced human CD40L or maturation cocktail (IL-6, IL-1β, TNF-α and PGE_2). Autologous CD8⁺ T cells were purified by negative selection (Miltenyi Biotec) and co-cultured with MoDCs at 3:1 (T:DC) ratio. On day 10, cells were restimulated with MAGE-3 peptide in the presence of GolgiStop (BD Bioscience). 3 hours later, cells were fixed and permeabilized, and intracellular proteins were stained with anti-human IFN-γ, perforin and IL-12p70 mAbs. Percentage of IFN-γ or perforin-expressing CD8⁺ T cells is depicted in the plots of the top two rows. Percentage of IL-12p70-expressing HLA-DR⁺ MoDCs is depicted in the bottom row. Similar results were obtained for two different donors.

Supplementary Figure 12. (Spencer)

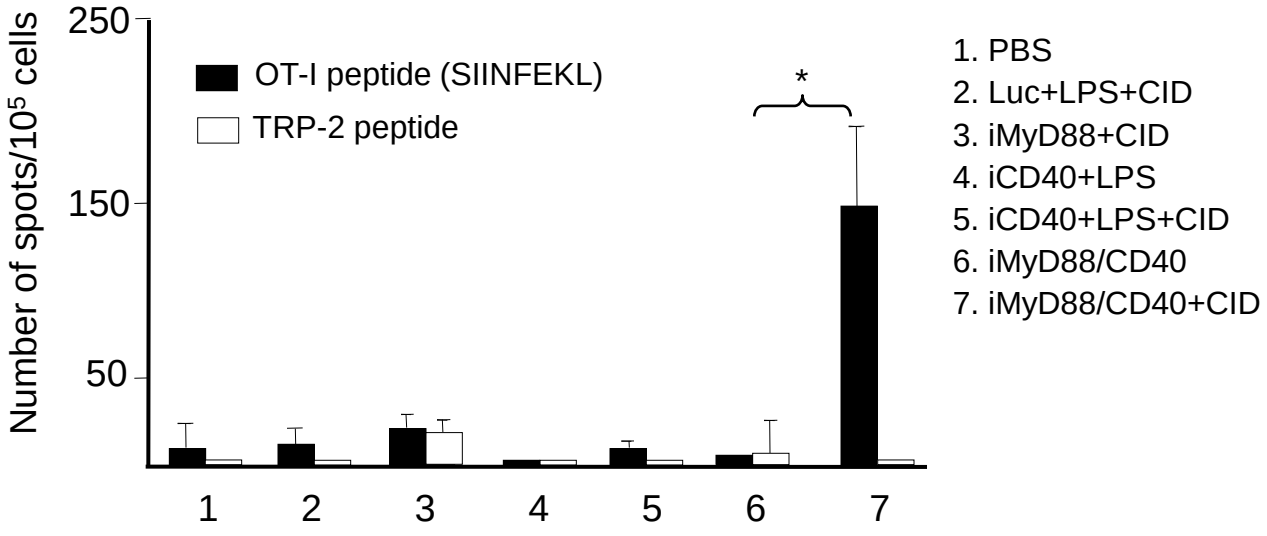
iMyD88/CD40 transgene expression in BMDCs.



(a) 1x10^6 BMDCs were transduced with 1,250, 2,500, 5,000, 10,000 or 20,000 VP/cell of Ad5-iMyD88/CD40 using 5 μl of Gene Jammer transfection reagent (Agilent Technologies). The intracellular expression of HA-tagged transgene was analyzed by flow cytometry with anti-HA-FITC mAb. **(b)** Histogram of percent HA$^+$ cells (green line represents HA$^+$). Dashed line represents untransduced cells.

Supplementary Figure 13. (Spencer)

iMyD88/CD40-DCs induce a higher frequency of antigen-specific IFN-γ-producing $CD8^+$ T cells.



Splenocytes from E.G7-OVA tumor-bearing mice, vaccinated with BMDCs as described in **Fig. 5c.** were tested by IFN-γ ELISpot assay. Cells were stimulated with 1 μg/ml of SIINFEKL peptide (OT-I) and TRP-2 (irrelevant H2-K^b-restricted) peptide. The number of IFN-γ-producing lymphocytes was evaluated in triplicate wells, *P < 0.05 (two-tailed). Assays were performed twice.