Supplemental Experimental Procedures

Flow cytometry and cell sorting. For the purification of DCs, single cell suspensions were incubated with biotin conjugated anti-CD11c antibody (Biolegend) followed by streptavidin conjugated MicroBeads (Miltenyi), and then positively selected on a magnetic field according to the manufacturer's instruction (Miltenyi). For the purification of DCs from tolerized animals (Figure 1and Supplemental Figure 1), positively selected DCs (described above) were stained with monoclonal antibodies against CD11c, CD11b, and Gr-1. $CD11c^{+}CD11b^{+}Gr\text{-}1^{+}$ cells were sorted on a high-speed MoFlo (Dako). Cell purity was about 85–95%.

For isolation of $Foxp3^{+}$ Tregs, spleen and LNs isolated from *Foxp3gfp*.KI mice, were enriched for $CD4^{+}$ T cells (Miltenyi microbeads). $CD4^{+}$ $Foxp3^{+}$ or $Foxp3^{-}$ cells were sorted on a FACS ARIA III (BD Biosciences). Cell purity was above 95%. For isolation of *Il-10*$^{-/-}$ Tregs, splenocytes and LNs were enriched for $CD4^{+}$ T cells (Miltenyi microbeads). $CD4^{+}CD25^{high}$ cells were sorted on a FACS ARIA III (BD Biosciences). Cell purity was above 95%. Foxp3 was expressed on >95% of $CD4^{+}CD25^{high}$ cells as verified by intracellular staining of Foxp3. For isolation of CTLA4 iKO Tregs, splenocytes and LNs were enriched for $CD4^{+}$ T cells (Miltenyi microbeads). $CD4^{+}CD25^{high}GITR^{+}$ cells were sorted on a FACS ARIA III (BD Biosciences). Cell purity was above 95%. Foxp3 was expressed on >95% of $CD4^{+}CD25^{high}GITR^{+}$ cells as verified by intracellular staining of Foxp3.

For isolation of OVA specific T cells, spleen and LNs isolated from OTII TCR transgenic mice were stained with antibodies against CD4, Va2 and CD25 and sorted on a FACS ARIA III (BD Biosciences). $CD4^{+}Va2^{+}CD25^{-}$ were isolated as Tn and $CD4^{+}Va2^{+}CD25^{high}$ as Tregs. Cell purity was above 95%.

For isolation of MOG_{35-55}-specific T cells spleen and LNs isolated from 2D2 TCR transgenic mice were stained with antibodies and $7AAD^{-}CD4^{+}V\alpha3.2^{+}V\beta11^{+}$ were sorted on a FACS ARIA III (BD Biosciences).

For isolation of human DCs peripheral blood mononuclear cells (PBMCs) were isolated as described below and cells were sorted as $CD14^{-}CD11c^{+}$HLA-DR^{+} on a FACS ARIA III (BD Biosciences).

For phospho protein staining, cells were permeabilized and stained using the Intracellular Fixation and Permeabilization Buffer Set kit, according to manufacturer instructions (eBioscience).

Histological analysis and Immunofluorescence. For autophagy immunofluorescence, sorted DCs were seeded in coverslips pretreated with poly lysine, fixed with 4% PFA for 15 min in room temperature followed by 10 min of fixation with ice cold methanol in -20o C, washed twice with PBS and ice cold methanol. Cells were permeabilized by using 0.1% saponin (Sigma-Aldrich) and stained with mouse anti-LC3 antibody (1:20, 5F10 nanoTools), rat anti-Lamp-1 (1:400, 1D4B Santa Cruz Biotechnology), rabbit anti-p62 (1:500, MBL), followed by incubation with Alexa fluor® 555 anti-mouse IgG (1:500, Molecular Probes), Alexa fluor® 647 anti-rabbit IgG (1:200, Molecular Probes), Alexa fluor® 488 anti-rat IgG (1:250, Molecular Probes). For visualization of the nuclei DAPI (Sigma-Aldrich) was used. Samples were coverslipped with moviol and visualized using inverted confocal live cell imaging system Leica SP5.

Puncta of LC3/cell and puncta of p62/cell were calculated using a macro developed in Fiji software.

For visualization of phagocytosis of antigen loaded-beads, Polybeads® carboxylated beads (3 μm, Polysciences Inc.) were incubated with LPS (0.5 μg/ml) and OVA-

Alexa fluor® 488 (50 μg/ml, Molecular Probes) for 2 h in 37 ^{o}C. Beads were thoroughly washed to remove excess of antigen and then incubated with MACS purified CD11c^{+} cells at 10:1 ratio for 20 min in 37 ^{o}C. Cells were then fixed and protocol for LC3 immunofluorescence staining was performed as described above. Co-localization of OVA-Alexa fluor® 488 and LC3 was calculated using cross-correlation analysis with volocity software (1)

For FoxO1 staining DCs were seeded in coverslips as described above and stained with FoxO1 rabbit monoclonal ab (C29H4) (1:100, Cell Signaling) followed by Alexa fluor® 555 anti-rabbit IgG (1:400 Molecular Probes). For visualizing nuclei Dapi (Sigma-Aldrich) was used. Relative FoxO1 intensity was calculated using Fiji software gating on nuclei.

For visualization of pULK-1, DCs were seeded in coverslips as described above and stained with pULK-1 rabbit monoclonal ab (DH14) (1:100, Cell Signaling) followed by Alexa fluor® 647 anti-rabbit IgG (1:200 Molecular Probes) and dapi. Puncta of pULK-1/cell was calculated using a macro developed in Fiji software.

Quantitative PCR analysis. Specific primers were as follows: mouse: *Hprt* 5'-GTGAAACTGGAAAAGCCAAA-3' (forward), *Hprt* 5'-GGACGCAGCAACTGACAT-3' (reverse); *Atg5* 5'-AGCTCTGGATGGGACTG-3' (forward), *Atg5* 5'-CTCCGTCGTGGTCTGAT-3' (reverse);

Atg16L1 5’-CCCGTGATGACCTGCTAAA-3’ (forward), *Atg16L1* 5’-CCATCAGGGCTGAAGACAAC-3’ (reverse) *Becn1* 5’-GGACAAGCTCAAGAAAAACCAATG-3’ (forward), *Becn1* 5’-TGTCCGCTGTGCCAGATGT-3’ (reverse), *Lc3b* 5’-CCCACCAAGATCCCAGTAG-3’ (forward), *Lc3b* 5’-CCAGGAACTTGGTCTTGTCCA-3’ (reverse), human GAPDH 5’-GCAAATTCCATGGCACCGT-3’ (forward), GAPDH 5’-TCGCCCCACTTGATTTTGG-3’ (reverse), *Lc3b* 5’-CTGTTGGTGAACGGACACAG-3’ (forward), *Lc3b* 5’-CTGGGAGGCATAGACCATGT-3’ (reverse).

Autophagic flux. BMDCs were treated with 0.5 μg/ml LPS and 100 μg/ml CTLA4-Ig (Abatacept-Orencia, Bristol-Myers Squibb) with 50μM chloroquine (Sigma Aldrich) or 100nM Bafilomycin A1 (enzo lifesciences) for 4hrs.

Western Blot analysis. Whole-cell lysates (40 μg protein) were subjected to SDS-PAGE electrophoresis on 12% gels and then transferred to Immobilon-Psq membrane (Millipore). Membranes were blocked with 5% skimmed milk, 1% BSA in TBST and then incubated with anti-LC3 Ab (1:1000, Novus Biologicals), anti-p62 (1:6000, MBL), anti-phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) (1:1000, Cell Signaling), anti-phospho-mTOR (Ser2448) (1:1000, Cell Signaling), anti-PI3 Kinase p85α (Z-8) (1:200, Santa Cruz Biotechnology), anti-mTOR (H-266) (1:200, Santa Cruz Biotechnology) anti-phospho-ULK-1 (ser555) (D1H4) (1:1000, Cell Signaling), anti-ULK-1 (D8H5) (1:1000, Cell Signaling), anti-FoxO1 (C29H4) (1:1000, Cell Signaling), and anti-actin Ab (1:5000, Millipore) as a loading control. Detection was performed using HRP-linked antibodies (Cell signaling Technology Inc.) and ECL detection reagents (Amersham). As control sample, protein extract from Neuro 2A

cell line was used. For obtaining Cytoplasmic/ Nuclear Extracts, cells were washed twice with ice cold PBS and the cell pellet was re-suspended in 200 µl of CE Buffer (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.3% NP-40, protease inhibitors). Cell suspension was incubated for 10 min on ice by vortexing periodically. Centrifugation at 3000 rpms for 5 min was followed by transferring the supernatant (cytoplasmic protein extracts) into new tubes. The pellets were resuspended in 100 µl CE Buffer without NP-40 and washed once. After centrifugation, supernatants were discarded and nuclear pellets were resuspended in equal volume of NE Buffer (20 mM Hepes pH7.9, 0.4 M NaCl, 1 mM EDTA, 25% Glycerol, protease inhibitors), followed by incubation for 10 min at 4oC, agitating periodically. Supernatants were collected after centrifugation at 14000 rpm at 4oC for 10 min and kept at -80oC as nuclear protein extracts. Relative intensity of bands was calculated using the Quantity One® analysis software (Bio-rad).

Chromatin Immunoprecipitation assay. $15x10^6$ cells were cross-linked with 1% (vol/vol) formaldehyde (followed by extensive wash with PBS) and lysed with 120 µl lysis buffer (1% (wt/vol) SDS, 10 mM EDTA and 50 mM Tris-HCl, pH 8.1, 1 × protease inhibitor 'cocktail' (Roche), 1 mM PMSF). Chromatin was sheared by Covaris Sonicator System to 200-400 bp fragments. Supernatants were collected after centrifugation and diluted at least 5 volumes in Dilution Buffer (1% (vol/vol) Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris-HCl, pH 8.1). 5 µg anti-FoxO1A antibody (ab39670, abcam) or normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) were incubated with diluted chromatin overnight. After preclearing. Immunoprecipitation continued by incubation with protein G Dynal magnetic beads (Invitrogen) for at least 3 hours at 4^0C. Magnetic bead–immunoprecipitated chromatin complexes were then washed twice with Low Salt Wash Buffer (0.1% SDS, 1%

Triton X-100, 20 mM Tris pH8, 2 mM EDTA, 150 mM NaCl), twice with High Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 20 mM Tris pH8, 2 mM EDTA, 500 mM NaCl), twice with LiCl Wash Buffer (0.7% Sodium Deoxycholate, 1% NP-40, 20 mM Tris pH8, 1 mMEDTA, 500 mM LiCl) and twice with TE Buffer. Immunoprecipitated chromatin was then eluted from Magnetic beads with Proteinase K (Roche) Digestion Buffer and heated at 50 °C for 30 minutes under agitation. Eluates were incubated at least for 6 h at 65°C for reversal of crosslinking, treated simultaneously with RNase A (Sigma Aldrich). DNA fragments were purified with NucleoMag beads kit (MN) and analyzed by SYBR Green Quantitative Real-time PCR (Kappa). The following primer pairs were used: mLc3bChIP_A1 5'–CATGCCTTGGGACACCAGAT-3' (forward), mLc3bChIP_A1 5'- ACCTTCTTCAAGTGCTGTTTGT-3' (reverse), mLc3bChIP_A2 5'- CCTCAGCTGGCTAAGAGCAT-3' (forward), mLc3bChIP_A2 5'- CCCAAGGATCTCAACCAAAC-3' (reverse).

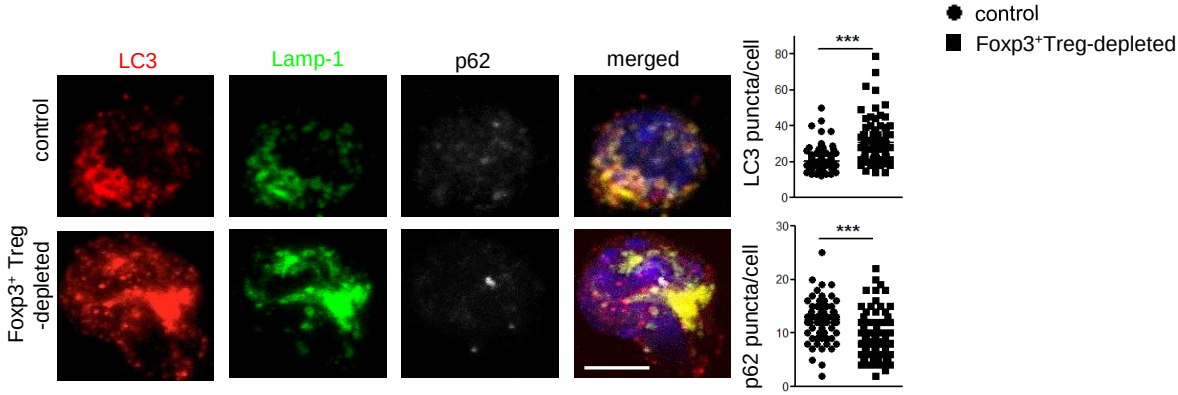
Immunoprecipitation assay. Approximately $10x10^6$ cells were washed 2 times with PBS. Cells were lysed with RIPA buffer for 30 min at 4^0C under constant agitation. Centrifugation was followed by collection of the supernatants, which contained the protein extracts. A fraction of extracts was kept as input. The rest of the extracts were precleared with magnetic beads and BSA. Then, the extracts were

diluted 5 times with IP Incubation Buffer and incubated either with PI3 Kinase p85α (6G10) mouse mAb (#13666 – Cell Signaling) or normal mouse IgG (sc-2025, Santa Cruz Biotechnology) (as a negative control) overnight at 4^0C. Immunocomplexes were then incubated with protein G Dynal magnetic beads (Invitrogen) for at least 4 hours at 4^0C. Extensive washes of immunocomplexes (4 times) with IP Incubation Buffer was followed by elution at 95^0C under periodical agitation. Supernatant collection after separation of beads through magnet was followed by Western blot

analysis of the immunocomplexes with B7-2/CD86 antibody (NB110-55488 Novus Biologicals).

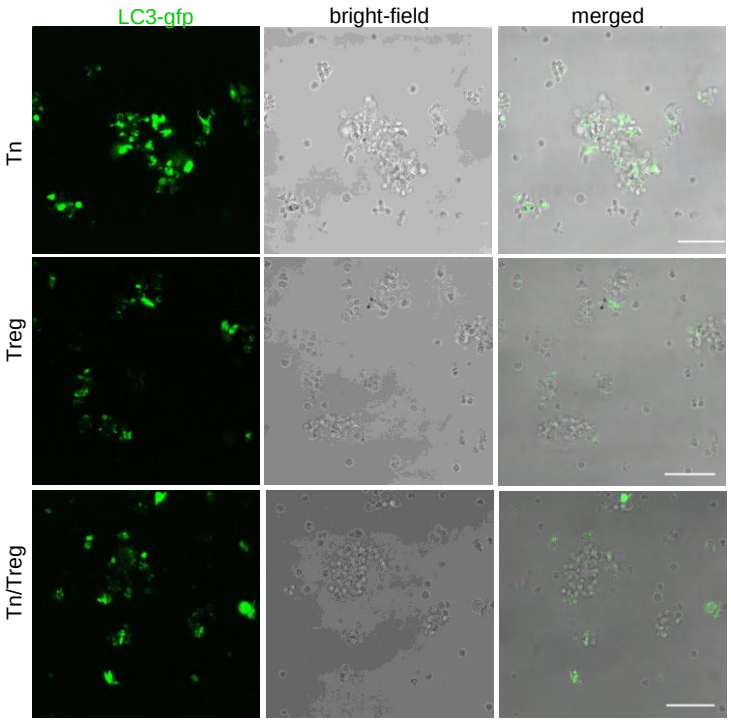
Supplemental References

1. Costes SV, Daelemans D, Cho EH, Dobbin Z, Pavlakis G, and Lockett S. Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophys J*. 2004;86(6):3993-4003.



Supplemental Figure 1. Depletion of Foxp3⁺ Tregs up-regulates autophagic pathway of DCs. Immunofluorescence confocal microscopy for LC3 (red), Lamp-1 (green), p62 (silver white) and DAPI (blue) in DCs of control and Foxp3⁺ Treg-depleted mice. One representative experiment of three is shown (5 μm scale bar). LC3 puncta/cell (****P*<0.0001) and p62 puncta/cell (****P*<0.0001) are depicted. Results are expressed as mean ± SEM; *n* = 4 mice per group, three independent experiments.

A



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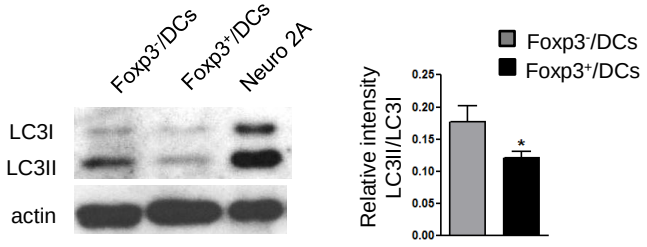
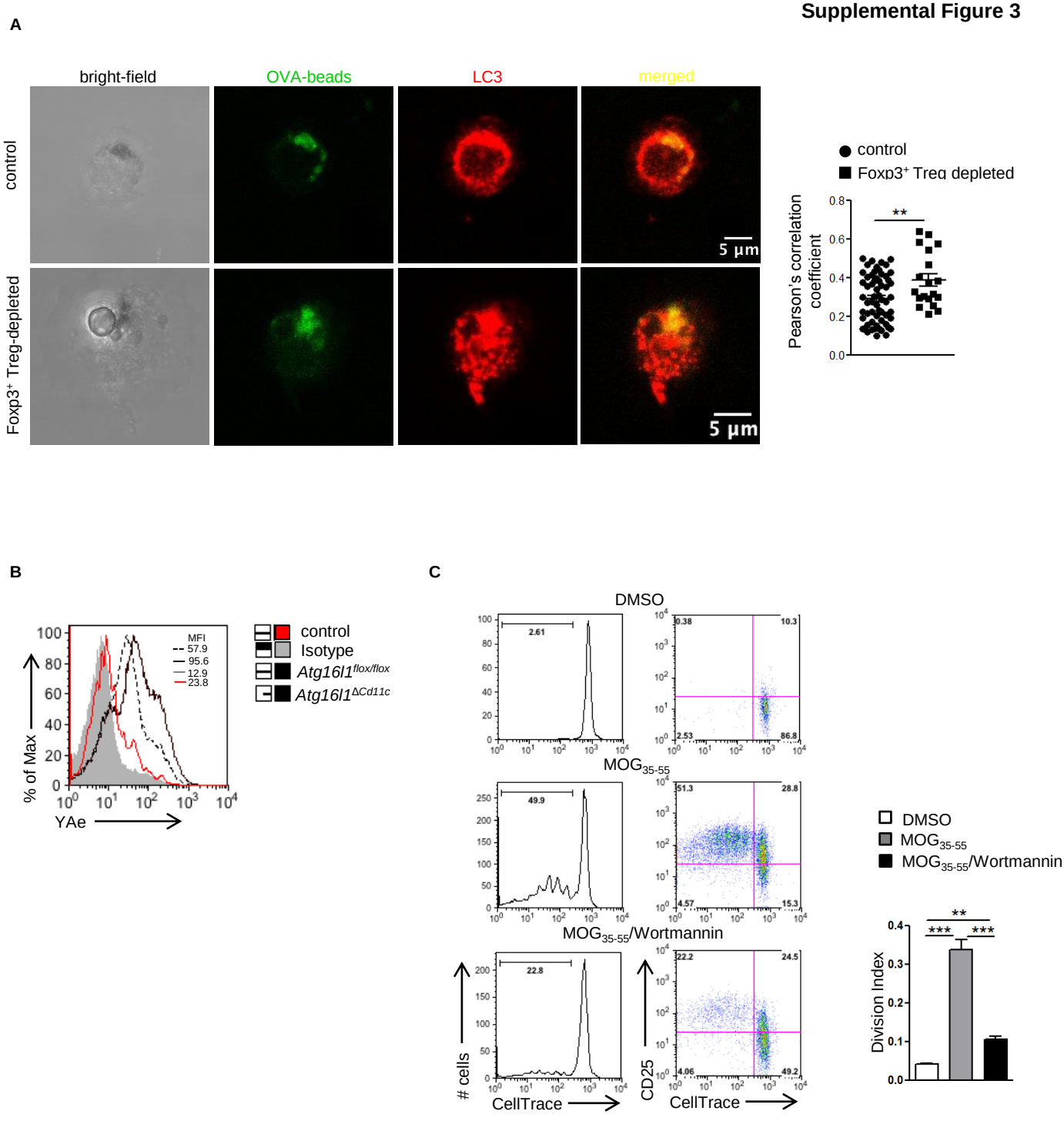
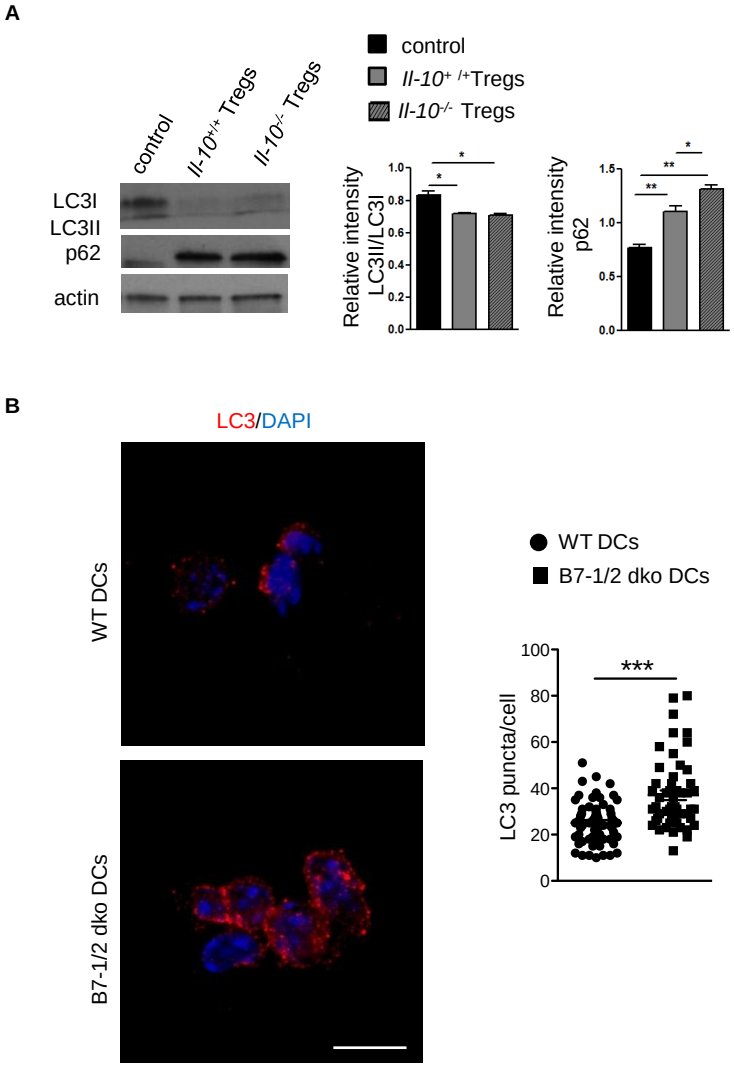


Supplemental Figure 2. Foxp3$^+$ Tregs diminish LC3 lipidation in DCs. (**A**) Confocal microscopy for co-cultures of OTII Tn or Tregs or a mixture of Tn/Treg (4:1 ratio) with sorted DCs from syngeneic LC3-gfp mice in the presence of $OVA_{323-339}$ peptide. Representative images from three individual experiments are depicted (50 µm scale bar). (**B**) Western blot analysis for LC3 and actin in CD11c$^+$ DCs from dLNs and spleens of *Rag1*$^{-/-}$ immunized mice, adoptively transferred with Foxp3$^+$ or Foxp3$^-$ CD4$^+$ T cells. Protein extracts from Neuro 2A cell line was used as a control. One representative experiment of three is depicted. Relative intensity of LC3II/LC3I (*P=0.0436) is depicted. Results are expressed as mean ± SEM; n = 6 mice per group, three independent experiments.

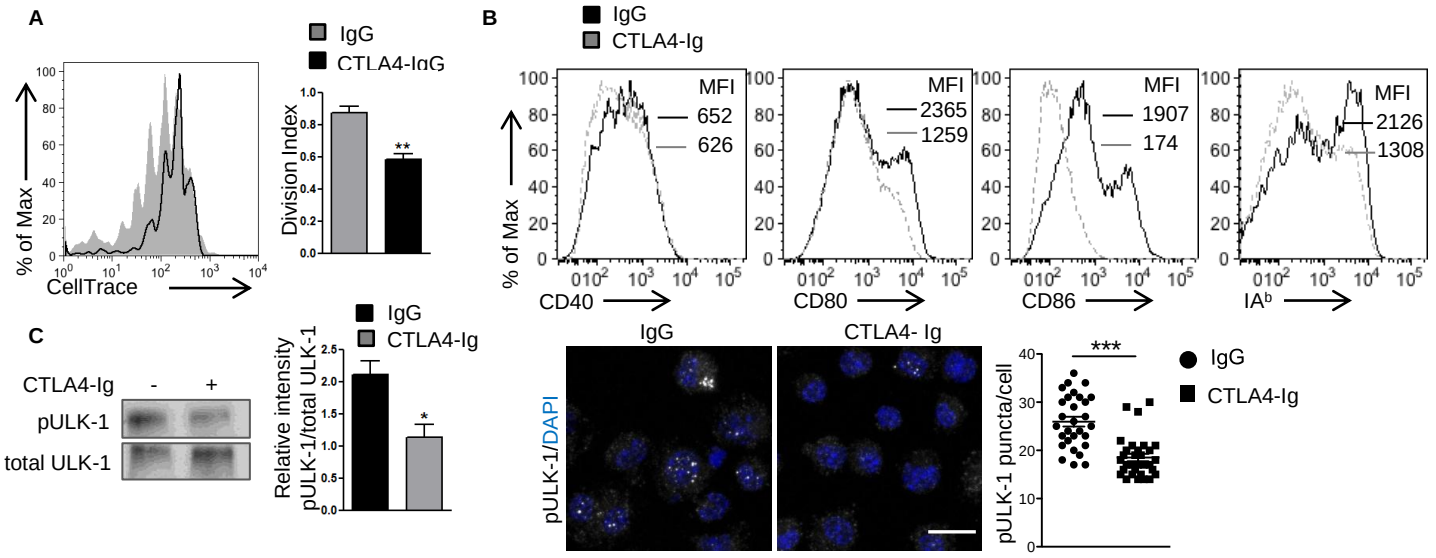
Supplemental Figure 3



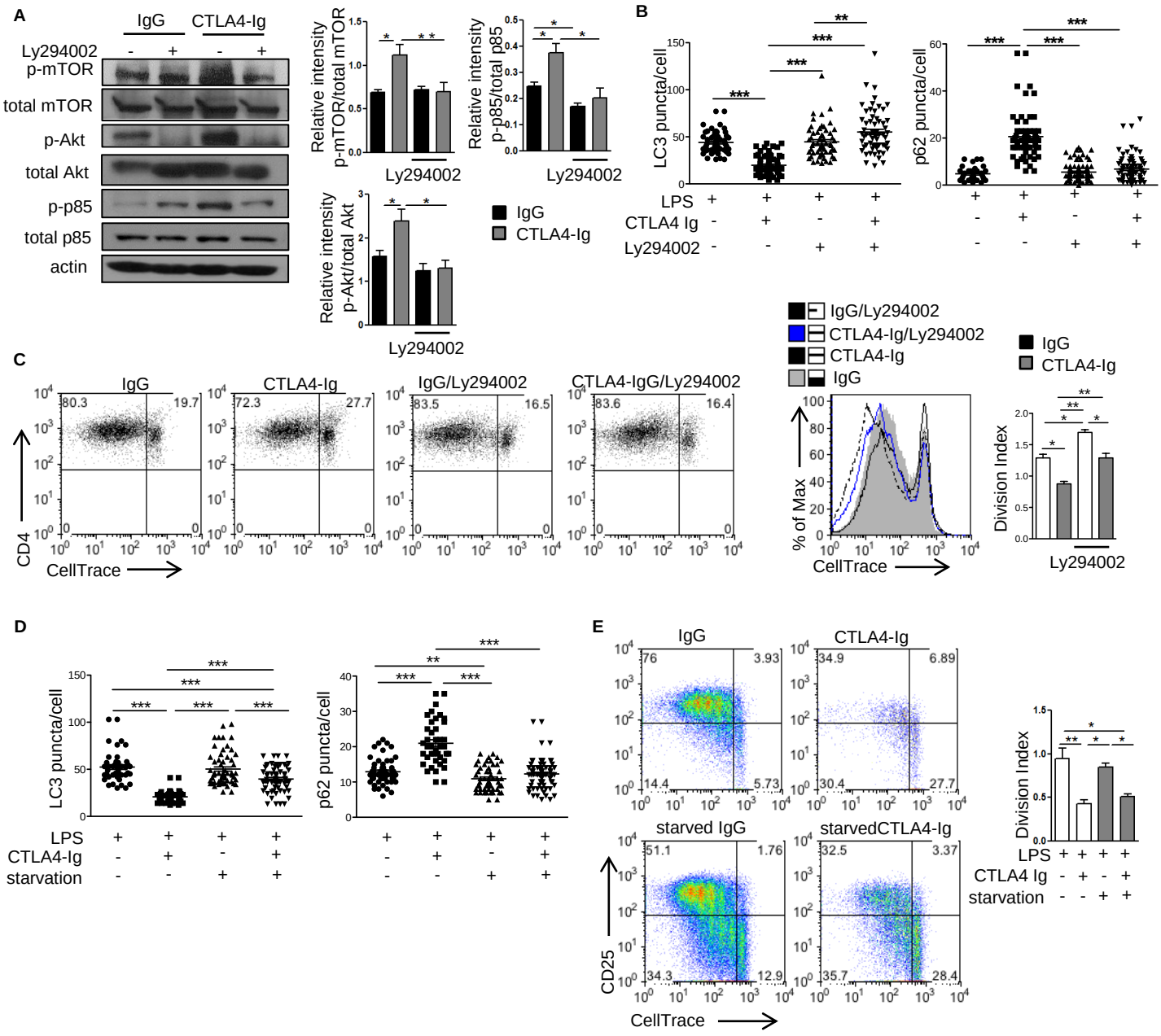
Supplemental Figure 3. Inhibition of autophagy in DCs suppresses antigen presentation of MOG_{35-55} peptide. (**A**) Carboxylated beads conjugated to LPS/OVA-Fluor® were internalized by DCs, isolated from dLNs and spleens of control or Foxp3⁺ Treg-depleted mice and analyzed by confocal microscopy after labeling with LC3 (red). Representative images from three individual experiments are depicted (5 µm scale bar). Pearson's Correlation coefficient (***P*=0.0059). (**B**) DCs from *Atg16l1*$^{\Delta Cd11c}$ or *Atg16l1*$^{flox/flox}$ mice were pulsed with Ea peptide for 4hrs. YAe expression analyzed by flow cytometry (where control is unpulsed DCs). (**C**) BMDCs were pulsed with MOG_{35-55}, treated with Wortmannin for 6hrs and cultured with Cell-Trace-labeled Vα3.2⁺Vβ8.1⁺CD4⁺ 2D2 T cells. Cell-Trace dilution, CD25 expression and division index (***P*=0.0011, ****P*=0.0002, ***P*=0.0001). One representative experiment of four is depicted.



Supplemental Figure 4. Foxp3⁺ Tregs modulate autophagy in DCs in an IL-10 independent but B7.1/2-dependent fashion. (**A**) Western blot analysis for LC3, p62 and actin in DCs isolated from dLNs and spleens of *Rag1*$^{-/-}$ immunized mice, adoptively transferred with *Il10*$^{+/+}$ or *Il10*$^{-/-}$ CD4^{+} Treg cells. One representative experiment of three is depicted. Relative intensity of LC3II/LC3I (**P*=0.0129, **P*=0.0245) and p62 (**P*=0.0086, ***P*=0.0035, **P*=0.0288) are depicted. Results are expressed as mean ± SEM; *n* = 4 mice per group, three independent experiments. Statistical significance was obtained by two-way ANOVA. (**B**) Immunofluorescence confocal microscopy for LC3 (red) and DAPI (blue) in DCs isolated from co-cultures of OTII Tregs and WT or B7-1/2 dko DCs in the presence of $OVA_{323-339}$ peptide (10 μm scale bar). One representative experiment of three is shown. LC3 (****P*<0.0001) is depicted. Results are expressed as mean ± SEM; *n* = 4 mice per group, three independent experiments. Statistical significance was obtained by unpaired Student's *t*-test.



Supplemental Figure 5. CTLA4 suppresses canonical autophagy in BMDCs and renders them less immunogenic. BMDCs were treated with CTLA4-Ig or IgG for 16-20hrs. (**A**) BMDCs pulsed with MOG_{35-55} for 6hrs, cultured with Cell-Trace-labeled 2D2 T cells. Division index (***P*=0.0050). One representative experiment of four is depicted. (**B**) CD40, CD80, CD86 and Ia^b expression is depicted. One representative experiment of four is depicted (**C**) Western blot analysis for pULK-1 (**P*=0.0140) and total ULK-1 in BMDCs. One representative experiment of four. Immunofluorescence confocal microscopy for pULK-1 (silver white), and DAPI (blue) (10 μm scale bar). One representative experiment of three. pULK-1 puncta/cell (****P*<0.0001). Results are expressed as mean ± SEM; *n* = 4 mice per group, three independent experiments. Statistical significance was obtained by unpaired Student's *t*-test.



Supplemental Figure 6. CTLA4 mediated suppression of autophagy in BMDCs is operated by PI3K/Akt/mTOR signaling cascade. BMDCs were treated with CTLA4-Ig or IgG for 16-20hrs in the presence or absence of Ly294002 (**A-C**) or in the absence of serum (starvation **D-E**). (**A**) Western blot for p-mTOR *(*P*=0.0430, **P*=0.0050) total mTOR, p-p85 *(*P*=0.0430, *P*=0.0456, *P*=0.0113), total p85, p-Akt *(*P*=0.0430, *P*=0.05) and total Akt in BMDC lysates. One representative experiment of four. (**B**) LC3 (***$P<0.0001$, **$P=0.0084$) and p62 (***$P<0.0001$) puncta/cell are depicted. (**C**) BMDCs were pulsed for 6 hrs with MOG_{35-55} and then cultured with Cell-Trace-labeled 2D2 T cells. Cell-Trace dilution and division index (*$P=0.0104$, *$P=0.0494$, **$P=0.0049$, **$P=0.0070$, *$P=0.0356$) is depicted. One representative experiment of three. (**D**) LC3 (***$P<0.0001$) and p62 (***$P<0.0001$, **$P=0.0091$) puncta/cell are depicted. (**E**) BMDCs were pulsed for 6 hrs with MOG_{35-55} and then cultured with Cell-Trace-labeled 2D2 T cells. Cell-Trace dilution and division index (**$P=0.0089$, *$P=0.05$, *$P=0.0210$, *$P=0.0149$) is depicted. One representative experiment of four. Results are expressed as mean ± SEM; $n = 4$ mice per group. Statistical significance was obtained by two-way ANOVA.

Supplemental Table 1: **Autophagy deficient DCs fail to induce EAE**. Disease incidence, Time of EAE onset and maximum EAE severity (clinical score) are shown.

Experimental group	Disease incidence	Time of onset (d)	Maximum score
Atg16l1$^{flox/flox}$	8/9 (89%)	11.13 ± 0.2950 (N=8)	3.889 ± 0.5321 (N=9)
Atg16l1$^{\Delta Cd11c}$	4/9 (44.4%)	11.25 ± 0.8539 (N=4)	1.111 ± 0.5576 (N=9)**

One representative experiment of two is depicted.

Abbreviations: EAE: experimental autoimmune encephalomyelitis

Disease incidence is presented as diseased mice/total mice; time of onset and maximum score is presented as mean ± s.e.m.

Statistics for maximum score of EAE: ***P*=0.0024, unpaired t test.

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