SUPPLEMENTAL INFORMATION

Figures S1 – S7

Experimental Procedures

Supplemental References

SUPPLEMENTAL FIGURES

Figure S1, related to Figure 1. Generation of *Clec16a* KD NOD mice

(A) *Clec16a* KD target sequences KD3 and KD5 were validated by luciferase reporter assay, using a dual-luciferase vector that incorporates the *Clec16a* cDNA. Data are representative of 3 experiments. (B) GFP expression in lymphocytes from KD3 transgenic mice that were used for all experiments denoted as KD. KD5 mice have comparable expression levels (not shown). (C) Relative expression of *Clec16a* mRNA in the spleen of WT and KD3 mice. P = 0.0002, data are representative of 5 experiments. (D) Expression of *Clec16a* protein in the thymus of WT (n=3) and KD3 (KD, n=3) mice. Similar results were obtained for spleen (not shown).

Figure S2, related to Figure 2. *Clec16a* KD causes T cell hyporeactivity

(A) Proliferation of CD4 (left panel) or CD8 (right panel) T cells from WT or KD mice in response to CD3 antibody. (B) Proliferation of CD4 T cells from WT, KD3 or KD5 mice in response to stimulation with CD3/CD28 antibody-coated beads. Results show mean ± SEM of triplicate measurements and are representative of 2 to 3 experiments.

Figure S3, related to Figure 3. *Clec16a* KD does not affect beta cell-derived antigen presentation

(A) Blood glucose measurements in WT (n = 13), KD3 (n = 6) and KD5 (n = 6) NOD mice injected intraperitoneally with 2 g glucose / kg body weight. (B) Insulin levels measured in the serum of WT (n = 6) and KD3 (n = 6) NOD mice before and after intraperitoneal glucose injection. (C) Total pancreatic insulin content measured in WT (n = 7), KD3 (n = 6) and KD5 (n = 4) NOD mice by ELISA. (D, E) Comparison of *Clec16a* mRNA content by quantitative PCR in purified islets from WT, KD3 (D) and KD5 (E) NOD mice. n = 6 mice per group for each data set. * $P < 0.05$. (F) Representative flow cytometry data (left panel) and proliferation index (right panel) of BDC2.5 T cells in the pancreatic lymph node (PLN) 3 days after transfer into WT (n = 9), KD3 (n = 9) or KD5 (n = 4) NOD mice. BDC2.5 T cells were loaded with proliferation dye prior to transfer into recipient mice to allow both their identification and the quantification of their proliferation. Representative data from the inguinal lymph node (ILN) is also shown for comparison. No BDC2.5 T cell proliferation was detected in the spleen or in other peripheral lymph nodes (not shown).

Figure S4, related to Figure 4. *Clec16a* KD does not affect DP thymocyte reactivity *in vitro*

Differentiation of sorted DP thymocytes *in vitro* by antibody stimulation. Cells were stimulated with plate-bound anti-TCRβ alone or anti-TCRβ and anti-CD5 antibody. After 24 h, cells were either analysed directly, or transferred into new cell culture plates without antibody for another 24 h prior to analysis. Results are representative of 2 experiments.

Figure S5, related to Figure 5. *Clec16a* KD modifies thymic selection

(A, B) Frequency of thymocyte populations distinguished by TCR and CD69 expression in WT (n = 6) and KD5 (n = 5) mice (A) or in WT (n = 3) and KD3 (n = 3) BDC2.5 TCR transgenic mice (B). TCR Vβ4 chain antibody was used in B. *, $P < 0.05$; **, $P < 0.01$.

(C, D) Average frequency (C) and representative flow cytometry data (D) for CD4SP1, CD4SP2 and CD4SP3 populations in BrdU pulse labeled mice (n = 3 mice per group for each time point). Thymocytes from BrdU-injected mice were stained for CD4, CD8, CD24 and CCR7, and the cells shown were pre-gated on the $BrdU^+CD4SP$ population. CD4SP1, CD4SP2 and CD4SP3 populations are defined by $CD24^+CCR7^-$, $CD24^+CCR7^+$ and $CD24^-CCR7^+$, respectively, where the CD24/CCR7 quadrant was set relative to DP cells that are $CD24^+CCR7^-$. $P = 0.0743$ for CD4SP1; $P = 0.26$ for CD4SP2; $P = 0.0319$ for CD4SP3; two-tailed paired t-test.

Figure S6, related to Figure 6. *CLEC16A* KD disrupts autophagy in human cells

(A, D) Relative expression of *CLEC16A* and *ATG5* mRNA in HEK293 (A) or HeLa (D) cells transduced with *CLEC16A* and *ATG5* shRNA, respectively, compared with cells transduced with a control (CTRL) shRNA. (B, C, E, F) LC3 and p62 quantification in HEK293 (B, C) or HeLa (E, F) cells transduced with control (CTRL), *CLEC16A* (KD) or *ATG5* shRNA. Cells were starved in the presence of bafilomycin (B, E), or in the presence or absence (-) of rapamycin (R) or 3-MA (M) that induces or inhibits autophagy, respectively (C), or in the presence of Pepstatin A (F). Results are representative of 2-4 similar experiments.

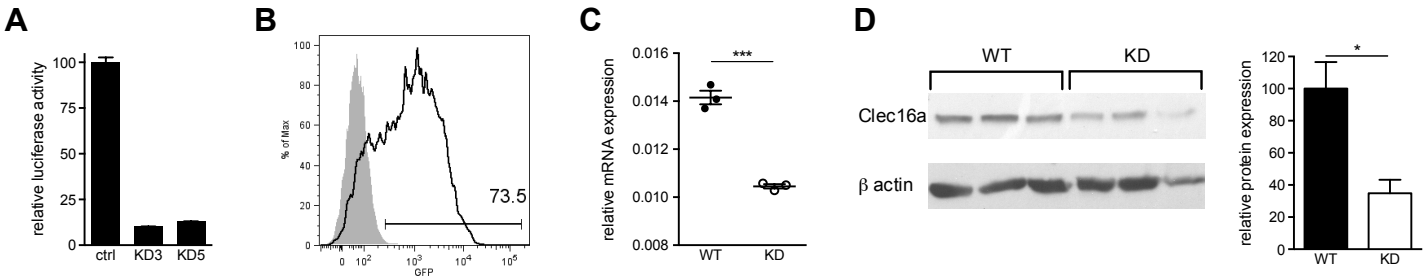
Figure S7, related to Figure 7. *Clec16a* modifies the stimulatory capacity of MJC1 cells

(A) Left panel: Flow cytometry profile of source cells used for the generation of the MJC1 cell line. Thymic cells had been pre-sorted to isolate $CD45^{-}EpCAM^{+}$ cells. These sorted cells (shown) were then sorted a second time to isolate $Ly\text{-}51^{+}UEA\text{-}1^{-}$ cells (circled region). Right panel: EpCAM and Ly-51 expression on MJC1 cells after the 14^{th} passage in culture.

(B) Representative images of control (ctrl) and *Clec16a* KD (KD3 and KD5) MJC1 cells cultured with (top row, 'fed') or without (bottom row, 'starved') serum. Cells were fixed and stained with DAPI (blue) and anti-LC3 antibody (red), showing the accumulation of LC3 in autophagosomes at 60-fold magnification.

(C) Representative flow cytometry data for the differentiation of DP cells into CD4SP cells in culture with control (ctrl) or *Clec16a* KD (KD3 and KD5) MJC1 cells or without MJC1 cells for 4 h. (D) Representative data for upregulation of CD69 on thymocytes cultured with control (ctrl) or *Clec16a* KD (KD3 and KD5) MJC1 cells, or without MJC1 cells. All data are representative of 3 experiments. (E) PD1 expression on DP or CD4SP cells 24 h after culture of immature DP thymocytes with MJC1 cells. (F) PD1 expression on DP or CD4SP thymocytes from WT (n = 9) or *Clec16a* KD mice (n = 9). CD4SP cells were subdivided into SP1, SP2 and SP3 based on their expression of CCR7 and CD24, as in Figure S5. * $P < 0.05$, ** $P < 0.01$.

Figure S1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50
25
0
ctrl
KD3
KD5
B
% of Max
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73.5
0
10^2
10^3
10^4
10^5
GFP
C
relative mRNA expression
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0.014
0.012
0.010
0.008
***
WT
KD
D
WT
KD
Clec16a
β actin
relative protein expression
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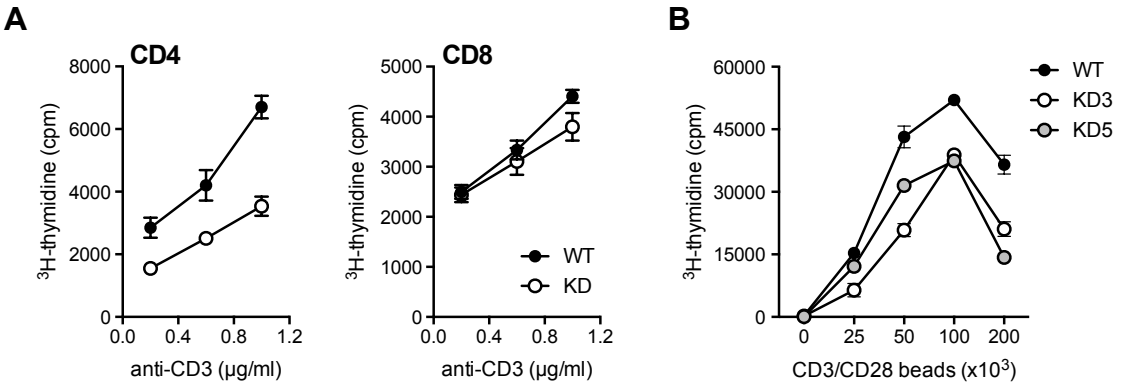
80
60
40
20
0
*
WT
KD

Figure S2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CD8
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anti-CD3 (µg/ml)
WT
KD
B
WT
KD3
KD5
CD3/CD28 beads (x10^3)

Figure S3

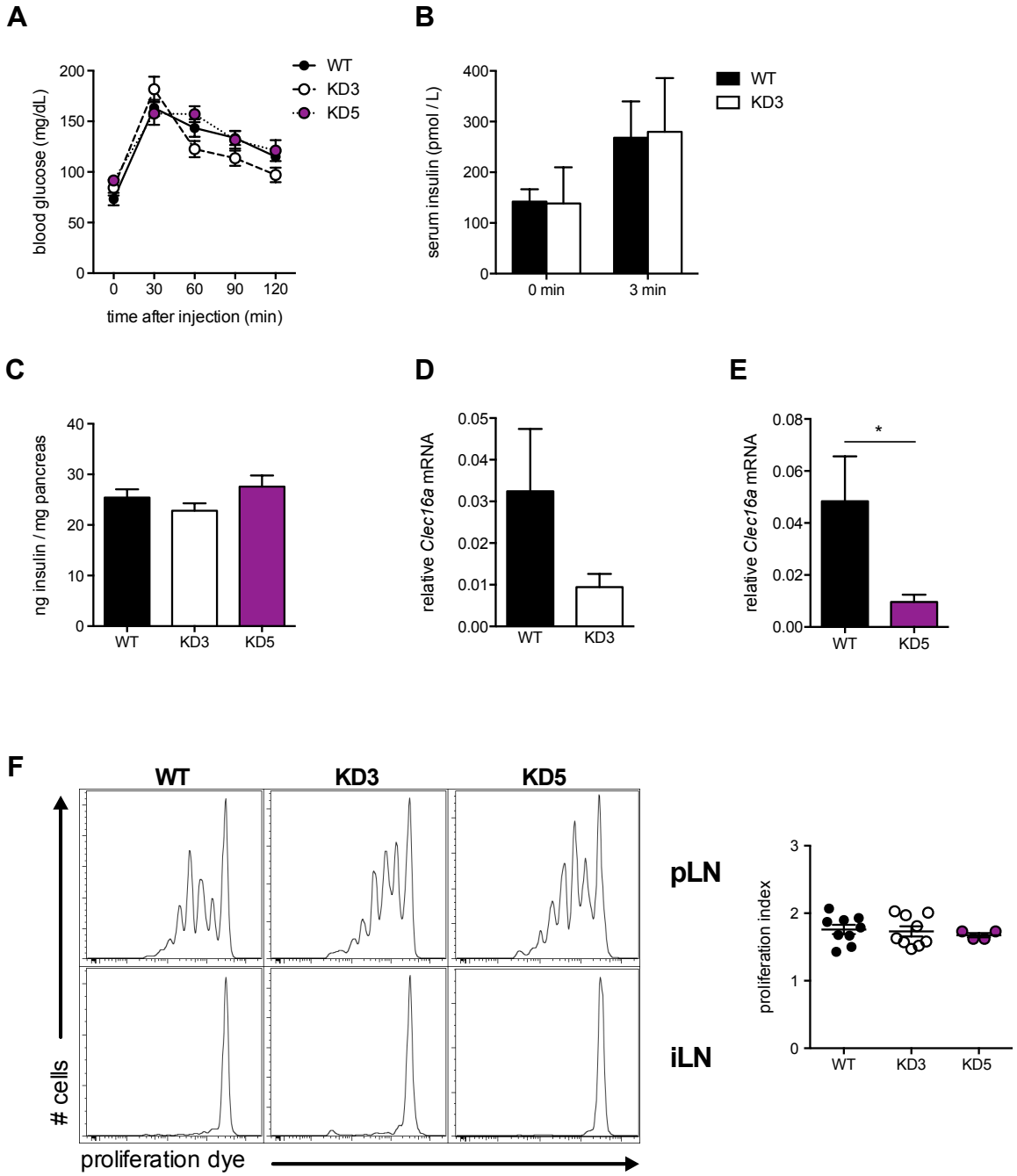
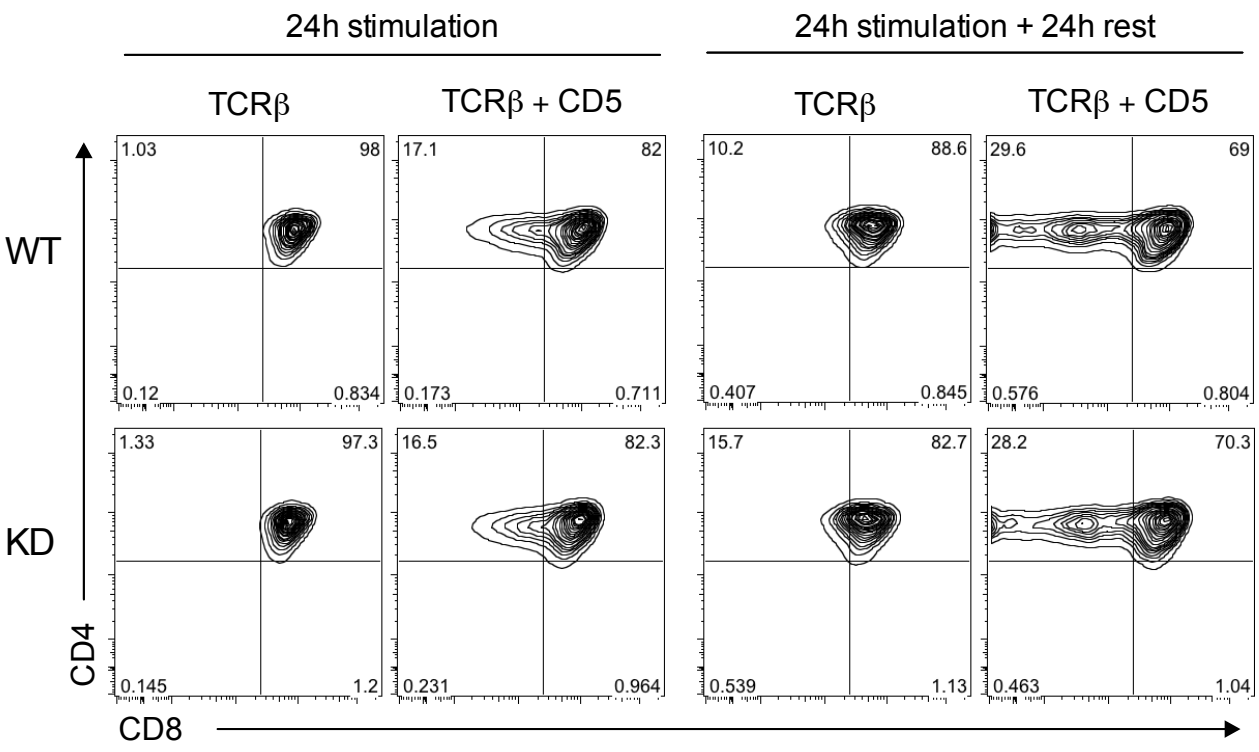

A
B
blood glucose (mg/dL)
time after injection (min)
WT
KD3
KD5
serum insulin (pmol / L)
0 min
3 min
C
D
E
ng insulin / mg pancreas
relative Clec16a mRNA
*
F
WT
KD3
KD5
cells
proliferation dye
pLN
iLN
proliferation index

Figure S4


24h stimulation
24h stimulation + 24h rest
TCRβ
TCRβ + CD5
TCRβ
TCRβ + CD5
WT
KD
CD4
CD8
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98
0.12
0.834
17.1
82
0.173
0.711
10.2
88.6
0.407
0.845
29.6
69
0.576
0.804
1.33
97.3
0.145
1.2
16.5
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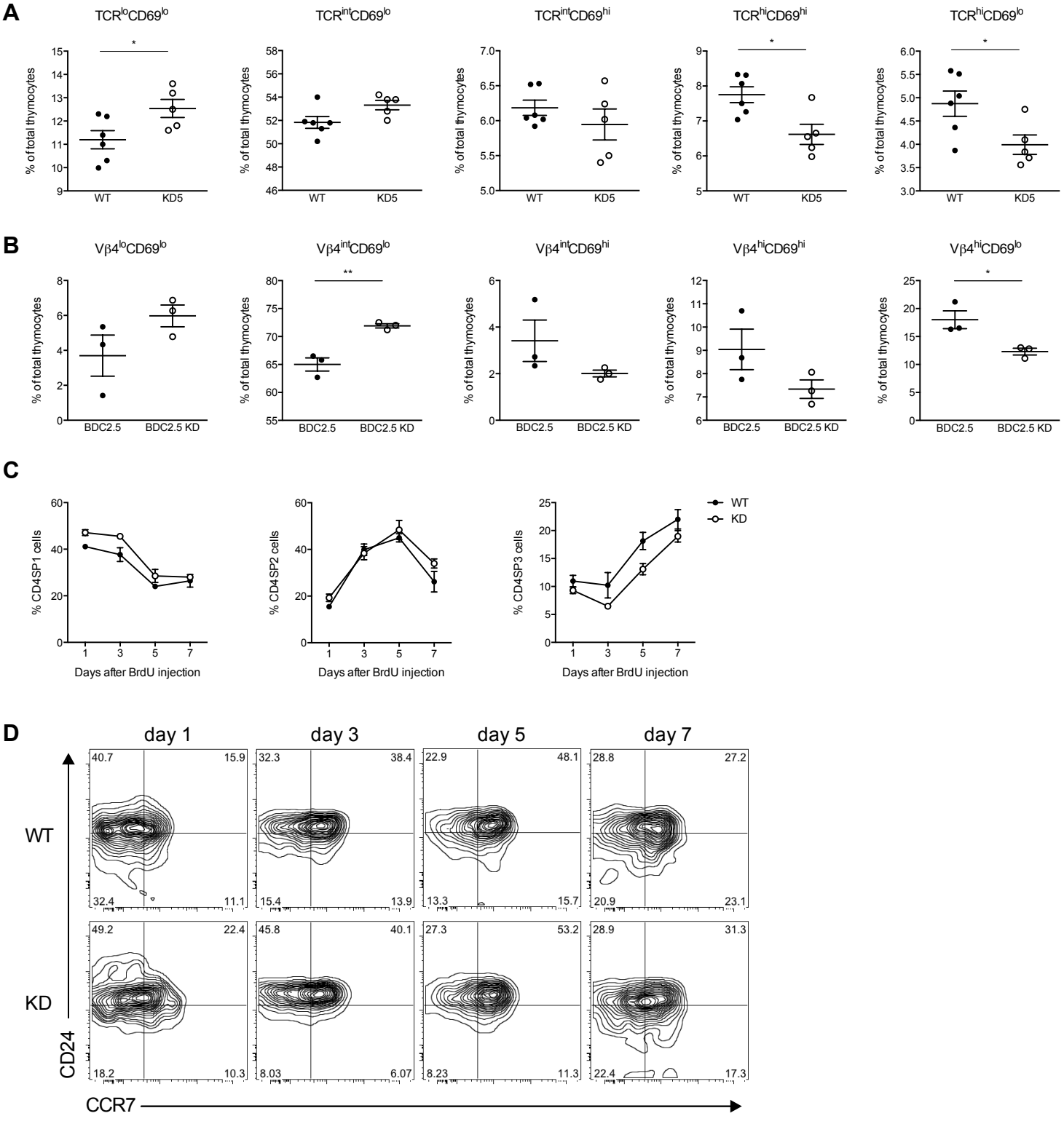
28.2
70.3
0.463
1.04

Figure S5


A
TCRloCD69lo
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TCRintCD69hi
TCRhiCD69hi
TCRhiCD69lo
% of total thymocytes
WT
KD5
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Vβ4loCD69lo
Vβ4intCD69lo
Vβ4intCD69hi
Vβ4hiCD69hi
Vβ4hiCD69lo
BDC2.5
BDC2.5 KD
C
% CD4SP1 cells
% CD4SP2 cells
% CD4SP3 cells
Days after BrdU injection
WT
KD
D
day 1
day 3
day 5
day 7
WT
KD
CD24
CCR7
40.7
15.9
32.4
11.1
32.3
38.4
15.4
13.9
22.9
48.1
13.3
15.7
28.8
27.2
20.9
23.1
49.2
22.4
18.2
10.3
45.8
40.1
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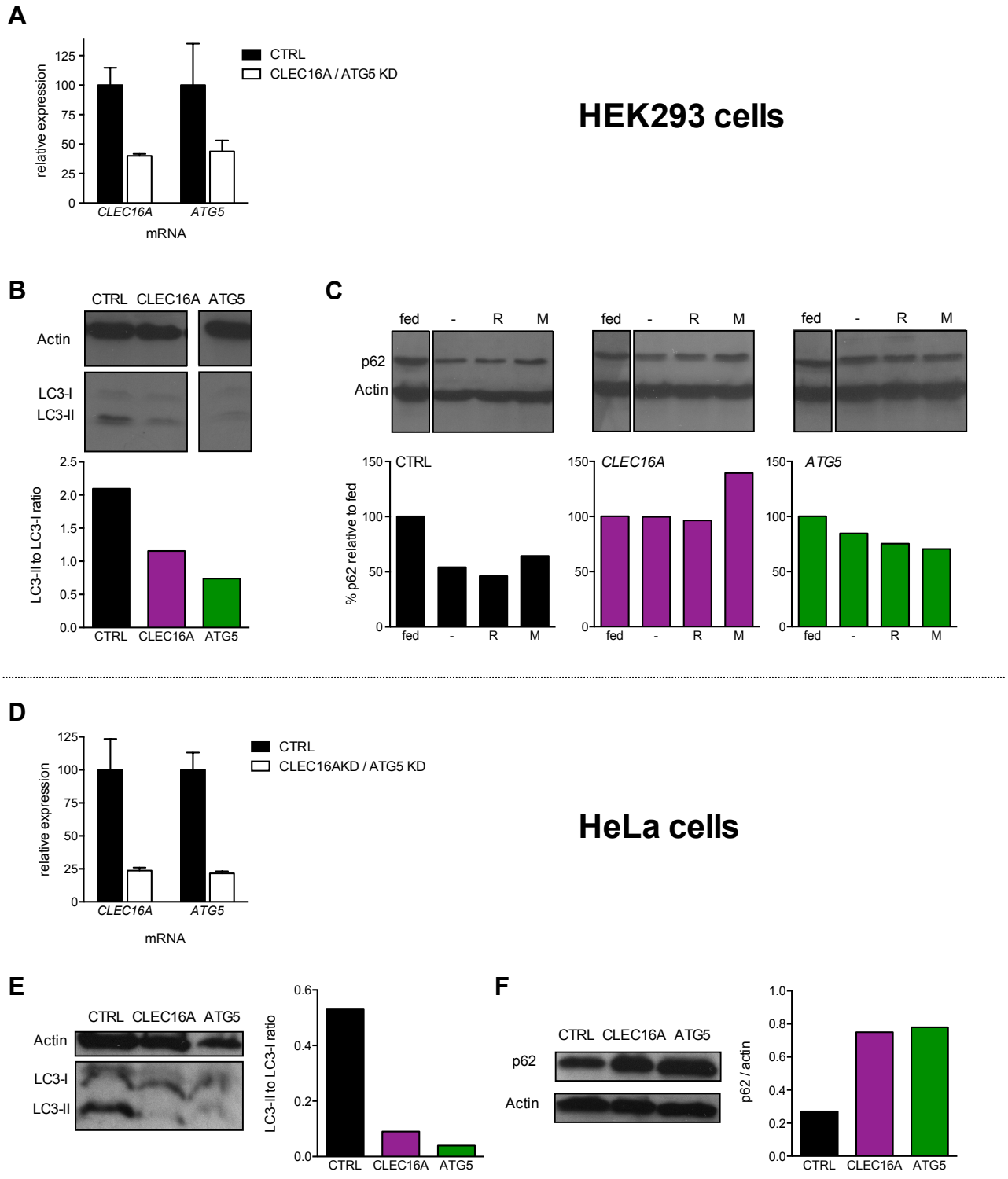
27.3
53.2
8.23
11.3
28.9
31.3
22.4
17.3

Figure S6


A
CTRL
CLEC16A / ATG5 KD
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100
75
50
25
0
CLEC16A
ATG5
mRNA
HEK293 cells
B
CTRL CLEC16A ATG5
Actin
LC3-I
LC3-II
LC3-II to LC3-I ratio
2.5
2.0
1.5
1.0
0.5
0.0
CTRL CLEC16A ATG5
C
fed - R M
p62
Actin
% p62 relative to fed
150
100
50
0
CTRL
CLEC16A
ATG5
D
CTRL
CLEC16AKD / ATG5 KD
relative expression
CLEC16A
ATG5
mRNA
HeLa cells
E
CTRL CLEC16A ATG5
Actin
LC3-I
LC3-II
LC3-II to LC3-I ratio
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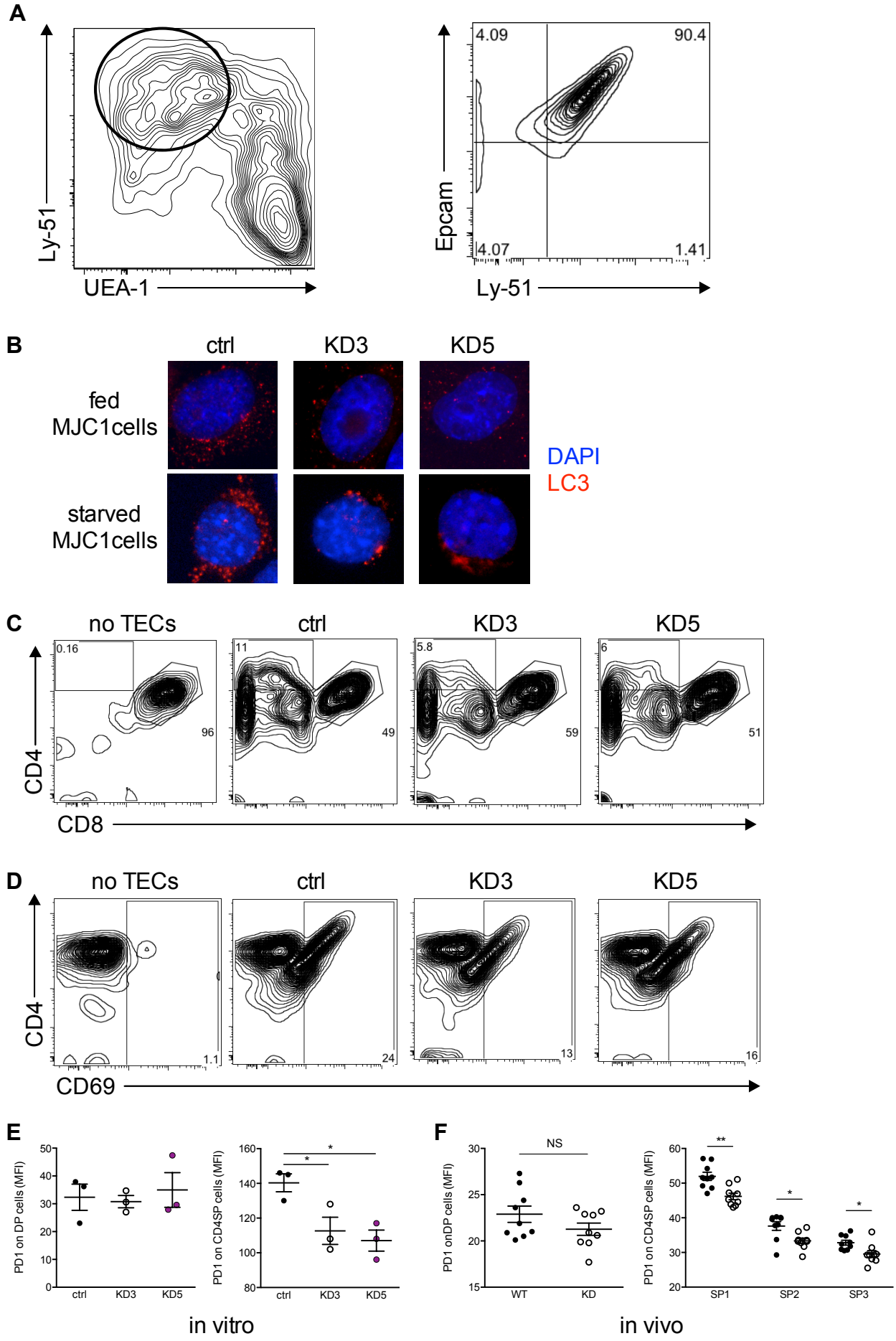
0.4
0.2
0.0
F
p62
Actin
p62 / actin
1.0
0.8

Figure S7


A
Ly-51
UEA-1
4.09
90.4
4.07
1.41
Epcam
Ly-51
B
ctrl
KD3
KD5
fed MJC1cells
starved MJC1cells
DAPI
LC3
C
no TECs
ctrl
KD3
KD5
0.16
96
11
49
5.8
59
6
51
CD4
CD8
D
no TECs
ctrl
KD3
KD5
1.1
24
13
16
CD4
CD69
E
PD1 on DP cells (MFI)
ctrl
KD3
KD5
PD1 on CD4SP cells (MFI)
ctrl
KD3
KD5
in vitro
F
PD1 onDP cells (MFI)
NS
WT
KD
PD1 on CD4SP cells (MFI)
SP1
SP2
SP3
in vivo

EXPERIMENTAL PROCEDURES

Mice

Clec16a KD mice were generated by lentiviral transgenesis in the NOD mouse strain as described previously (Kissler et al., 2006). Briefly, NOD zygotes were microinjected into the pervitelline space with high-titer (>10^8 infectious particles/ml) lentivirus encoding the GFP reporter protein together with shRNA sequences expressed within a microRNA structure from the 3' UTR of GFP. KD3 mice were generated using the vector pLBM described previously (Gerold et al., 2011), and the target sequence in *Clec16a* is CACCTTGTACGTCATTTCTATA. KD5 mice were generated using the vector pUGM, which was cloned by inserting the GFP-mir30 cassette from pLBM into the pUTG (Herold et al. 2008; Zheng and Kissler, 2013) vector, such that GFP and *Clec16a* shRNA are under the control of a ubiquitin promoter, instead of the CMV promoter present in pLBM. The KD5 target sequence in *Clec16a* is GAGTGTCCACCTTGTACGTCAT. Transgenic mice were maintained as homozygous breeders. WT NOD mice used for comparison in all experiments were bred and housed in the same facility and in the same room as transgenic mice. NOD BDC2.5 TCR transgenic mice were purchased from Jackson Laboratories, and bred with *Clec16a* KD mice to generate the NOD BDC2.5/Clec16a KD line. OT-II TCR$\alpha^{-/-}$ mice were kindly provided by T. Serwold. For all animal studies, no specific randomization or blinding was used. Experiments were performed with age- and sex-matched mice. All experimental procedures in animals were approved by the Regional Government of Lower Franconia or by the Institutional Animal Care and Use Committee, for experiments performed at the University of Wurzburg and at the Joslin Diabetes Center, respectively.

Diabetes frequency studies

All disease studies were performed with age-matched, contemporary cohorts of mice. Onset of diabetes was monitored by weekly (for spontaneous disease) or thrice-weekly (for cyclophosphamide studies and cell transplantation studies) measurements of glycosuria using Diastix (Bayer). Mice with two consecutive readings > 250 mg / dL were considered diabetic. For cell transplantation experiments, splenocytes or magnetically-sorted lymphocytes were injected intravenously (4-10x10^6 cells / mouse) into NOD.SCID or *Clec16a* KD (KD3) NOD.SCID mice, as indicated. For cyclophosphamide-accelerated disease, mice were injected with 200 mg / kg cyclophosphamide (Sigma-Aldrich) intraperitoneally. NOD.SCID mice reconstituted with splenocytes or purified lymphocytes were injected with cyclophosphamide 3 weeks after cell transplant.

Generation of bone-marrow chimeras

For the generation of bone-marrow chimeras, irradiated NOD.SCID mice (250 rad) or NOD mice (900 rad) were injected intravenously with lineage-depleted bone-marrow cells (3-5x10^5 / mouse) from WT or KD3 mice. Lineage depletion was performed using magnetic separation with a MACS lineage-depletion kit (Miltenyi Biotech) according to the manufacturer's instructions. Measurements in bone-marrow chimeras were performed 6-8 weeks after cell transplantation.

Thymic transplantations

Thymectomies of NOD WT mice were performed by Surgical Services at Jackson Laboratories at 4 weeks of age. Mice were irradiated and reconstituted with NOD WT lineage-cell depleted bone marrow as described above. Two weeks later, NOD WT or Clec16a KD E14 thymic lobes were transplanted under the kidney capsule. Transplanted mice were injected with 200 mg / kg cyclophosphamide (Sigma-Aldrich) intraperitoneally 6 weeks later to accelerate diabetes onset.

Glucose tolerance test

Mice were fasted overnight. Blood glucose levels were determined at the indicated time points before and after intraperitoneal injection of 2 g glucose / kg using a Contour blood glucose monitoring system (Bayer). Blood samples were collected from the tail vein at the indicated time points to measure serum insulin levels by ELISA.

***In vivo* BDC2.5 T cell proliferation**

Splenocytes from WT or *Clec16a* KD BDC2.5 TCR transgenic mice were labeled with a proliferation dye (Cell Proliferation Dye eFluor® 450, eBioscience). $3x10^6$ cells were injected intravenously into NOD WT or *Clec16a* KD mice. Inguinal and pancreatic lymph nodes were harvested 3 days after cell transfer and cell proliferation of $BDC2.5^+$ T cells was analysed by flow cytometry.

T cell *in vitro* assays

For cell proliferation assays, $CD4^+$ T cells were purified either magnetically ($CD4^+$ T cell kit, Miltenyi Biotech) or by cell sorting on a FACSAria cell sorter (BD Biosciences). T

cells ($5x10^4$ / well) were stimulated with CD3 antibody in the presence of irradiated T cell-depleted splenocytes ($3x10^5$), with CD3/CD28 antibody-coated beads (Invitrogen/Life Technologies) at the indicated concentrations/ratios or with PMA (25 ng/ml) and Ionomycin (1 μg/ml). Alternatively, cells isolated from BDC2.5 transgenic mice were stimulated with the BDC2.5 mimotope peptide (Judkowski et al., 2001) (RTRPLWVRME) in the presence of irradiated T cell-depleted splenocytes at the indicated concentrations. Proliferation was measured at 72 h following the addition of ^{3}H-thymidine (0.5 mCi / well) for the last 16 h of culture with a Microbeta plate reader (Perkin-Elmer). Results are expressed in counts per minute and show the mean +/- SEM of triplicate measurements. For Treg cell suppression assays, WT $CD4^+CD25^-$ T cells ($5x10^4$ / well) were co-cultured at the indicated ratio with WT or KD3 $CD4^+CD25^+$ T cells isolated magnetically with a MACS $CD4^+CD25^+$ isolation kit (Miltenyi Biotech), in the presence of irradiated splenocytes ($4x10^5$) and CD3 antibody (1 mg / ml, clone 2C11, eBioscience). All T cell assays were performed in RPMI-1640 cell culture medium supplemented with 2-mercaptoethanol, 10 mM HEPES,1 mM Sodium Pyruvate , antibiotics (penicillin / streptomycin) (all from Invitrogen/Life Technologies) and 10% fetal bovine serum.

Flow cytometry

Flow cytometry measurements were performed using a FACSCantoII or LSRII instrument (BD Biosciences). Cell sorting was performed with a FACSAriaIII instrument (BD Biosciences). Data were analysed with FlowJo software (TreeStar Inc.). Fluorescently conjugated CD4, CD8, TCRβ, CD69, CD25, Helios, FoxP3, CD24, CCR7,

PD-1, TCR Vβ4, CD45, EpCAM, Ly-51, CD80, I-A^k, I-A/I-E and TCR Vβ-specific antibodies were purchased from Biolegend, eBioscience and BD Biosciences. An UEA I-Biotin antibody was purchased from Vector Laboratories. Intracellular staining was performed with a FoxP3-labelling kit (eBioscience). All surface staining was performed in PBS in the presence of 1% fetal bovine serum and cells were pre-incubated with the CD16/32 Fc-receptor blocking antibody prior to labeling with fluorescently-conjugated antibodies. CCR7 staining was performed in pre-warmed PBS at 37^o C.

***In vivo* thymocyte depletion with anti-CD3**

For stimulation of DP thymocytes *in vivo*, mice were injected with 20 mg purified (no azide / low endotoxin) CD3 antibody (clone 2C11, eBioscience) intravenously. Mice were euthanized 48 h after injection for analysis of thymocytes by flow cytometry.

BrdU pulse labeling

Mice were injected once with 1.6 mg BrdU (Sigma-Aldrich) intraperitoneally. Thymocytes were dissected at the indicated time points and labeled with fluorescently-conjugated anti-BrdU antibody using a BrdU flow kit (BD Biosciences) following the manufacturer's instructions.

Immunohistochemistry

The thymus was frozen in OCT (Fisher Scientific) prior to cryosectioning into 5 mm slices that were then fixed in cold acetone (Sigma-Aldrich) onto glass slides and left to dry at room temperature. Slides were washed twice in PBS with 0.1% Tween-20 (PBS-

T), and incubated for 30 min with PBS-T supplemented with 1% bovine serum albumin (Sigma-Aldrich) and 10% goat serum (Invitrogen/Life Technologies). Slides were then incubated 1 h at room temperature with chicken Keratin 8 antibody (abcam) or biotinylated UEA-I lectin (GeneTex), and rabbit LC3 antibody (Fisher Scientific). Slides were washed 3 times in PBS-T and incubated 45 min with secondary antibodies (goat anti-chicken Alexa fluor 594 or streptavidin Alexa fluor 594, and goat anti-rabbit Alexa fluor 488, all from Invitrogen/Life Technologies). Finally, slides were washed, dried and sealed prior to analysis with an Axioplan2 microscope (Zeiss). Image analysis was performed with ImageJ64 software. Autophagosomes (LC3 punctae that co-localized with keratin-8 or UEA-I positive cells) were quantified both manually and by automated counting (with ImageJ64, with a threshold of 3-50 pixel square for recognition of LC3 punctae) within equally sized regions with equivalent cell density (as measured by epithelial cell staining coverage of the image). 5 independent slides for each mouse were analysed and 5 mice per group were compared.

Insulitis

Pancreatic tissue was frozen in OCT (Fisher Scientific) prior to cryosectioning into 7 mm slices. Sections were fixed in 10 % formalin at -20°C. After washing in PBS sections were stained with hematoxylin (Fisher Scientific) for 1 min. Sections were rinsed in water and hematoxylin staining was fixed in acid water. After washing with distilled water, sections were rinsed in 0.2% ammonium hydroxide solution. Sections were then first washed with distilled water and then rinsed with 95% ethanol before counter-stained with eosin (alcoholic Eosin Y, Fisher Scientific) for 30 sec. Sequential dehydration steps

were performed in 95% (2x) and 100% (3x) ethanol. Finally sections were preserved in xylene, covered and mounted with Cytoseal60 mounting medium (Electron Microscopy Sciences). Sections were acquired on a light microscope (Olympus BX-60) equipped with a digital camera Olympus DP70 using software (DPManager). Pancreatic islets were scored as having no infiltration, moderate infiltration or severe infiltration.

Measurement of pancreatic insulin content

Pancreata of NOD WT or *Clec16a* KD mice were homogenized in 75% ethanol + 1.5% HCl followed by incubation on ice overnight. Samples were pelleted by centrifugation, the supernatant collected and the remaining pellet was homogenized again in 75% ethanol + 1.5% HCl. After centrifugation, the supernatants were combined and insulin was measured by ELISA.

Thymic epithelial cell isolation

Thymus epithelial cells were enriched using a Percoll (Fisher Scientific) density gradient as described previously (Nedjic et al., 2008), following a protocol kindly provided by Dr. Ludger Klein. Briefly, thymic tissue was cut in a collagenase (Fisher Scientific)/DNase (Roche) solution and incubated at 37^{o} C for 30 min to disaggregate cells and connective tissue. EDTA (10 mM) was added and cells were filtered (40 mm filter) and washed after an additional 5 min incubation at 37 degrees. Cells were then centrifuged through Percoll layers of increasing density (PBS, Percoll 1.06 and Percoll 1.115) at 1350 g for 30 min. Cells at the interface between PBS and Percoll 1.06 were collected, washed and used for immunoblotting and analysis by flow cytometry.

For RNA isolation from thymic epithelial cells, thymi were sequentially digested with Liberase TM (Roche) and DNase (Roche). CD45 cells were depleted using MACS depletion (Miltenyi Biotec) and further enriched for EpCAM+ cells using MACS (Miltenyi Biotec).

Immunoblotting for LC3 and p62

For immunoblotting, cells were lysed (20 mM Tris-HCl pH 7.5, 10 mM NaCl, 2% Triton-X-100, protease inhibitors (Roche)) and lysates were cleared by centrifugation prior to protein concentration measurement using a Pierce 660 nm Protein Assay kit (Fisher Scientific) with a Synergy Mx Microplate Reader (Biotek). Samples were mixed with Laemmli buffer (Boston BioProducts) and boiled for 5 min prior to loading onto a 12% SDS-PAGE gel for size separation. Protein was transferred onto a nitrocelluose membrane and detection was performed with mouse actin antibody (Santa Cruz Biotechnology), rabbit LC3 antibody (GeneTex), rabbit p62 antibody (Enzo Life Sciences), and HRP-conjugated anti-mouse or anti-rabbit antibodies (Fisher Scientific). Band density was quantified using ImageJ64 software on blot images scanned in 16-bit gray scale at 600 dpi.

Immunoblotting for CLEC16A

Cells were lysed in RIPA buffer (Abcam) supplemented with protease inhibitors (Roche). Samples were loaded onto an 8% SDS-PAGE gel. Detection was performed with mouse actin antibody (Santa Cruz Biotechnology) and rabbit Clec16a antibody (Novus Biologicals), and HRP-conjugated anti-mouse or anti-rabbit antibodies (Fisher

Scientific). Band density was quantified using ImageJ64 software on blot images scanned in 16-bit gray scale at 600 dpi.

Immunoblotting for phosphorylated proteins following T cell stimulation

$CD4^+$ T cells were purified magnetically ($CD4^+$ T cell isolation kit, Miltenyi Biotec) and cells were incubated at 37°C in pre-warmed serum-free media. After washing, cells were labeled with biotinylated anti-CD3 and anti-CD4 antibodies on ice. Cells were activated by streptavidin mediated crosslinking for the indicated time points at 37°C. Cells were immediately lysed in RIPA buffer (Abcam) supplemented with protease inhibitors (Roche). Samples were loaded onto a 12% or 8% SDS-PAGE gel. Detection was performed with mouse actin antibody (Santa Cruz Biotechnology), rabbit phospho-ZAP70 (GenScript), rabbit phospho-PLCγ (Cell Signaling Technologies), rabbit phospho-ERK1/2 antibody (Cell Signaling Technologies), and HRP-conjugated anti-mouse or anti-rabbit antibodies (Fisher Scientific). Band density was quantified using ImageJ64 software on blot images scanned in 16-bit gray scale at 600 dpi.

Generation of the MJC1 cell line

The thymus of newborn (4-6 day old) C57BL/6 mice was digested and disaggregated with a mixture of collagenase/dispase/DNase, and cells were stained with EpCAM, CD45 and Ly-51 antibody, and with UEA-1. $EpCAM^+CD45^-$ cells were sorted with a FACSAria instrument (BD Biosciences), and sorted cells were subjected to a second sort to isolate $Ly\text{-}51^+UEA\text{-}1^-$ cortical TECs. TECs were then cultured with irradiated mouse embryonic fibroblasts in DMEM / F12 medium containing hydrocortisone, cholera toxin

and Y-27632. After the 9th passage, cells grew independently of growth factors, and were frozen as stock in liquid nitrogen after the 14th passage.

Lentiviral cell line transduction

HEK293, HeLa and MJC1 cells were infected with lentivirus encoding neomycin resistance and shRNA that targets *CLEC16A* and *ATG5* (HEK293, HeLa), or puromycin resistance and shRNA that targets *Clec16a* and *Atg5* (MJC1), respectively. The target sequence for *CLEC16A* KD was GACTGATGATGTCCTGGATCTG. The target sequence for *Atg5/ATG5* KD was GCAGAACCATACTATTTGCT, and had been characterized previously (Lum et al., 2005). The target sequences for *Clec16a* KD were CACCTTGTACGTCATTTCTATA (KD3) and GAGTGTCCACCTTGTACGTCAT (KD5). Control (CTRL HEK293, CTRL HeLa and ctrl MJC1) cells were infected with lentivirus encoding puromycin resistance and an shRNA that targets a luciferase gene not present in either human or mouse cells.

Cell starvation experiments

Cells were cultured for 24 h in complete media (DMEM supplemented with 10% FCS, 2 mM Glutamine and Penicillin/Streptomycin) or Earle's Balanced Salt Solution (Sigma-Aldrich). Where indicated, cells were treated with 0.5 mM Rapamycin or 5 mM 3-methyladenine (3-MA) (all from Sigma-Aldrich). For measurement of autophagic flux by inhibition of protein degradation, cells were treated with 100 nM Bafilomycin A1 (InvivoGen) or 10 mM E64d (Sigma-Aldrich) for 4 h prior to cell lysis.

Thymocyte differentiation assays

DP thymocytes were sorted by CD4/CD8 staining (antibody-differentiation assays) or CD4 / CD8 / TCR / CD69 staining (TEC-differentiation assays, gating on DP, $TCR^{lo/int}CD69^{-}$) using a FACSAria instrument (BD Biosciences). For antibody stimulation, 48-well cell-culture plates were pre-coated with CD3 (10 mg / ml) and CD5 (10 mg / ml) antibody prior to deposition of cells. For TEC-stimulation, MJC1 cells ($2x10^4$) pre-incubated with IFN-γ for 3 days (10 U / ml, PeproTech) were washed and mixed with sorted DP cells ($2x10^4$) on 96-well plates. Thymocytes were removed, fluorescently labeled and analysed by flow cytometry at the indicated time-points. MHC II levels on MJC1 cells were analyzed by flow cytometry.

LC3-OVA constructs

The dsRed-LC3 encoding fragment of a dsRed-LC3-GFP construct previously published (Sheen et al., 2011) was amplified by PCR and cloned into the pUGM lentiviral vector using the BamHI and NheI restriction sites. The LC3 fragment was amplified using a forward primer that incorporates the sequence for the OVA323-339 peptide (ISQAVHAAHAEINEAGR) together with reverse primers that either left LC3 intact or introduced a 1 amino acid mutation at position 120 (Glycine > Alanine), as described in ref. 31. These OVA-LC3 fragments (native or mutated G120A) were subcloned into the dsRed-LC3 lentiviral vector using the XhoI and NheI restriction sites to to generate vectors encoding dsRed-OVA-LC3 or dsRed-OVA-$LC3_{G120A}$.

Quantitative RT-PCR

RNA was isolated using the RNeasy Mini Kit (Qiagen) or the High Pure RNA isolation Kit (Roche) followed by cDNA synthesis using the Superscript III cDNA synthesis Kit (Invitrogen). RT PCR was performed using Sybr Green (Fisher Scientific). The following primers were used:

Human:

CCCCGGTTTCTATAAATTGAGC and CACCTTCCCCATGGTGTCT (*GAPDH*)

ATGCTGCACTACATCCGAGAT and TCGAGTTCGATCACATGGCTC (*CLEC16A*)

AAAGATGTGCTTCGAGATGTGT and AGGTGTTTCCAACATTGGCTC (*ATG5*).

Mouse:

CCAGCCTTCCTTCTTGGGTAT and TGTTGGCATAGAGGTCTTTACGG *(Actin)*

CCTGATTTGGGGCGATCAAAA and CATAACGGCCTGATTTCTGCC *(Clec16A)*

Statistics

Data were analysed with the Prism software (Graphpad). Diabetes frequency comparisons were carried out using the Log-rank test, except for the experiment shown in Fig. 1e, where Fisher's exact test was used. All other comparisons were performed using a two-tailed t-test, with $P < 0.05$ considered significant. P values indicated by asterisks were as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Variances for t-test comparisons were determined with the Prism software, and were found not to be significanty different. Data met the assumption of the test used, i.e. that data were normally distributed and had similar variance. Sample sizes were approximated in initial experiments, and adjusted to increase power as needed in replicate experiments, based on initially observed effect size.

SUPPLEMENTAL REFERENCES

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