Autophagy links antimicrobial activity with antigen presentation in Langerhans cells

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Conflict of interest: The authors have declared that no conflict of interest exists.

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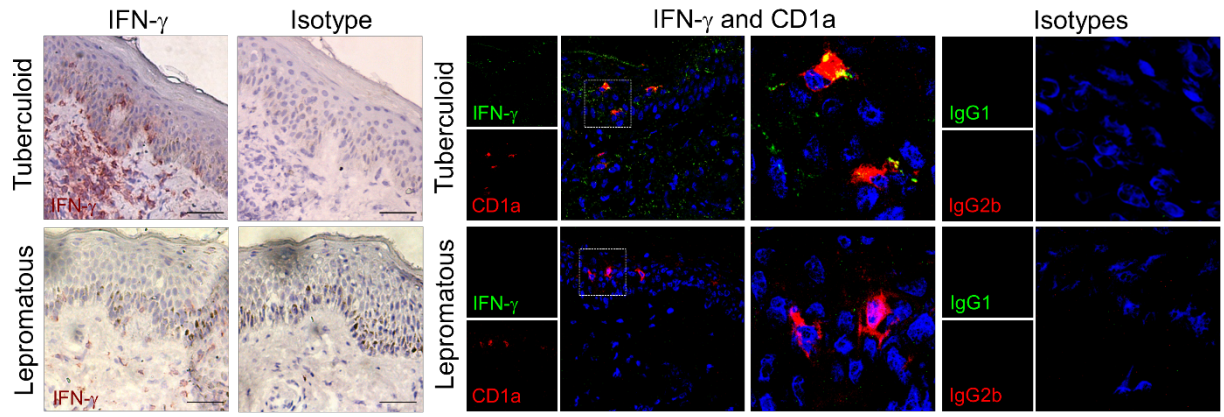
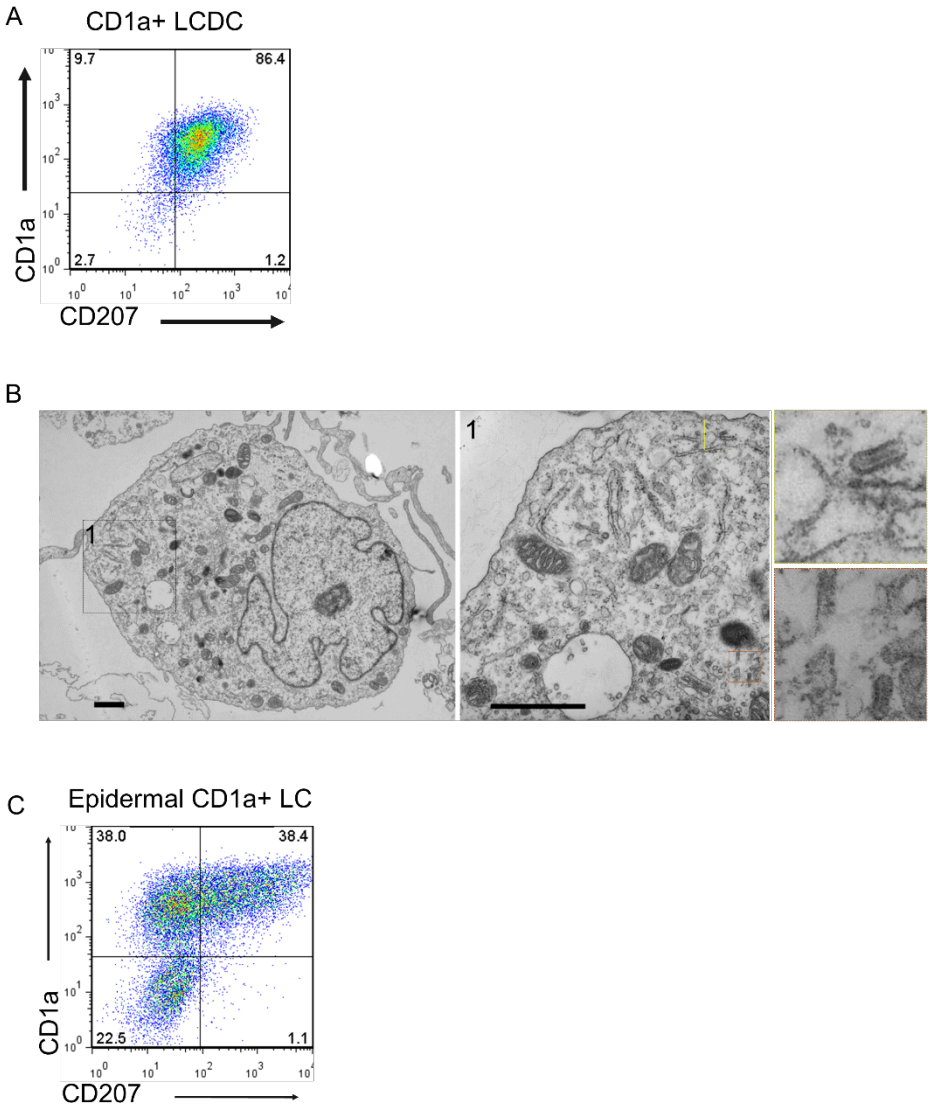
Supplementary Materials:

Supplementary Figure 1



Supplementary Figure1. IFN-γ expression and isotype control in leprosy lesions (T-lep and L-lep); one representative labeled section is shown out of four individuals at 20X; scale bar equal 30 μm. Immunofluorescence labeling of IFN-γ (green) and CD1a (red), or isotype controls, in T-lep and L-lep lesions. Data are representative of three individual T-lep or L-lep samples. Lower magnification images captured on a 20X lens. Higher magnification images captured on a 63X lens.

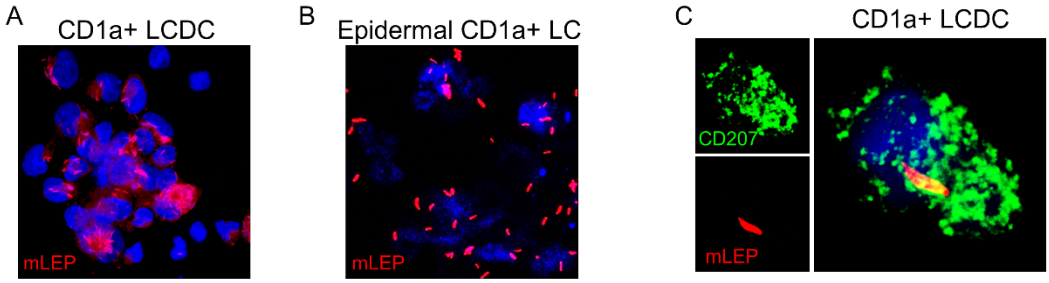
Supplementary Figure 2



Supplementary Figure 2. (**A**) Human CD1a$^+$ LCDC were positively selected following two weeks of culture. CD1a$^+$CD207$^+$ LCDC cell purity, consistently ≥ 90%, was determined by flow cytometry. Representative flow staining of eight independent experiments is shown. (**B**) CD1a$^+$ LCDC were positively selected following two weeks of culture, fixed and processed for electron microscopy. Scale bar represents 1 μm. Micrograph labeled (1) corresponds with magnification shown. Yellow and red boxes indicate Birbeck granules. Representative images of three independent experiments are shown. (**C**) CD1a$^+$ cells were positively selected from human epidermis. CD1a$^+$CD207$^+$ LCDC cell purity,

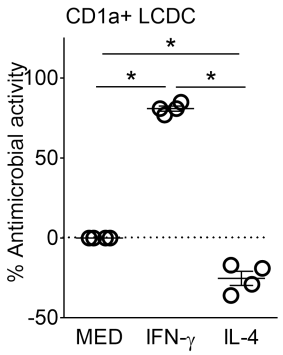
consistently ≥ 70%, was determined by flow cytometry. Representative flow staining of four independent experiments is shown.

Supplementary Figure 3



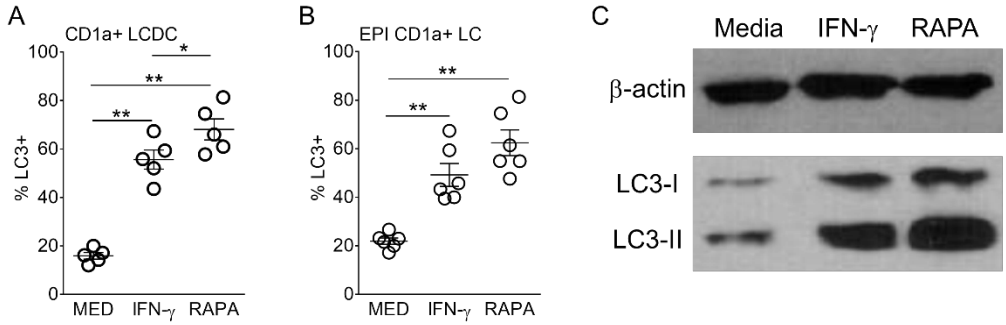
Supplementary Figure 3. (**A**) Human LCDC were infected with PKH26-*M. leprae* (red) at a multiplicity of infection of 10 overnight, fixed, and stained with DAPI (blue). Representative images of three independent experiments are shown. (**B**) Human primary LC were infected with PKH26-*M. leprae* (red) as in (A). Representative images of three independent experiments are shown at 63X. (**C**) Human LCDC were infected with PKH26-*M. leprae* (red) at a multiplicity of infection of 10 overnight, fixed, and stained with anti-CD207 antibody (green) and DAPI (blue). Representative images of five independent experiments are shown. Image captured on a 63X lens, with 6X zoom.

Supplementary Figure 4



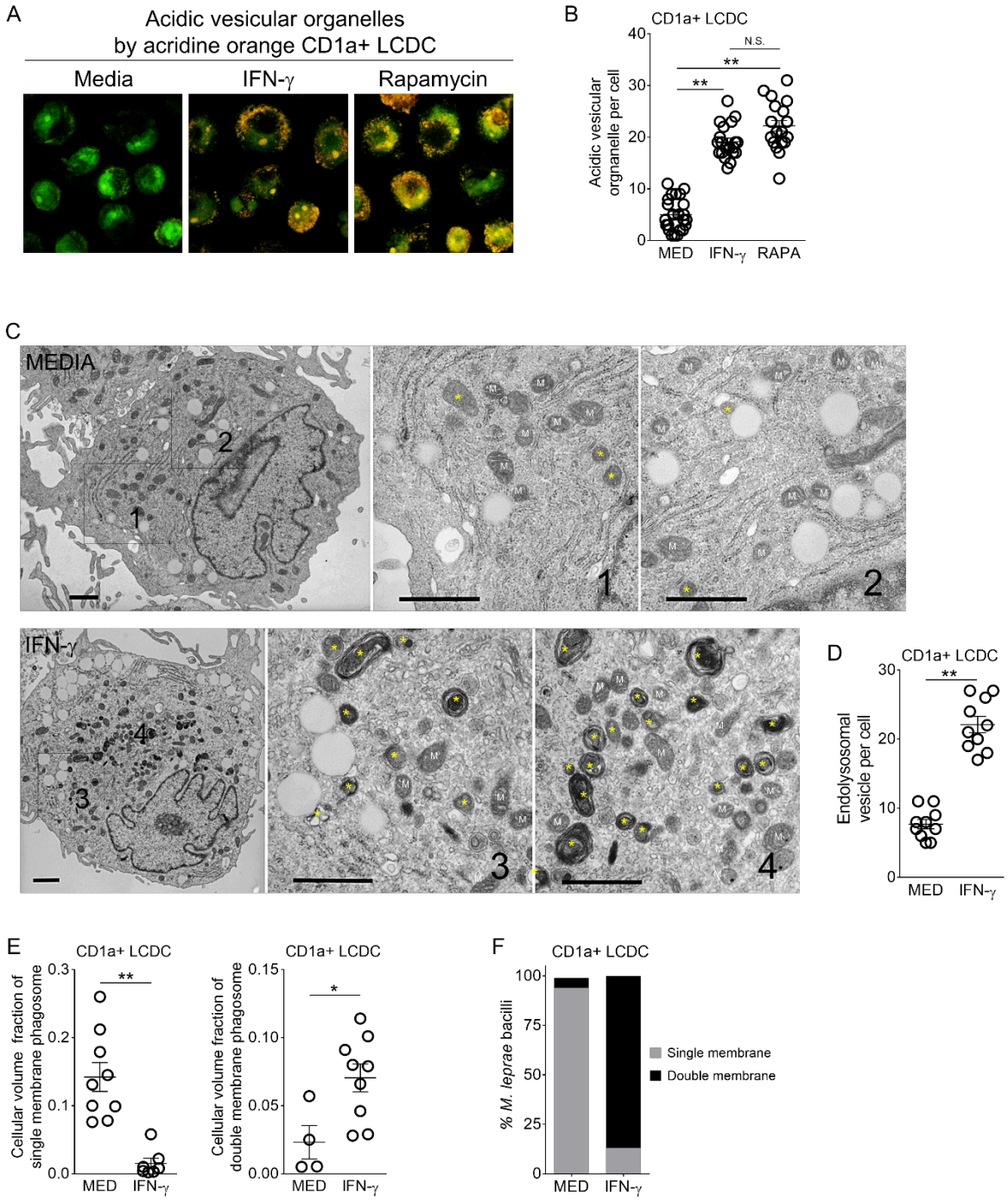
Supplementary Figure 4. Human LCDC were stimulated with rIFN-γ or rIL-4 for four hours, washed and infected with *M. leprae* at a multiplicity of infection of 10 overnight, washed and stimulated with rIFN-γ or rIL-4 for an additional four days. Viability of mLEP was calculated by the ratio of bacterial 16S RNA and DNA (RLEP) detected by qPCR, and percent increase or decrease relative to no treatment (media) was determined. Data are represented as mean ± SEM, n = 4. *$P < 0.05$. Repeated measures one-way ANOVA.

Supplementary Figure 5



Supplementary Figure 5. (**A**) Human LCDC were cultured with rIFN-γ, rapamycin (RAPA) or DMSO (MED) overnight in 10% vitamin D-sufficient human serum, fixed and immunolabeled with anti-LC3 antibody (green) and anti-CD207/langerin antibody (red). Nuclei were stained with DAPI (blue). Representative image depicted in Figure 2A. LC3 punctated cells were quantified. Data are represented as mean of percent positive cells ± SEM, n = 5. (**B**) Human primary $CD1a^+$ LC were cultured as in (A). Representative image depicted in Figure 2C. LC3 punctate cells were quantified. Data are represented as mean of percent positive cells ± SEM, n =6. $*P < 0.05$. $**P < 0.01$. (**C**) LCDC were cultured as in (A). Representative immunoblot images of three independent experiments are shown. Two-tailed student's t-test, or repeated measures one-way ANOVA.

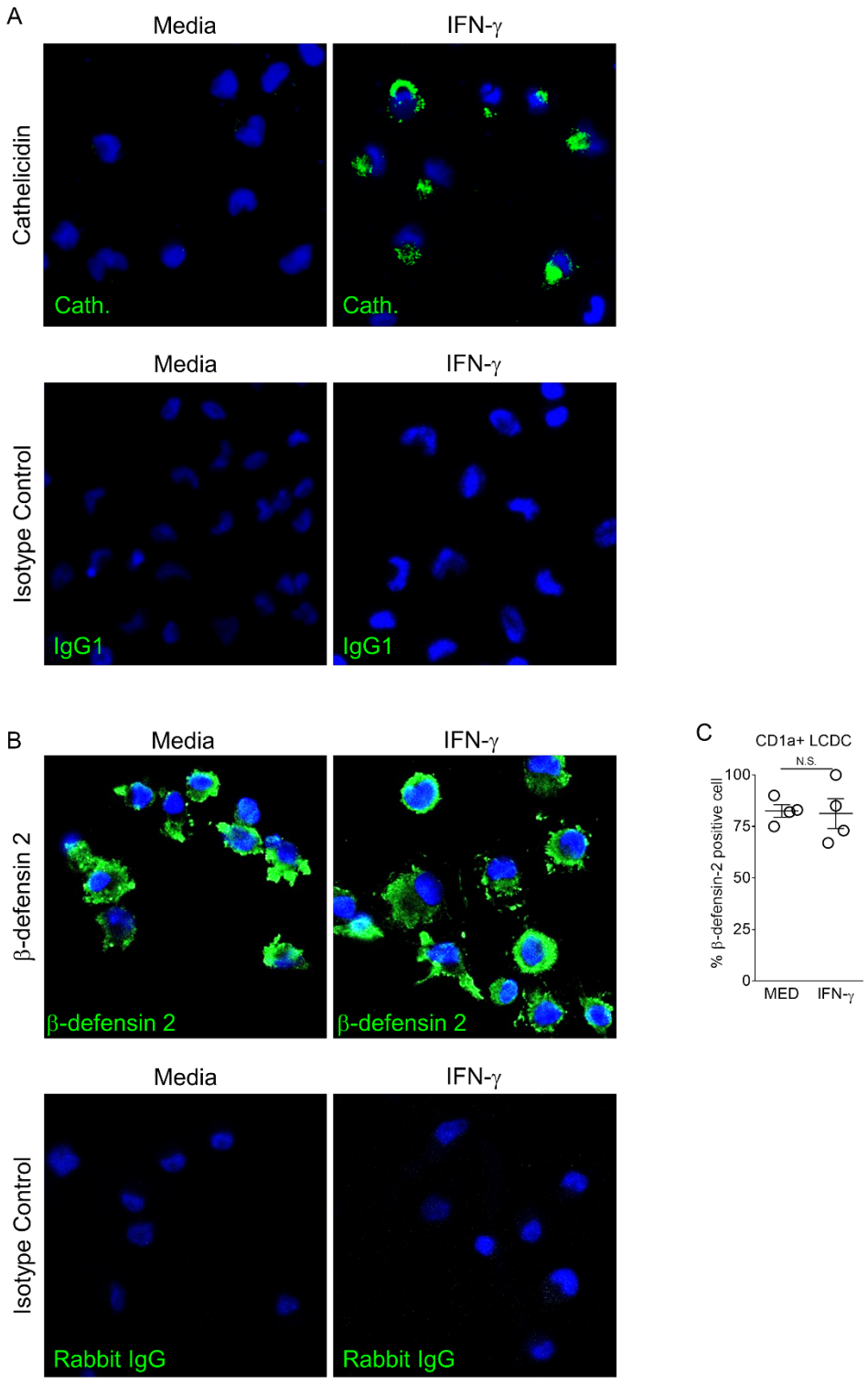
Supplementary Figure 6



Supplementary Figure 6. (**A**) Human LCDC were cultured with rIFN-γ, rapamycin (RAPA) or DMSO (MED) overnight in 10% vitamin D-sufficient human serum, stained with 1 μg/ml acridine orange for 15 minutes. Representative images of cells from three independent experiments are shown at 63X. (**B**) The

number of acidic vesicles (orange puncta) per cell were quantified. Data are represented as mean puncta per cell ± SEM, n ≥ 20 cells. (**C**) LCDC were stimulated with rIFN-γ or medium overnight in 10% vitamin D-sufficient human serum, fixed, and processed for electron microscopy. *M* denotes mitochondria. Yellow * denotes endolysosomal vesicle. Representative images of three independent experiments are shown. (**D**) The number of endolysosomal vesicles per cell was quantified from electron microscopy samples. Data are represented as mean endolysosomal vesicle per cell ± SEM, n = 10 cells. (**E**) The volume fractions of electron microscopy images were calculated by dividing the sum of the area occupied by single or double membrane autophagic compartments by the area of the cell. Data are represented as the mean volume fraction per cell ± SEM, n = 9 cells per condition. $*P < 0.05$. $**P < 0.01$. Two-tailed student's t-test, or repeated measures one-way ANOVA. (**F**) Human LCDC were stimulated with rIFN-γ for 4 hours, washed and infected with PKH26-*M. leprae* (red), at a MOI of 10 overnight, washed and stimulated with rIFN-γ for an additional 4 hours. Infected cells were fixed and processed for electron microscopy. Bacterial bacilli in single membrane phagosomes or double membrane autophagosomes were counted from 4 electron microscopy images.

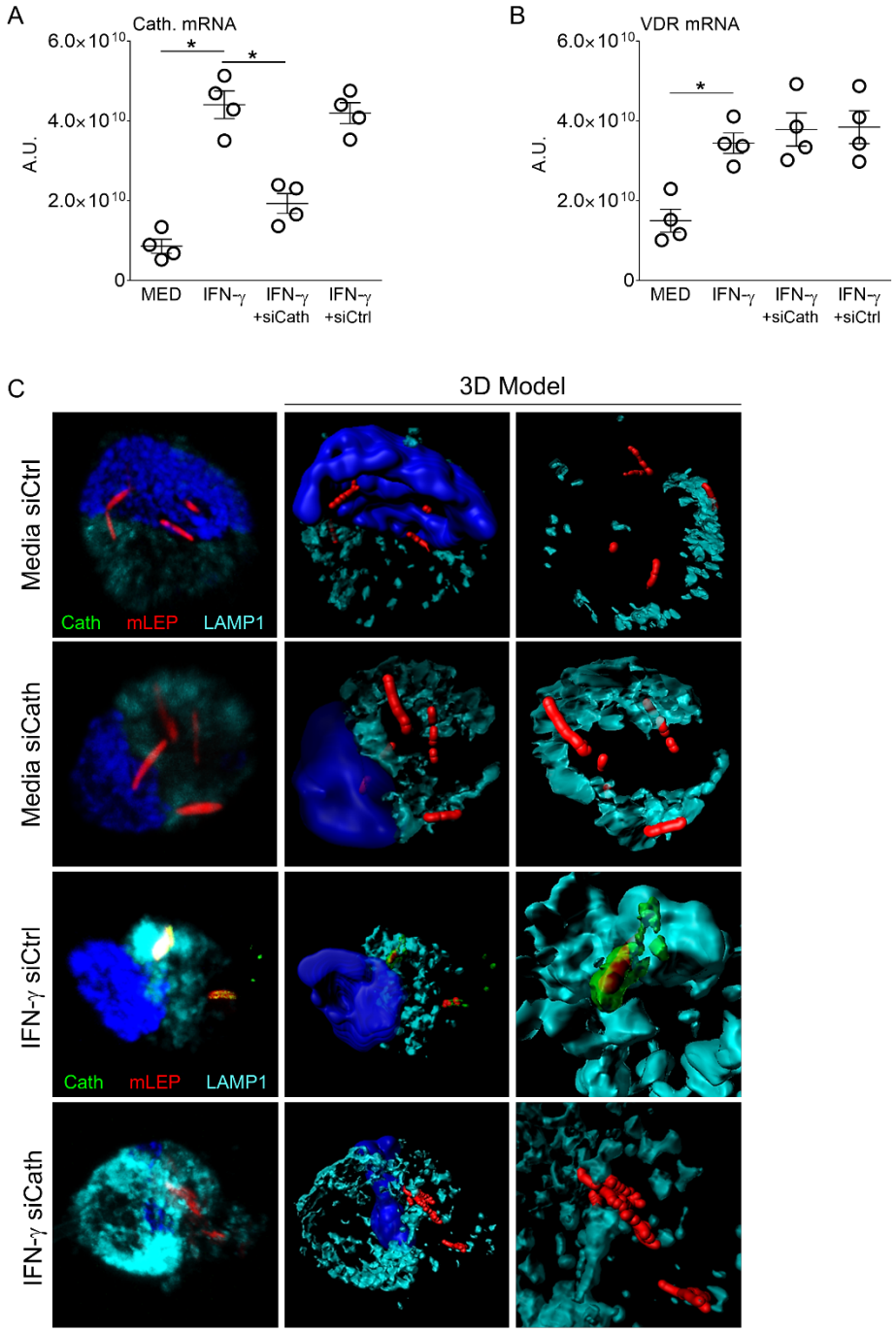
Supplemental Figure 7



Supplementary Figure 7. (**A**) Human LCDC were stimulated with rIFN-γ overnight with Golgi blocker, fixed, and immunolabeled with anti-cathelicidin antibody (green) and DAPI (blue). Representative of

three independent experiments are shown at 63X. (**B**) LCDC were stimulated with rIFN-γ overnight, fixed, and immunolabeled with anti-β-defensin 2 antibody (green) and DAPI (blue). Representative of three independent experiments are shown at 63X. (**C**) The percentage of positive cells were quantified. Data are represented as mean of percent positive cells ± SEM, n = 4. Two-tailed student's t-test.

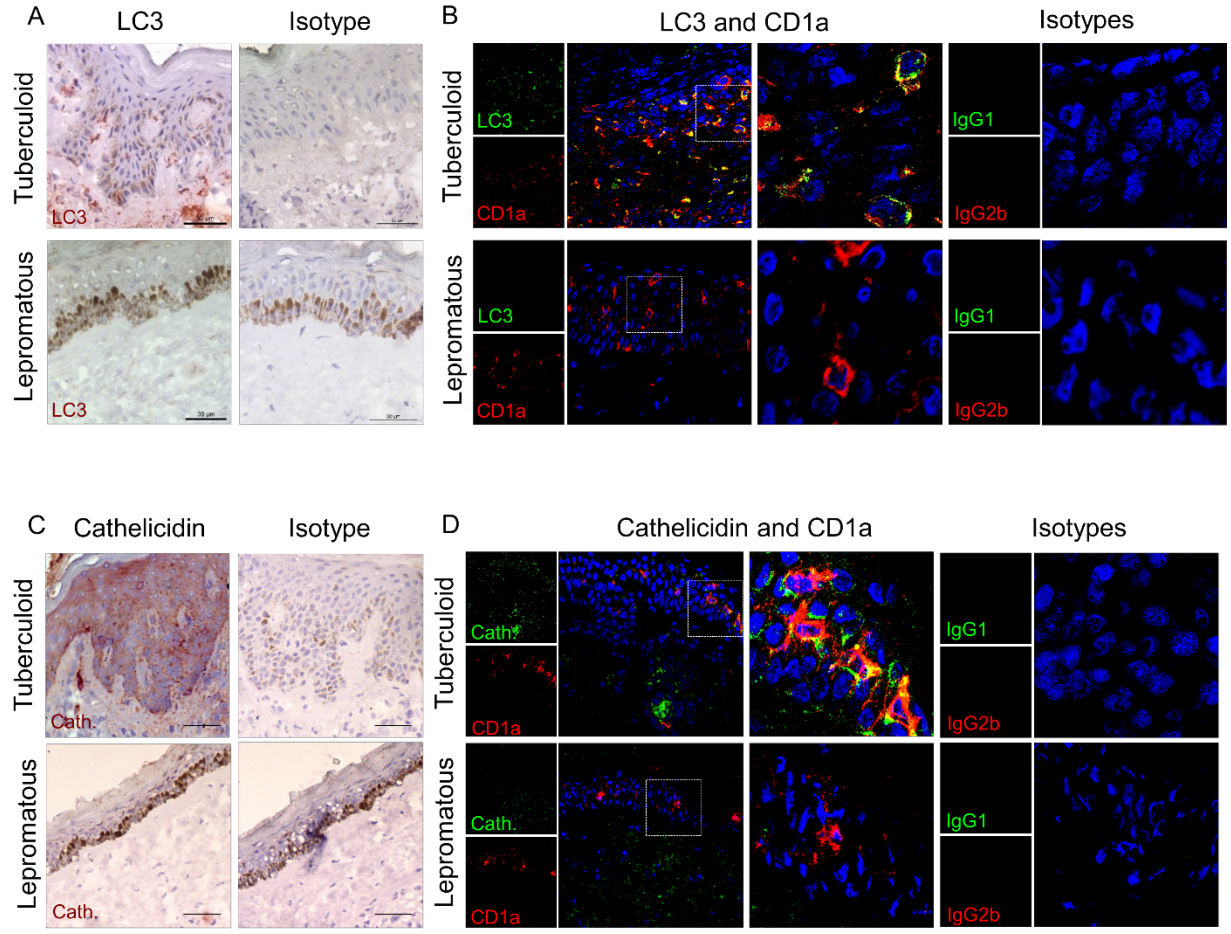
Supplementary Figure 8



Supplementary Figure 8. (**A**) Human LCDC were transfected with siRNA oligos specific for cathelicidin (siCath) or nonspecific (siCtrl) and then treated with rIFN-γ in 10% human vitamin D-sufficient serum for 2 hours. Cathelicidin gene expression was assessed by qPCR. Data are represented as arbitrary units (AU) ± SEM, n = 4. (**B**) LCDC were transfected and stimulated as in (A). Vitamin D receptor (VDR)

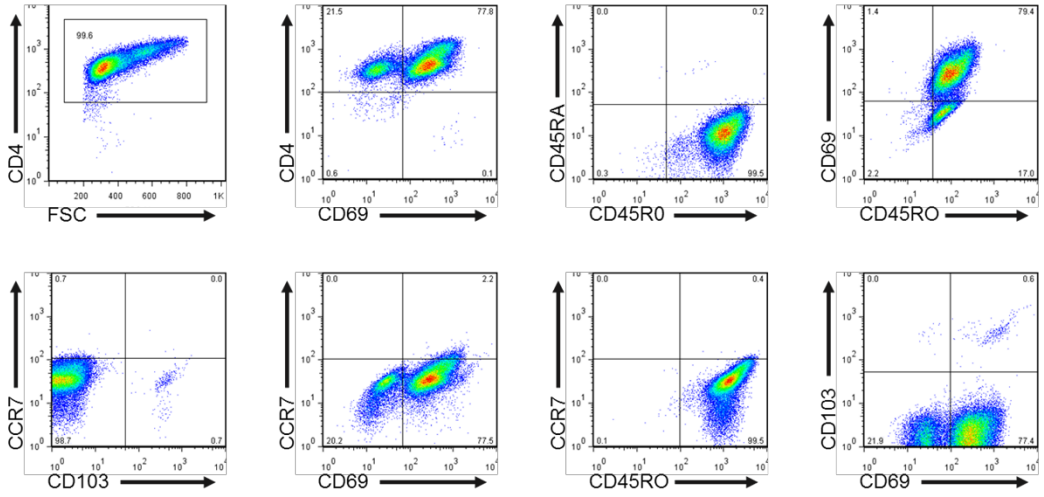
gene expression was assessed by qPCR and was unaffected by siCath transfection. Data are arbitrary units (AU) ± SEM, n = 4. *$P < 0.05$. Repeated measures one-way ANOVA. (**C**) LCDC were transfected with siRNA oligos specific for cathelicidin (siCath) or nonspecific (siCtrl) and then treated with rIFN-γ or medium in 10% human vitamin D-sufficient serum for four hours, washed and infected with PKH26-*M. leprae* (red) overnight, washed and transfected with siRNA oligos and rIFN-γ for an additional four hours, and immunolabeled with anti-cathelicidin antibody (green) and anti-LAMP1 antibody (cyan). Representative immunofluorescence images of three independent experiments are shown. Images captured on a 63X lens, with 6X zoom.

Supplementary Figure 9



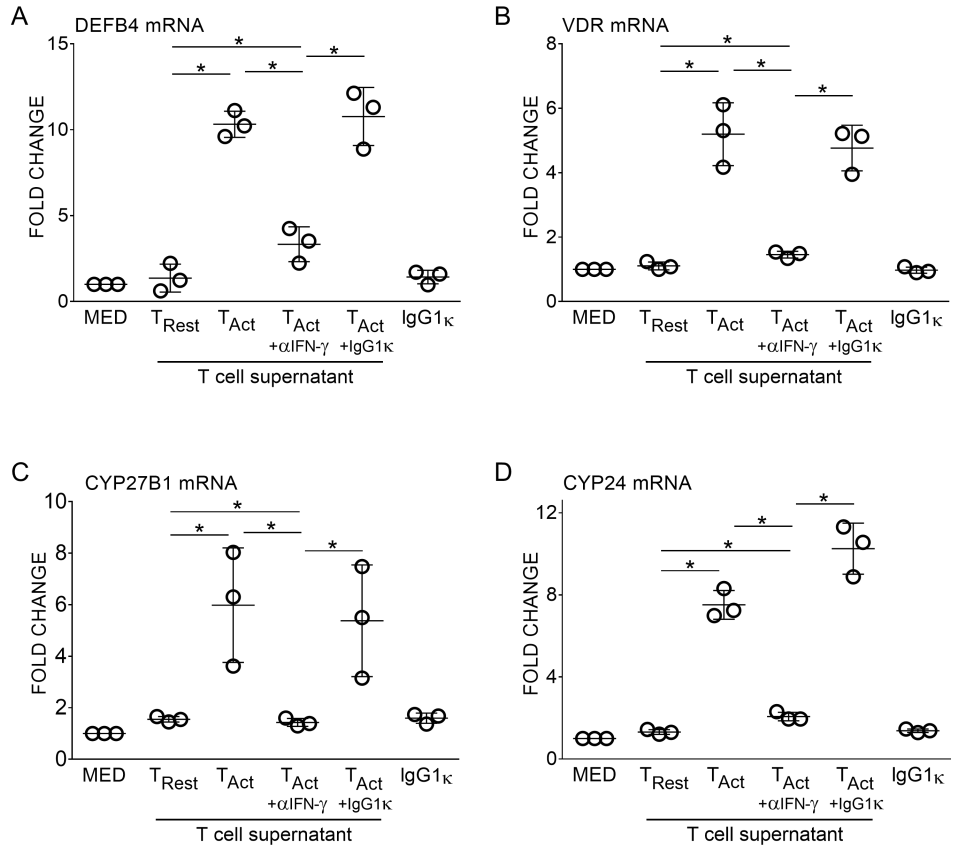
Supplementary Figure 9. (**A**) LC3 expression and isotype control in leprosy lesions (T-lep and L-lep). Data are representative of three individual T-lep or L-Lep samples at 20X; scale bar equal 30 µm. (**B**) Immunofluorescence labeling of LC3 (green) and CD1a (red), or isotype controls, in T-lep and L-lep lesions. Data are representative of three individual T-lep or L-Lep samples. (**C**) Cathelicidin expression and isotype control in leprosy lesions. Data are representative of four individual T-lep or L-Lep samples at 20X; scale bar equal 30 µm. (**D**) Immunofluorescence of cathelicidin (green) and CD1a (red), or isotype controls, in T-lep and L-lep lesions. Data are representative of four individual T-lep or L-lep samples. (**B and D**) Lower magnification images captured on a 20X lens. Higher magnification images captured on a 63X lens.

Supplementary Figure 10



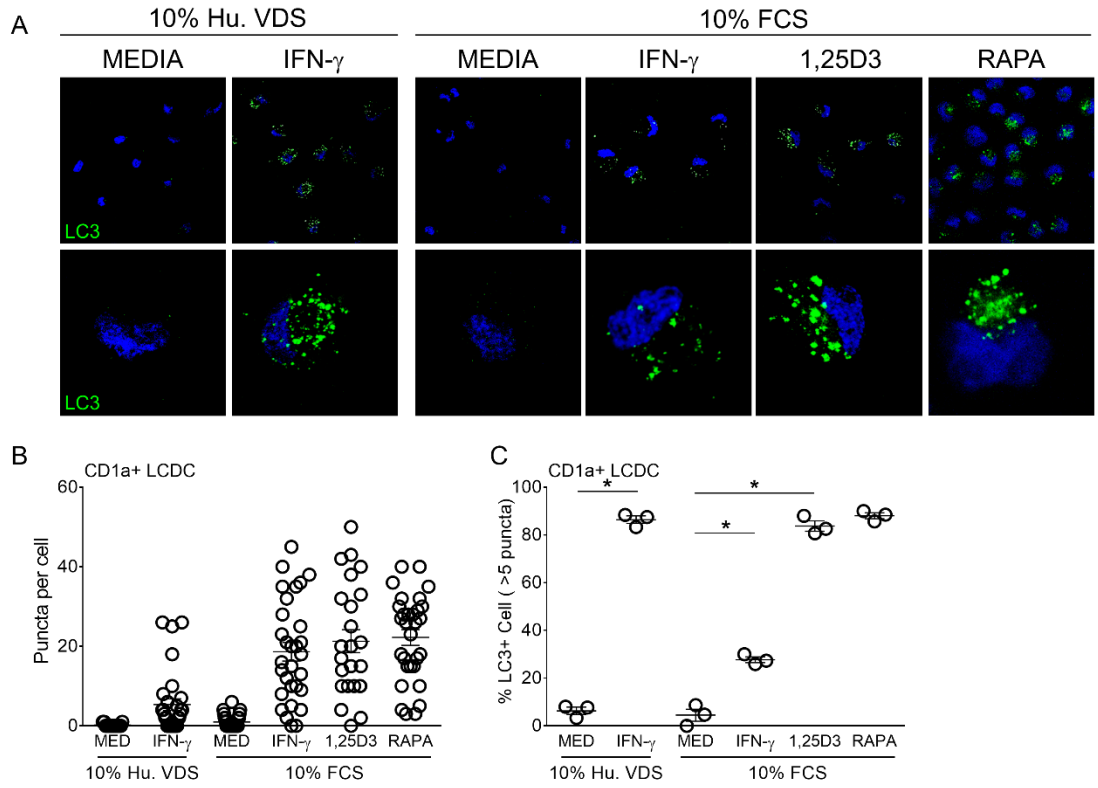
Supplementary Figure 10. Human $CD4^+$ T cells were isolated from a T-lep leprosy lesion. Expression of CD4, CD45RA, CD45RO, CD69, CD103, and CCR7 was analyzed by flow cytometry. Representative flow staining of three independent experiments are shown.

Supplementary Figure 11



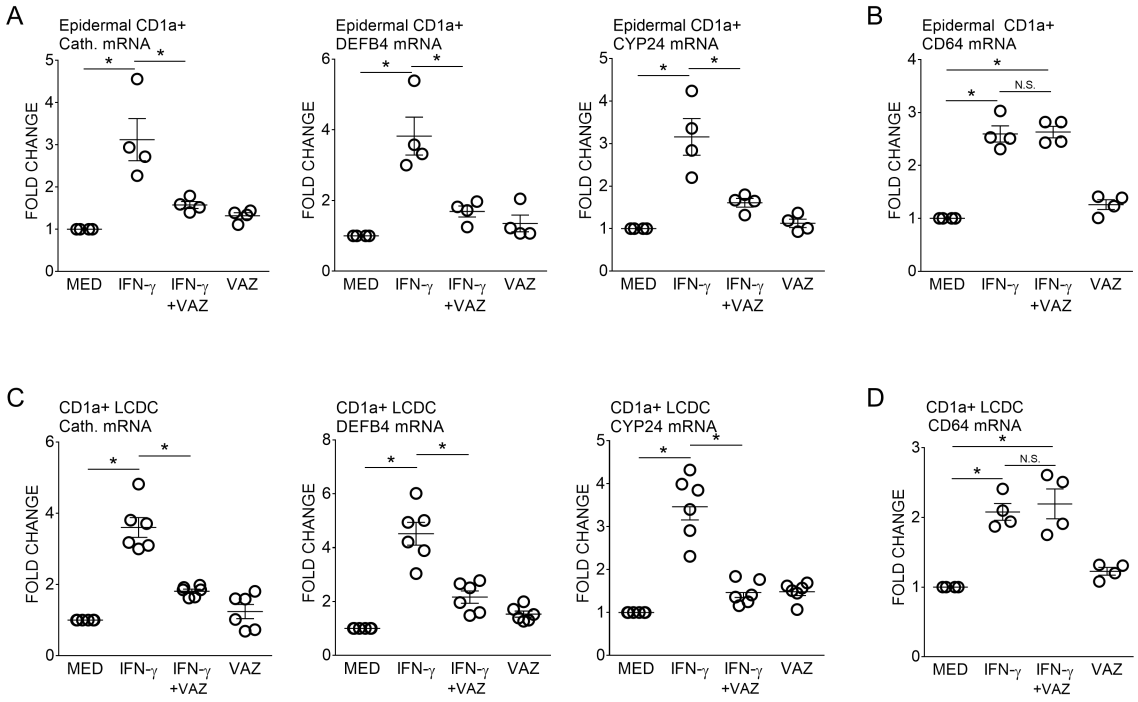
Supplementary Figure 11. Human LCDC were pretreated with blocking mAB to IFN-γ or isotype control antibody for 30 minutes prior to addition of T cell supernatants in 10% human vitamin D-sufficient serum. (**A**) DEFB4 gene expression was assessed by qPCR. Data are represented as mean fold change ± SEM, n = 3. (**B**) VDR gene expression was assessed by qPCR. Data are represented as mean fold change ± SEM, n = 3. (**C**) CYP27B1 gene expression was assessed by qPCR. Data are represented as mean fold change ± SEM, n = 3. (**D**) CYP24 gene expression was assessed by qPCR. Data are represented as mean fold change ± SEM, n = 3. **P* < 0.05. Repeated measures one-way ANOVA.

Supplementary Figure 12



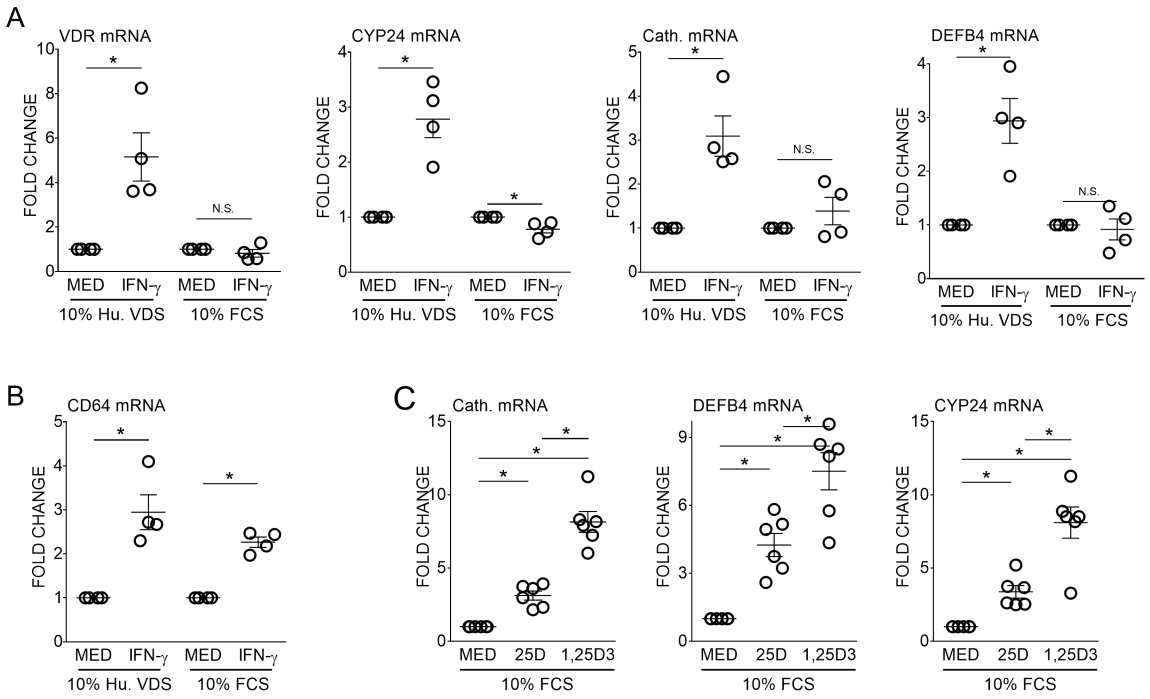
Supplementary Figure 12. (**A**) Human LCDC were cultured with rIFN-γ, or medium overnight in 10% vitamin D-sufficient human serum (25D = 100 nmol/L); or rIFN-γ, 1,25D3, or rapamycin (RAPA) in 10% vitamin D-insufficient FCS (25D = 20 nmol/L), fixed and immunolabeled with anti-LC3 antibody (green). Nuclei were stained with DAPI (blue). Representative immunofluorescence images of three independent experiments are shown. Lower magnification images captured on a 63X lens. Higher magnification images captured on a 63X lens, with 6X zoom. (**B**) LC3 punctated cells were quantified. Data are represented as mean of percent positive cells ± SEM, n = 3 donors. (**C**) The percentage of cell with ≥ 5 puncta per cell were quantified. Data are represented as mean percentage ± SEM, n ≥ 30 cells. **P* < 0.05. Repeated measures one-way ANOVA.

Supplementary Figure 13



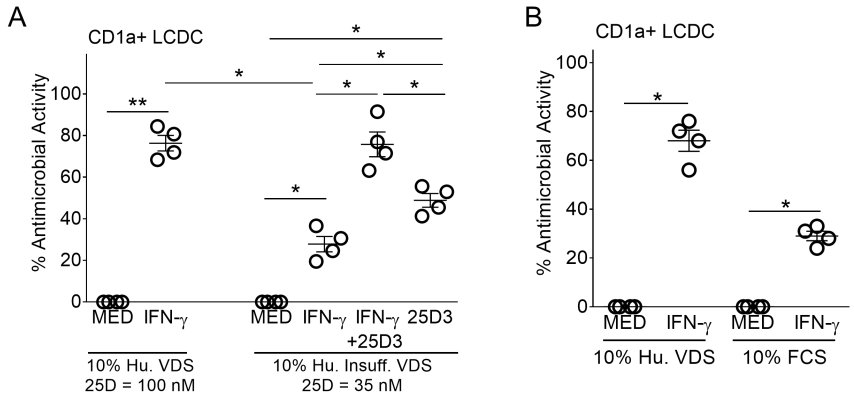
Supplementary Figure 13. (**A**) Human primary CD1a$^+$ LC were cultured in 10% vitamin D-sufficient serum, pretreated with the VDR agonist VAZ (ZK159222) for 30 minutes, and then stimulated with rIFN-γ for 2 hours. Cathelicidin, DEFB4, and CYP24 gene expression was assessed by qPCR. Data are represented as mean fold change ± SEM, n = 4. (**B**) Human primary CD1a$^+$ LC were cultured as in (A), CD64 gene expression was assessed by qPCR. Data are represented as mean fold change ± SEM, n = 4. *$P < 0.05$. (**C**) Human LCDC were cultured as in (A), cathelicidin, DEFB4, and CYP24 gene expression was assessed by qPCR. Data are represented as mean fold change ± SEM, n = 6. (**D**) Human LCDC were cultured as in (A), CD64 gene expression was assessed by qPCR. Data are represented as mean fold change ± SEM, n = 6. *$P < 0.05$. Repeated measures one-way ANOVA.

Supplemental Figure 14



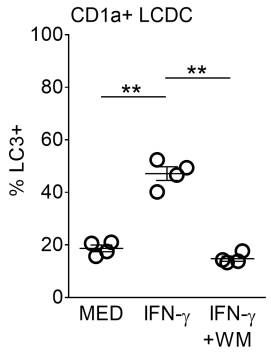
Supplementary Figure 14. (**A**) Human LCDC were stimulated rIFN-γ in 10% vitamin D-sufficient human serum or 10% vitamin D-insufficient FCS for 2 hours. VDR, CYP24, cathelicidin, and DEFB4 gene expression was assessed by qPCR. Data are represented as mean fold change ± SEM, n = 4. (**B**) Human LCDC were stimulated as in (A). CD64 gene expression was assessed by qPCR. Data are represented as mean fold change ± SEM, n = 4. (**C**) Human LCDC were stimulated with 25D or 1,25D in 10% vitamin D-insufficient FCS for 2 hours. Cathelicidin, DEFB4, and CYP24 gene expression was assessed by qPCR. Data are represented as mean fold change ± SEM, n = 6. *$P < 0.05$. Repeated measures one-way ANOVA.

Supplementary Figure 15



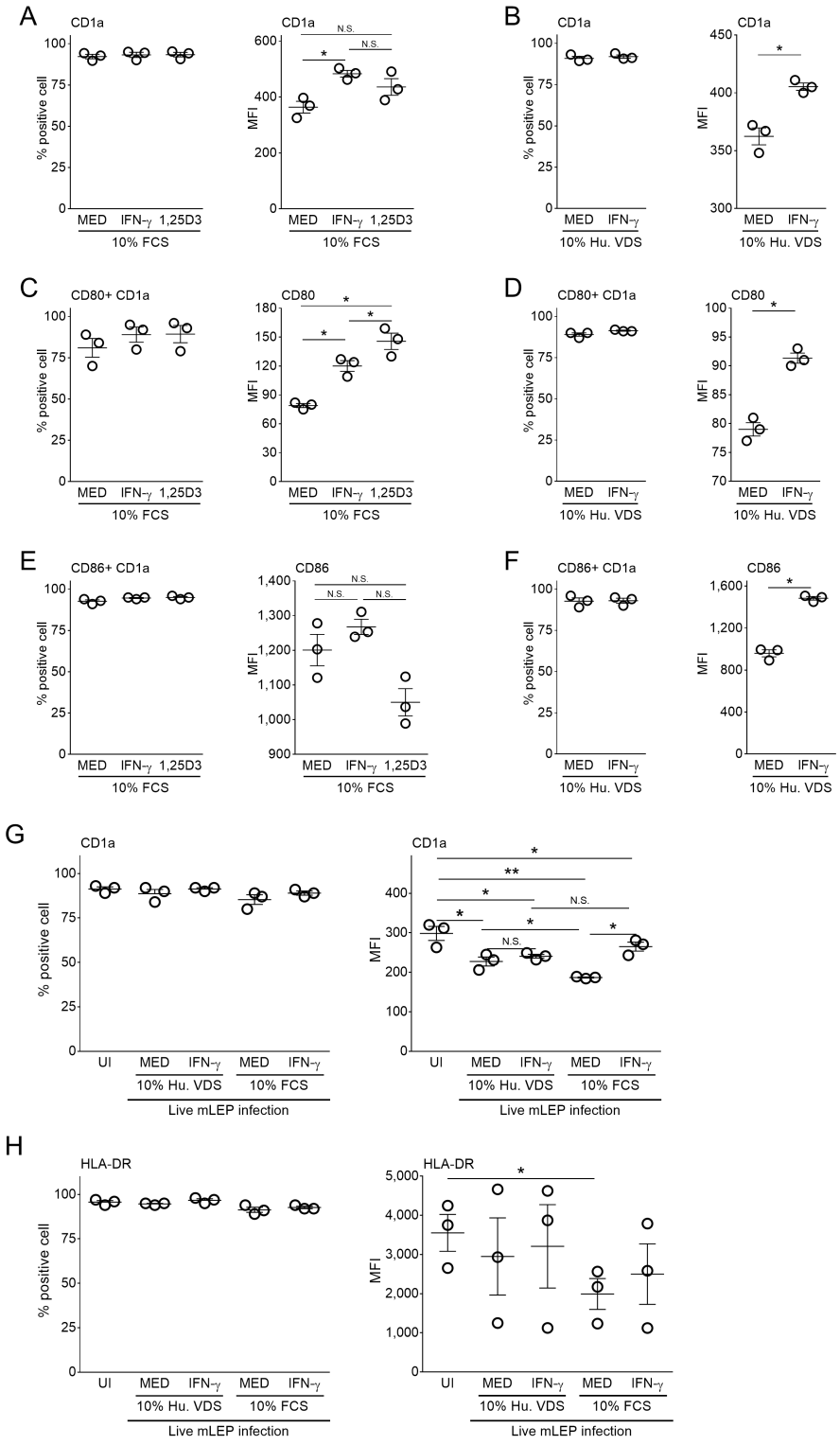
Supplementary Figure 15. (**A**) Human LCDC were stimulated with rIFN-γ for 4 hours in 10% vitamin D-sufficient human serum or vitamin D-insufficient human serum, with or without the addition of 25D3 to reach sufficient concentration; washed and infected with *M. leprae* at a multiplicity of infection of 10 overnight. Infected cells were washed and stimulated with rIFN-γ in 10% vitamin D-sufficient human serum or vitamin D-insufficient human serum, with or without the addition of 25D3 to reach sufficient concentration for an additional four days. Viability of mLEP was calculated by the ratio of bacterial 16S RNA and DNA (RLEP) detected by qPCR, and percent increase or decrease relative to no treatment (media) was determined. Data are represented as mean ± SEM, n = 4. (**B**) Human LCDC were stimulated with rIFN-γ for 4 hours in 10% vitamin D-sufficient human serum or vitamin D-insufficient FCS, washed and infected with *M. leprae* at a multiplicity of infection of 10 overnight. Infected cells were washed and stimulated with rIFN-γ in 10% vitamin D-sufficient human serum or vitamin D-insufficient FCS for an additional 4 days. Viability of mLEP was calculated as in (A). Data are represented as mean ± SEM, n = 4.*$P < 0.05$. **$P < 0.01$. Repeated measures one-way ANOVA.

Supplemental Figure 16



Supplementary Figure 16. Human LCDC were cultured with rIFN-γ, with or without pretreatment of the autophagy inhibitor wortmannin (WM) or DMSO (MED), or medium overnight in 10% vitamin D-sufficient human serum, fixed and immunolabeled with anti-LC3 antibody (green). Nuclei were stained with DAPI (blue). Representative image depicted in Figure 4D. LC3 punctate cells were quantified. Data are represented as mean of percent positive cells ± SEM, n = 4. $*P < 0.05$. $**P < 0.01$. Repeated measures one-way ANOVA.

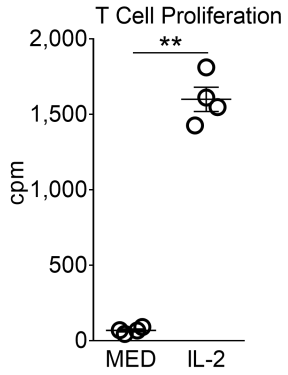
Supplementary Figure 17



Supplementary Figure 17. (**A**) Human LCDC were stimulated with rIFN-γ or 1,25D3 in 10% vitamin D-

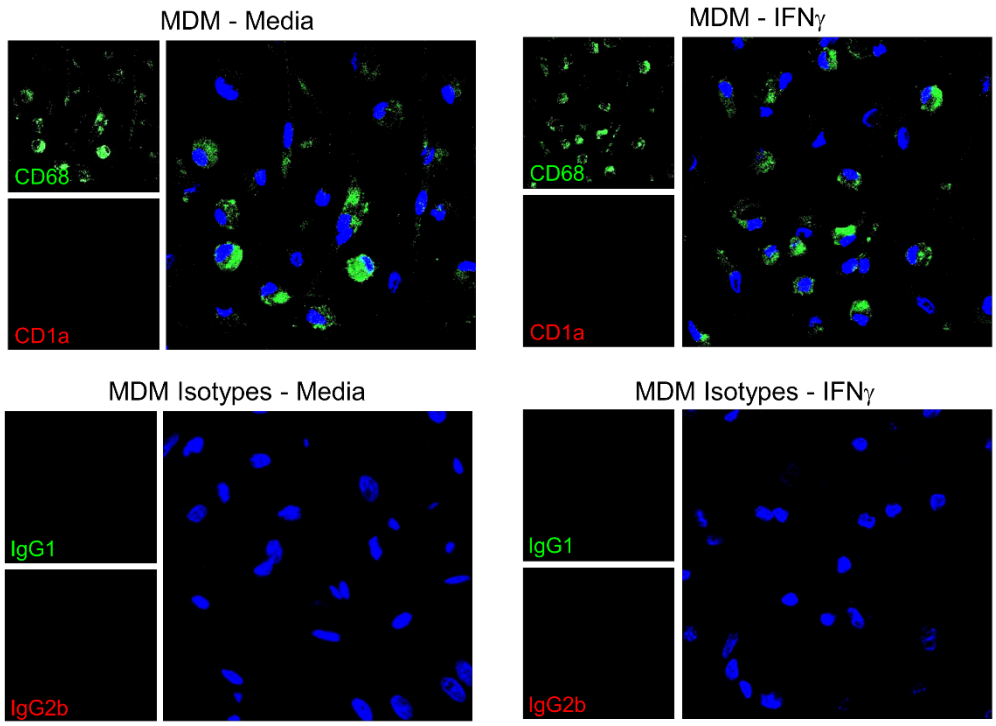
insufficient FCS overnight. Cells were fixed and expression of CD1a was analyzed by flow cytometry. (**B**) Human LCDC were stimulated with rIFN-γ or medium in 10% vitamin D-sufficient human serum overnight. Cells were fixed and expression of CD1a was analyzed by flow cytometry. (**C**) Human LCDC were stimulated with rIFN-γ or 1,25D3 in 10% vitamin D-insufficient FCS overnight. Cells were fixed and expression of CD80 was analyzed by flow cytometry. (**D**) Human LCDC were stimulated with rIFN-γ or medium in 10% vitamin D-sufficient human serum overnight. Cells were fixed and expression of CD80 was analyzed by flow cytometry. (**E**) Human LCDC were stimulated with rIFN-γ or 1,25D3 in 10% vitamin D-insufficient FCS overnight. Cells were fixed and expression of CD86 was analyzed by flow cytometry. (**F**) Human LCDC were stimulated with rIFN-γ or medium in 10% vitamin D-sufficient human serum overnight. Cells were fixed and expression of CD86 was analyzed by flow cytometry. (**G**) Human LCDC were stimulated with rIFN-γ or medium in 10% vitamin D-sufficient human serum or 10% vitamin D-insufficient FCS for 4 hours, washed, and infected with PKH26-*M. leprae* at a MOI of 10 overnight. Infected cells were washed and stimulated with rIFN-γ or medium in 10% vitamin D-sufficient human serum or 10% vitamin D-insufficient FCS for 4 hours. Cells were then fixed and CD1a was analyzed by flow cytometry. (**H**) Human LCDC were stimulated and infected as in (G). Infected cells were fixed and HLA-DR was analyzed by flow cytometry. Data are represented as mean percentage or MFI ± SEM, n = 3. $*P < 0.05$. Repeated measures one-way ANOVA.

Supplementary Figure 18



Supplementary Figure 18. LCD4.G were treated with 1 nM recombinant IL-2 as positive control. Companion for Figure 4K. Data are represented as mean ± SEM of triplicate cultures, n = 4. Two-tailed student's t-test.

Supplementary Figure 19



Supplementary Figure 19. Human monocyte-derived-macrophages (MDM) were stimulated with rIFN-γ overnight, fixed, and immunolabeled with anti-CD68 antibody (green) and anti-CD1a antibody (red). Nuclei were stained with DAPI (blue). Representative immunofluorescence images of four independent experiments are shown at 63X.

Supplementary Table 1

	Alleles	LCDC1	LCDC2	LCDC4	LCDC5	LCDC6	LCDC7	LCDC8	LCDC9	LCDC10	LCDC11	MDM42	MDM58	MDM241	MDM252	MDM273	MDM296
CD1a-restricted T cells	DRB1 04:03									X							X
	DRB1 11:04															X	
	DRB3 02:02:01G			X	X	X		X						X	X	X	
	DRB4 01:01:01G	X	X	X				X	X	X	X		X				X
	DQB1 03:01				X	X								X		X	
	DQB1 03:02									X	X						X

Supplementary Table 1. (**A**) Human LCDC, MDM, and LCD4.G T cells were genotyped for MHC class II alleles.