RESEARCH ARTICLE

CLEC16A regulates splenocyte and NK cell function in part through MEK signaling

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Abstract

CLEC16A is implicated in multiple autoimmune diseases. We generated Clec16a inducible knockout (KO) mice to examine the functional link between CLEC16A auto-inflammation and autoimmunity. Clec16a KO mice exhibited weight loss and thymic and splenic atrophy. Mitochondrial potential was lowered in KO mice splenocytes resulting in aggregation of unhealthy mitochondria in B, T, and NK cells. In Clec16a KO mice we detected disrupted mitophagy in splenic B and T cells. NK cells from Clec16a KO mice exhibited increased cytotoxicity. Incomplete mitophagy was attenuated with PI3K and/or MEK inhibition in Clec16a KO mice. Our results demonstrate a functional link between CLEC16A and disrupted mitophagy in immune cells and show that incomplete mitophagy predisposes the KO mice to inflammation. Taken together, loss of function variants in CLEC16A that are associated with decreased CLEC16A expression levels may contribute to inflammation in autoimmunity through disrupted mitophagy. Drugs modulating mitophagy reverse the process and may be effective in treating and preventing autoimmunity in individuals with risk associated CLEC16A variants.

Introduction

Genome-wide association studies (GWAS) have consistently identified associations between single nucleotide polymorphisms (SNPs) in the 16p13 locus harboring the C-type lectin-like domain family 16A (CLEC16A) gene and type-1 diabetes (T1D)[1–5]. CLEC16A locus polymorphisms have also been associated with the susceptibility to several other autoimmune diseases, including multiple sclerosis [6, 7], primary adrenal insufficiency [8], Crohn’s disease[8, 9], primary biliary cirrhosis[10], juvenile idiopathic arthritis[11, 12], rheumatoid arthritis[12]
CLEC16A and mitophagy

and alopecia areata[13], suggesting that CLEC16A could be a master regulator of aberrant immune responses in autoimmunity. Despite the strong associations of CLEC16A with numerous autoimmune and inflammatory disorders little is known about the function of CLEC16A or its role in disease pathogenesis. No study to date has investigated the possible connection of CLEC16A’s role in immune cells.

The first evidence of CLEC16A function came from studies on its drosophila ortholog-Ema, an endosomal membrane protein required for endosomal trafficking and maturation. Human CLEC16A expression was shown to rescue the drosophila null mutant demonstrating conserved protein function across species[14]. Ema is also required for normal autophagosomal growth and autophagy[15]. We recently showed that loss of Clec16a leads to an increase in Parkin, the master regulator of mitophagy, in mice with pancreas-specific deletion. Islets of these mice had abnormal mitochondria with reduced oxygen consumption and ATP concentration. Patients with the CLEC16A T1D risk variant, rs12708716-G, have reduced expression of CLEC16A in islets and attenuated insulin secretion[16], providing further evidence that CLEC16A could control β-cell function and contribute to diabetes risk through complex interactions between resident pancreatic cells, NK cells and potentially other immune cells where CLEC16A is known to play a key role[17]. Likewise, a role of CLEC16A in autophagy and neurologic diseases was reported in another independent mutant mouse model of Clec16a[18]. Thus, CLEC16A is becoming an attractive candidate for functional studies to explore the pathogenic mechanisms and therapeutic options through interventions at the protein level of CLEC16A in autoimmune diseases.

Given the association of CLEC16A to several autoimmune disorders we sought to understand the role of CLEC16A in immune cells. Mitochondrial dysfunction may contribute to the pathogenesis of autoimmune disease. Autoimmune diseases develop as a consequence of a synergistic combination of genetic predisposition, largely unknown environmental triggers, and immunologic events. We hypothesized that reduced expression of CLEC16A leads to disrupted mitophagy. In this study we used our novel whole body inducible Clec16a knockout model (UBC-Cre-Clec16a\textsuperscript{loxP}) along with autophagy pathway inhibitors to delineate the role of CLEC16A in mitophagy in immune cells contributing to immune dysregulation. We demonstrate that reduction in CLEC16A is associated with disrupted mitophagy and defective mitochondria, effects that were attenuated by PI3K and/or MEK inhibitors.

Materials and methods

UBC-Cre-Clec16a\textsuperscript{loxP/loxP} mice

The Institutional Animal Care and Use Committee (IACUC) of the Children’s Hospital of Philadelphia approved all animal studies. All methods were performed in accordance with the IACUC guidelines and regulations.

Mice were group-housed on an individually-ventilated cage rack system on a 12:12 light:dark cycle. Mice were fed standard rodent chow and water \textit{ad libitum}. Clec16a\textsuperscript{loxP} mice were generated by flanking exon 3 (Ozgene). Mice with targeted insertion in the Clec16a gene were crossed to the Flpo Deleter line (mouse Strain: 129S4/SvJae-Gt (ROSA) 26Sortm2(FLP\textsuperscript{ERT2}) Sor/J; The Jackson Laboratory) to achieve deletion of the FRT-flanked Neomycin cassette. Clec16a\textsuperscript{loxP} mice were mated to UBC-Cre-ERT2 mice (inducible cre recombinase driven by the human ubiquitin C promoter) obtained from The Jackson Laboratory to generate UBC-Cre-Clec16a\textsuperscript{loxP} mice. Mice were kept on mixed background C57BL/6-129S1.

Ten-week-old UBC-Cre-Clec16a\textsuperscript{loxP/loxP} mice were treated with tamoxifen (100 mg/kg/day) to induce knockout of Clec16a (Clec16a KO) or vehicle (10%: ethanol: 90% corn oil) for a control group by gavage at 24-hour intervals for five consecutive days. Clec16a\textsuperscript{loxP/loxP} mice were administered tamoxifen or vehicle and used as control groups.
All inhibitors were purchased from Sigma-Aldrich (St. Louis, MO, USA) with purity of greater than 98% confirmed by HPLC. For inhibitor experiments, ten-weeks-old UBC-Cre-Clec16a\(^{loxP}\) mice were treated with tamoxifen (100 mg/kg/day) or vehicle (10%: ethanol: 90% corn oil) for control group by gavage (10 ml/kg) and vehicle or inhibitor (i.p., 10 ml/kg) at 24-hour intervals for five consecutive days. Vehicle (specific for inhibitor) or inhibitor were injected 30 minutes prior to either vehicle or tamoxifen gavage. Vehicle for U0126 (10 mg/kg) and Wortmannin (2 mg/kg) was 1% DMSO: 10% Cremaphor: 89% of 0.09% saline and for Bafilomycin A1 (1 mg/kg) was 1% DMSO: 99% of 0.09% saline. Stock solutions were created for each compound and frozen. Fresh compound was made daily prior to injection. All animals were sacrificed according to humane endpoint or four weeks after initiation of the experiment.

**Quantitative real-time PCR**

Total RNA was isolated with Trizol, purified using the RNAeasy Mini Kit (Qiagen) and converted to cDNA by High Capacity RNA-to-cDNA Kit (Applied Biosystems). Human and murine CLEC16A RNA transcripts and control genes (\(\beta\)-Actin and HRPT1) were measured by real time PCR on a ViiA™ 7 Real Time PCR. Triplicates were used for all the samples. PCR runs were performed on ViiA™ 7 Real Time PCR System using ViiA7 RUO software v1.2.2 (Life Technologies).

**Transmission electron microscopy**

Purified B, T, and NK cells were prepared for transmission electron microscopy (TEM) as previously reported to analyze mitochondrial defects\[^19\]. Briefly, cells were washed with pre-warmed PBS twice, and fixed in 2% glutaraldehyde in PBS for 60 minutes. Then, cells were washed with PBS 3 times and post fixed with 1% osmium tetroxide in PBS for 1 hour. Cells were rinsed 3 times with distilled water and were further stained with 1% tannic acid for 1 hour and then infiltrated and embedded in Epon resin. Ultrathin sections of 70 nm were generated with an ultramicrotome (Ultracut 7, Leica Microsystems) and post-stained with 2% aqueous uranyl acetate and Reynold’s lead citrate for 10 minutes each. Samples were examined with a JEOL 2100 TEM at an accelerating voltage of 200 kV.

**Cell cultures**

The YAC-1 target was grown at 37˚C in 5% CO\(_2\) in complete RPMI medium (Gibco). Complete’ indicates supplementation with 10% FBS, L-glutamine, nonessential amino acids, sodium pyruvate, HEPES and penicillin-streptomycin (all from Gibco). Murine splenic NK, T and B cell suspensions were negatively selected using EasySep Mouse NK, T and B cell Enrichment Kits, following the manufacturer’s instructions (StemCell).

**Flow cytometry**

To assess mitochondria membrane potential in control and knockout, rmIL-15 activated splenocytes, cells were stained with MitoTracker® Deep Red FM (31.3nM) and analyzed by FACS. Cell-associated fluorescence was assessed with FACSCalibur flow cytometer (BD Pharmingen) and analyzed with FlowJo software.

**NK cell cytotoxicity assays**

Splenocytes from the control and Clec16a KO mice where specified were used as effector against \(^{51}\)Cr-labeled YAC-1 targets at effector-to-target cell (E: T) ratios of 50:1, 25:1, 12.5:1, 6.25:1, and 3.12:1. In-vitro IL-15 stimulation of murine splenocytes was performed by adding
100 ng/ml of rmIL-15 for 48hrs where indicated. All test conditions were performed in triplicate, and supernatants harvested after 4 hours of incubation at 37˚C were evaluated for the presence of $^{51}$Cr using a Top Count XL counter and Lumiplate scintillation system (Beckman Coulter). Cytotoxicity was defined as the lysis of YAC-1 target cells. Maximal release was determined by counts obtained after the incubation of target cells in 0.5% NP40.

**Western blot**

Briefly, lysis was performed with NP40 lysis buffer. The lysates were electrophoresed on 4–12% NuPAGE Bis-Tris gels in MOPS SDS running buffer and transferred onto nitrocellulose membranes (Invitrogen) overnight. The membranes were blocked in 3% BSA and incubated with indicated primary antibodies where specified for: mouse CLEC16A, PINK1 (Abgent), GFP, Nrdp1 (Novus Biologicals), TOM20 (ProteinTech), Parkin, p62/SQSTM1, LC3 I/II, ATG16L1, cytochrome c, caspase-9, p-ERK1/2 and EK1/2 (Santa Cruz), cleaved Caspase-3, p-Akt (ser473), p-Akt (Ser308) and total Akt (Cell signaling. The membranes were washed and incubated with a respective mouse/rabbit secondary antibody and bound antibody was detected with WesternBright ECL kit (Advansta). Membranes were stripped and reprobed for β-actin as loading control.

**Interferon-γ (IFN-γ) ELISA**

Human IFN-γ was detected using DouSet ELISA system (R&D System) as per manufactures instructions. EBV-immortalized lymphoblastoid cell lines generated from Type1 diabetes patients were activated with 100 ng/ml PMA plus 1 μg/ml ionomycin (PMA+I). Culture supernatants were collected after 48 hrs of induction. Supernatants were collected at indicated time points and IFN-γ was measured by DouSet ELISA system.

**Mouse Cytokine Array**

The Proteome Profiler Mouse Cytokine Array Kit, Panel A (ARY006, R&D Systems) was used to determine systemic chemokine/cytokine profile. Briefly, plasma was diluted and mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture was then incubated with the array membrane overnight at 4˚C. The membranes were washed and incubated with streptavidin-horseradish peroxidase followed by chemiluminescent detection. The array data were quantitated to generate a protein profile, and results are presented as average signal (pixel density) of the pair of duplicate spots representing each cytokine or chemokine analyzed using Image-J software (NIH).

**Statistical analysis**

All graphs denote mean values, and error bars represent the SE. Data were analyzed using unpaired Student’s t-test or factorial analysis of variance (ANOVA) as applicable using Prism 7 (GraphPad Software, Inc). Multiple sets of data were compared using one-way and two-way ANOVA Tukey’s multiple comparison test where appropriate. P values less than 0.05 were considered statistically significant.

**Results**

To investigate CLEC16A function, we generated inducible whole-body Clec16a knockout mice (UBC-Cre-Clec16aloxP). Adult mice (10 weeks old) were treated with tamoxifen to induce Clec16a Knockout shown in supplemental materials (S1 Fig). Control littermate mice received an equal volume of vehicle or tamoxifen. Recombination was confirmed by PCR using DNA
isolated from whole blood (Fig 1A). Knockout was confirmed by RT-PCR on RNA (Fig 1B) and Western blot for CLEC16A protein expression from control and KO mice splenocytes (Fig 1C). The Clec16a KO mice exhibit significant reduction in body weight as early as day 9 compared to controls. The decrease became more significant over the course of the study (Fig 1E). In addition to weight loss, Clec16a KO mice exhibit atrophy of the thymus (top panel) and

Fig 1. Characterization of Clec16a KO in tamoxifen-inducible UBC-Cre-Clec16a<sup>loxP/loxP</sup> mice. (A) PCR analysis of genomic DNA isolated from whole blood of KO and control (vehicle and TAM treated) mice show PCR products of 618bp in controls and 146bp in KO, confirming a removal of Clec16a exon 3 in KO mice. (B) Relative CLEC16A mRNA expression by RT-PCR in murine splenocytes normalized to HPRT1. RT-PCR results show means±SE of three experiments. (C) Representative Western blot of CLEC16A expression in murine splenocytes. (D) Quantitation graph depicting CLEC16A protein expression (n = 3 repeats). (E) Reduction in total body weight of Clec16a KO mice. Multiple t tests were used to analyze data. Statistical significance was determined using the Holm-Sidak method. ***P<0.001, **P<0.01, *P<0.05. (F) Thymus image from control (vehicle), control (TAM) and KO (top panel). Spleen image from control (vehicle), control (TAM) and KO mice (bottom panel). (G) Total splenocyte numbers from control and KO mice (n = 10). Significances of differences among groups were evaluated using an unpaired Student’s t-test ***P<0.001. (H) Thymus weight/body weight ratios for control (vehicle or TAM) and KO groups. (I) Spleen weight/body weight ratios for control (vehicle or TAM) and KO groups. Dashed line represents vehicle controls. Significances of differences were analyzed using multiple t-tests with discovery determined using the Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 1%. Computations assume that all rows are sample from populations with the same scatter (SD). Sidak’s multiple comparison populations with the same scatter (SD) ***P<0.001 and ****P<0.0001 between groups. Sidak’s multiple comparisons test *P<0.05, **P<0.01, and ***P<0.0001 compared to Day 0 within groups test.

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spleen (bottom panel) (Fig 1F) and significant reduction of total splenocyte numbers (Fig 1G). The thymus exhibited acute atrophy in KO mice. The Clec16a KO mice exhibited significant decrease in thymus weight and thymus/body weight ratios as early as day 9 compared to controls. The decrease became more significant over the length of the study (Fig 1H). Spleens showed a gradual regression in size. The Clec16a KO spleen weight/body weight ratio became significant by day 18 in comparison to controls (Fig 1I). Control vehicle and control-TAM littermates showed no atrophy of thymus and spleen.

Since essentially no thymus was left in Clec16a KO mice, to examine the possibility of a mitochondrial defect we measured the mitochondria membrane potential (MMP) in splenocytes isolated from Clec16a KO mice and control littermates. Resting and cells activated with murine recombinant interleukin-15 were evaluated for change in mitochondrial membrane potential using flow cytometry. Control and Clec16a KO unstimulated splenocytes showed no significant difference (S2A and S2B Fig). At 24h post activation we observed a 19% decrease in the percentage of active mitochondria in activated KO mice splenocytes compared to activated controls. This difference increased 48h post activation, reaching 29% compared to activated controls. Lowering of mitochondrial membrane potential is depicted as change in mean fluorescence intensity post IL-15 activation (Fig 2A and 2B). Clec16a KO was confirmed in an immunoblot analysis shown in supplement materials (S2C Fig). We also measured levels of P62 and LC3 I/II from resting and activated splenocytes lysates in an immunoblot analysis to assess autophagic flux. Resting Clec16a KO splenocytes showed significant increase in P62 and LC3-I/II expression in comparison to resting controls. With activation the levels of accumulated P62 and LC3 I/II further increased significantly in IL-15 activated splenocytes (S2D and S2E Fig). Activated control splenocytes showed no significant change for P62 and LC3-I/II. Thus, Clec16a KO activated splenocytes exhibit lowered mitochondrial membrane potential and defective autophagic flux.

We next performed Transmission Electron Microscopy (TEM) of splenic immune cells (B, T and NK cells) isolated from control and Clec16a KO mice to compare the mitochondrial morphology. Clec16a KO mice show dramatic qualitative changes in mitochondria and vacuolar morphology in immune cells (splenic B, T, and NK cells: control (Fig 2C–2E) vs Clec16a KO (Fig 2F–2H). Specifically, TEM images reveal changes in the mitochondrial membrane architecture and accumulation of disordered, disintegrated, elongated and fragmented mitochondria and vacuolated structures in the Clec16a KO mice in all immune cell types (B, T, and NK cells). Fragmented and elongated mitochondria with abnormal cristae, engulfed in vacuolated structures are clearly visible in zoomed in images of B (S3 Fig) and T cell (S4 Fig).

We next evaluated the whole splenocytes lysates from control (Vehicle) ±Bafilomycin and Clec16a KO±Bafilomycin treated mice in an immunoblot analysis for possible mitophagy/autophagy signaling defects. Bafilomycin A1, a selective inhibitor of the vacuolar-type H+ -adenosine triphosphatase blocks autophagic degradation. We observed reduced Nrdp1 expression in Clec16a KO mice compared to controls (Fig 2I and 2J). As anticipated, Clec16a KO mice splenocyte lysates showed increased expression of PINK1, Parkin and TOM20 and further accumulation upon bafilomycin treatment. Vehicle treated splenocyte lysates showed barely detectable levels of PINK, Parkin and P62. We found significant increase in p62 and LC3-II expression in Clec16a KO compared to control. P62 is degraded by autophagy and inhibition of autophagy increases its abundance. Bafilomycin treatment did not significantly alter the P62 and ATG16L1 levels. The rise in LC3-II following Bafilomycin treatment was small, suggesting a defect in autophagic flux. In light of decreased splenocyte numbers, we hypothesized there was increased cell death in Clec16a KO mice spleen. To test that, we examined the relative abundance of cytochrome-c and caspase-9 in Clec16a KO mice splenocytes to determine if disrupted mitophagy contributed to cell death. We detected significant increase in
Fig 2. *Clec16a* knockout disrupts mitophagy *in-vivo* in response to lowered mitochondrial membrane potential resulting in aggregation of fragmented mitochondria in splenic immune cells. (A) The histogram depicts lowered mitochondrial membrane potential in splenocytes from *Clec16a* KO mice (*n* = 7) upon *Clec16a* KO in comparison to control-vehicle treated (*n* = 6) littermates using MitoTracker Deep Red FM dye in a flow-based assay. (B) Mitochondrial membrane potential depicted as change in mean fluorescence intensity (MF) for two time points, 24h and 48h. (C-H) Representative Transmission Electron Microscopy images of immune cells (splenic B, T and NK cells: control (Vehicle) (C-E) vs *Clec16a* KO (F-H). (I) Representative immune blot images depicting Nrdp1/PINK1/Parkin.
both cytochrome-c and caspase-9. Further evaluation in immunoblot analysis revealed a significant increase in expression levels of cleaved caspase-3 in Clec16a KO mice in comparison to control littermates. Splenocyte lysates from Bafilomycin treated KO mice showed further increase in caspase-9 and cleaved caspase-3 (Fig 2I), providing evidence that the intrinsic pathway of cell death is activated in these settings. Control treated Bafilomycin splenic lysate showed significant increase in the expression of ATG16L1, P62 and LC3-I/II compared to untreated controls. Bafilomycin treatment in control mice did not alter the expression of CLEC16A, Nrdp1, PINK1, and Parkin (S2F and S2G Fig). Thus, Clec16a knockout lowers mitochondrial membrane potential and triggers defective mitophagy in vivo.

We hypothesized that reduced expression of Clec16a in mouse leads to disrupted mitophagy via Nrdp1/PINK/Parkin pathway. To examine the signaling underlying the morphologic changes we observed in splenic immune cells of Clec16a KO by TEM, we performed immunoblot analysis. Since B-cells (60%) and T-cells (27%) contribute to the majority of the cell population of the spleen, we specifically evaluated pure B and T cells isolated from murine splenocytes (Fig 3A and 3C). Western blot analysis of purified B (Fig 3A and 3B) and T cell lysates (Fig 3C and 3D) in Clec16a KO showed significantly elevated levels of PINK1 and Parkin. Control lysates showed barely detectable levels of PINK and Parkin for both cell types. We observed significant increase and accumulation of P62 in B cells (Fig 3A and 3B) and T cells of KO mice (Fig 3C and 3D). Increased accumulation of P62 indicate that mitophagy is disrupted and incomplete in Clec16a KO B and T cells. We also evaluated the T cell subpopulations from control and Clec16a KO mice (S5A–S5C Fig). We observed a significant decrease for both CD4+ and CD8+ effector cell population (S5B and S5C Fig). However, naive and memory T cells in CD4+ and CD8’ cells remained unchanged. The decrease in effector population possibly could be attributed to increased apoptosis.

Since our whole body inducible Clec16a mice displayed thymic atrophy, and T cells play a key role in regulating the immune responses, to specifically address the role of Clec16a in T cells we generated CD4-Cre Clec16alox/lox mice. We followed CD4-Cre Clec16alox/lox mice up to 30 weeks of age and found no significant difference between the control and CD4 Clec16a KO mice thymic and splenic T cell subpopulation (S6A and S6B Fig). These results suggest that the CLEC16A loss alone in T cell is not enough to produce the phenotype shown in the whole-body inducible knockout.

Given that NK cells constitute 1% of the total spleen immune cell population, and that the decrease we observed occurred in the total splenocyte numbers in KO mice, we performed functional evaluation of NK cells in Clec16a KO mice. We examined NK cytotoxicity in splenocytes isolated from Clec16a KO mice and controls with and without 48-hour rmIL-15 activation in a standard 4-hour 51Cr release assay. As expected, resting control splenocytes exhibited minimal killing of YAC-1 targets (7.8±1.8%; at 50:1 Effector: Target). By contrast, resting murine NK cells from Clec16a KO mice demonstrated increased killing (14.5±1.8%), a two-fold significant increase in cytotoxicity that remained significant at all effector:target ratios as compared to controls (Fig 4A). Cytotoxicity increased after IL-15 mediated activation in both the groups. At identical effector: target ratios, activated-control splenocytes demonstrated 30±2.5% cytotoxicity. Clec16a KO mice exhibited increase in cytotoxicity, with 45
Fig 3. Clec16a knockout mice exhibit disrupted mitophagy in splenic B and T cells. (A) Representative Immunoblot analysis depicting expression levels of CLEC16A, PINK1, Parkin, p62, and β-actin in pure B cells isolated from control (Vehicle) and KO mice splenocytes. (B) Graph of quantitation depicting protein expression levels. Data expressed as means±SE of three independent experiments. Significances of differences among groups were evaluated using two-tailed unpaired Student’s t-test. (C) Representative Immunoblot depicting expression levels of CLEC16A, PINK1, Parkin, p62, and β-actin in pure T cells isolated from control and Clec16a KO mice splenocytes. (D) Graph of quantitation depicting protein expression levels. Data expressed as means±SE of three independent experiments. Membranes were stripped and reprobed for β-actin as a loading control. *P<0.05, **P<0.01, ***P<0.001. Significances of differences among groups were evaluated using unpaired Student’s t-test.

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±2.7% that remained significant at all effector:target ratios. Thus, Clec16a knockout results in increased NK cytotoxicity in Clec16a KO mice.

We previously reported that the minor allele (A) at SNP rs2903692 is protective against T1D and associates with higher relative abundance of CLEC16A mRNA in NK cells. We predicted that possessing this allele will result in restrained NK cell functions. To test this, we evaluated EBV cell lines generated from type-1 diabetes patients homozygous for protective alleles (A/A) with higher levels of CLEC16A for IFN-γ production compared to the G/G homozygotes. As anticipated, the EBV cell lines generated from type-1 diabetes patients homozygous for protective alleles (A/A) with higher levels of CLEC16A show significantly reduced IFN-γ production compared to the G/G homozygotes (Fig 4B). Collectively, these results demonstrate that under normal conditions, CLEC16A plays a critical role in shaping the initial pro-inflammatory response possibly by constraining major NK cell functions.

In light of enhanced intrinsic cell death and mitophagy defect observed above experiments were designed to determine whether PI3K inhibitor-Wortmannin and MEK inhibitor-U0126 could rescue the disrupted mitophagy observed in Clec16a KO mice. We treated mice with Wortmannin or U0126 and interrogated the mitophagy pathway in an immunoblot analysis for rescue in whole splenocytes lysates. In Clec16a KO, Nrdp1 expression significantly decreased in comparison to control (Fig 5A). Wortmannin alone had no effect on Nrdp1, PINK1, Parkin, P62, LC3-I/II and caspase-9 protein expression. The small decrease observed in CLEC16A expression with Wortmannin is unclear if this effect is related to Nrdp1 and could suggest a Nrdp1-independent role of CLEC16A. Parkin, P62, and Casp-9, and LC3-II showed significant increase in expression in Clec16a KO mice compared to control. Wortmannin-treated Clec16a KO mice showed a significant reversal in expression of PINK1, Parkin, P62, LC3-I/II and caspase-9 in comparison to KO (Fig 5A). To further confirm the specificity and involvement of PI3K signaling, we probed for p-Akt and total Akt in controls: Wortmannin and Clec16a KO+Wortmannin treated mice. In Clec16a KO, we observed significant increase in p-Akt levels in comparison to control (Fig 5C and 5D). Wortmannin treatment significantly decreased the Akt phosphorylation in keeping with induced activation of Akt signaling in Clec16a KO mice.

Fig 4. NK cytotoxicity of mouse splenic NK cells and IFN-γ secretion in human EBV-immortalized lymphoblastoid cell lines. (A) Cytotoxicity of resting and mIL-15 (100ng/ml) activated splenocytes depicting killing of 51Cr-labeled YAC-1 targets from control (C) and Clec16a KO mice. Results include means±SE of five experiments (n = 5). (B) Interferon-γ release in T1D EBV cell lines measured by ELISA. EBV-immortalized lymphoblastoid cell lines generated from Type1 diabetes patients were activated with 100 ng/ml PMA plus 1 μg/ml ionomycin (PMA+I). Culture supernatants were collected after 48 hrs of induction and IFN-γ was measured. “G/G” represents non-protective allele and “A/A” represents the protective allele for Type 1 diabetes. Results are means±SE of three independent repeats. C vs. KO (‘P<0.05, **P<0.01, ***P<0.001), C vs. C+IL-15 (*P<0.05, **P<0.01, ***P<0.001), KO vs. KO+IL-15 (*P<0.05, **P<0.01, ***P<0.001), C+IL-15 vs. KO+IL-15 (P<0.05, **P<0.01) by two-way ANOVA with Tukey’s multiple comparison test.

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Fig 5. Wortmannin and U0126 reverse the disrupted mitophagy in Clec16a KO mice. (A) Representative western blot from splenocyte lysates of Control±Wortmannin and KO±Wortmannin treated mice depicting expression levels of CLEC16A, Nrdp1, PINK1, Parkin, P62, LC3 I/II, caspase-9 and rescue of disrupted by Wortmannin. Membranes were stripped and reprobed for β-actin as a loading control. (B) Quantitation graph depicting expression levels of CLEC16A, Nrdp1, PINK1, Parkin, P62, LC3 I/II and Caspase-9 normalized to β-actin. Data expressed as means±SE of three independent experiments. (C) Representative western blot image of p-Akt (Ser473 or Ser308) and total Akt. (D) Quantitation graph depicting expression levels of p-Akt (Ser473 or Ser308). (E)
We next evaluated rescue of disrupted mitophagy with the MEK inhibitor, U0126. We undertook a cell fractionation approach and performed immunoblot analysis on cytosol, nuclear and mitochondrial fraction (Fig 5E). The cytosolic fraction from Clec16a KO splenocytes showed detectable levels of PINK1, Parkin and p62. The mitochondrial fraction in KO showed significant up-regulation of PINK1, Parkin, and P62 and reverted the LC3-II expression similar to control mice. PINK, Parkin and P62 were barely detectable in the cytosol and mitochondrial fractions of control mice. PINK1, Parkin, P62 and LC3-I/II were not detected in nuclear fractions of any mice (Fig 5E). U0126 inhibits MEK1 and MEK2, and thus ERK activation. To confirm the signaling involved and specificity of inhibitor, we probed splenocytes lysate for p-ERK and total ERK in control (Vehicle), Clec16a KO and Clec16a KO+U0126 treated mouse. As anticipated, in Clec16a KO, we observed significant increase in the levels of phosphorylated ERK 1/2 in comparison to control vehicle treated mice (Fig 5G and 5H). U0126 treated Clec16a KO mice showed significantly decreased ERK1/2 phosphorylation to basal level, suggesting the activation of ERK1/2 signaling in Clec16a KO with disrupted mitophagy. Control treated U0126 mice showed no significant increase or decrease in protein expression compared to control alone (S7A and S7B Fig). Thus, Clec16a knockout disrupts mitophagy in splenic immune cells. Taken together, these results suggest that Wortmannin and U0126, are capable of rescuing the disrupted mitophagy signaling defect in Clec16a KO mice.

Increased cytokines/chemokine levels reflect the inflammatory mechanism utilized during the development, progression and pathogenesis of various autoimmune and inflammatory diseases. To gain insight in the inflammatory mechanism we profiled plasma from control ±U0126 and KO±U0126 treated mice for cytokines and chemokines using Mouse Cytokine Array panel (S8A and S8B Fig). Plasma from Clec16a KO mice showed more robust significant upregulation of Th1 cytokines (TNF-α, IL-1, & IL-16), vs. Th2 (IL-10 & IL-13) and elevated levels of key chemokines (GM-CSF, KC (CXCL1), M-CSF, MCP-1(CCL2), MCP-5 (CCL12), MIG (CXCL9), MIP-1β) in comparison to control mice (Fig 6). U0126 treatment in Clec16a KO mice significantly reversed all the up-regulated cytokines and chemokines. U0126 treatment in control significantly reduced basal expression of IL-1α, CXCL1 and TNF-α. These results suggest the inflammatory mechanism involved in autoimmune diseases is mediated by disrupted mitophagy and can be attenuated in part by ERK1/2 inhibitor therapy.

**Discussion**

CLEC16A is a well-established T1D susceptibility gene[1–5] and has also been convincingly implicated in MS[6, 7], PAI[8], CD[9], PBC[10], JIA[12], RA[12, 20], and AA[13]. More recently, CLEC16A has become an attractive candidate for studies addressing potential therapeutic targets. Despite this interest, little is known about CLEC16A function in humans. We previously reported in mice with pancreas-specific deletion of Clec16a that CLEC16A is required for normal glucose-stimulated insulin release through its effect on mitophagy[16].
The clinical significance of this work is supported by findings in patients with the \textit{CLEC16A} T1D risk variant, rs12708716-G, who have reduced expression of CLEC16A in islets and attenuated insulin secretion. This work, however, does not address the complete role of CLEC16A in immune cell function, a critical component of autoimmunity.

Recent studies suggest that \textit{CLEC16A} may control the HLA-II antigen presentation pathway via late endosomal maturation in antigen-presenting cells (APC)\cite{21} and that \textit{CLEC16A} variation impacts thymic selection playing a role in thymic epithelial cell (TEC) autophagy\cite{22}. These data suggest that an increase in CLEC16A is provocative for autoimmune disease rather than a decrease in CLEC16A; that is, the previously proposed mechanism is at odds with the effect of the autoimmune risk variant, rs12708716-G, which results in reduced expression of CLEC16A\cite{16}. By contrast, our work illustrates that decrease in CLEC16A expression via genetic \textit{Clec16a} KO in mice led to disrupted/incomplete mitophagy, cell death and immune dysfunction.

To our knowledge, this is the first study elucidating the role of CLEC16A action in immune cells. We employed UBC-Cre-Clec16a\textsuperscript{loxP} mice—an inducible \textit{Clec16a} KO model to study CLEC16A’s role on a whole organism level. We chose this model to circumvent possible embryonic lethality and determine the effect of CLEC16A loss on immune cells in adult mice.
Our whole body Clec16a KO mice exhibit atrophy of the thymus and spleen and rapid weight loss with no decrease in food intake. The atrophy observed in thymus is acute and in spleen is gradual. Spleen in KO mice showed reduced size and total splenocyte numbers suggesting increased cell death. Splenic immune cells of Clec16a KO mice exhibit lowered mitochondrial membrane potential. TEM images reveal changes in the mitochondrial membrane architecture and accumulation of disordered, disintegrated, elongated, and fragmented unhealthy mitochondria in splenic immune cells. Our data shows that lowered mitochondrial membrane potential initiates Nrdp1/PINK/Parkin1 dependent, incomplete mitophagy processing and activation of intrinsic pathway of cell death if impaired mitochondria are not disposed effectively.

Autophagic flux was reduced in splenocytes suggesting incomplete mitophagy in Clec16a KO mice as indicated by modest increase in LC-3 I/II and lack of change in P62 with Bafilomycin treatment. P62 protein is itself degraded by autophagy and serves as a marker to study autophagic flux. Pure B and T-cells in Clec16a KO revealed the same reduced autophagic flux as indicated by accumulation of P62. Our whole-body inducible Clec16a KO murine model demonstrates that post-developmental Clec16a knockout results in increased NK cytotoxicity in Clec16a KO mice independent of NK cell activation, consistent with immune dysfunction. Hyperactive NK cell may be most influential at the initiation of the autoimmune response through interactions with T and B cells. To our knowledge, no one has shown the link between CLEC16A expression and immune dysfunction. Failure to remove dysfunctional mitochondria leads to hyper-activation of inflammatory pathways and enhancement of the inflammatory function in major auto-inflammatory and autoimmune diseases[23]. Dead cells constitute a source of novel antigens and proinflammatory molecules that can provoke autoimmune response. Exposure of novel antigens to the immune system may be the lead trigger of abnormal immune response caused by polarized innate immune cells and other cells of the local environment. As a consequence, the resulting activation and polarization of innate and adaptive immune cells towards a Th-1 type immune response may be among key factors contributing to the pathogenesis. This is further supported by the fact that conditional targeting of T cells in CD4 Cre Clec16a<sup>loxP</sup> mice showed no difference in T cell repertoire and pathological phenotype.

Malfunctioning autophagy, either too little or too much can be detrimental to cells. Harmful imbalances in autophagic regulation are conceptualized as a state of autophagic stress in many human diseases including cancer, neurodegenerative, cardiac, infectious, inflammatory and autoimmune diseases[24–27]. Autophagy and apoptosis are important and interconnected stress response mechanisms. The effect of autophagy on disease progression has not yet been discovered, and the identification and development of new drug targets is still a key focus. Many lines of evidence support the existence of cross-talk between PI3K and ERK and the potential for PI3K to act as an upstream activator of ERK [28, 29]. ERK activity has been associated with classical markers of apoptosis execution, such as effector caspase-3 activation, characterized by release of cytochrome-c from mitochondria and activation of initiator caspase-9. Inhibition of autophagy leads to enhanced cell death and inflammation as observed in our Clec16a KO mice. PI3K inhibitor-Wortmannin and MEK inhibitor-U0126 attenuated the mitophagy defects observed in the Clec16a KO murine model and provide evidence in support of the future therapies that depend on ability to correct disease related factors that promote autophagic stress and contribute to pathological imbalances in the system.

Our study underscores a critical role of CLEC16A action in immune cells signaling through the Nrdp1/PINK/Parkin pathway. A delicate balance of CLEC16A activity appears to be needed for cellular homeostasis. In patient populations harboring variants that result in CLEC16A hypofunction, drugs with modulatory effects on mitophagy could compensate for
the attenuated CLEC16A activity and present formidable candidates for targeted interventions.

Supporting information
S1 Fig. Generation of Clec16a KO UBC-Cre-Clec16a^loxp/loxp^ mice. Schematic representation of Cre-mediated recombination of the Clec16a locus. (TIFF)

S2 Fig. Clec16a knockout disrupts mitophagy in-vivo in response to lowered mitochondrial membrane potential resulting in aggregation of fragmented mitochondria in splenic immune cells. (A) Representative histogram depicts the basal mitochondrial membrane potential in control and Clec16a KO splenocytes at 24 and 48hrs without stimulation using MitoTracker Deep Red FM dye in a flow-based assay. (B) Mitochondrial membrane potential depicted as change in mean fluorescence intensity for two time points, 24h and 48h in unstimulated control and Clec16a KO splenocytes. (C) Immunoblot depicts CLEC16A expression in control and Clec16a KO splenocytes at 24 and 48hrs. (D) Representative immune blot image from control±IL-15 and KO±IL-15 splenocyte lysate depicting levels of P62 and LC3-I/II. (E) Quantitation graph depicting expression levels of P62 and LC3-I/II. (F) Representative immune blot images from splenocyte lysate depicting CLEC16A Nrdp1, Parkin, P62, TOM20, ATG16L1, LC3I/II, Cytochrome-c, and Caspase-9 expression in Control±Bafilomycin treated mice. (G) Quantitation graph depicting expression levels of CLEC16A, Nrdp1, Parkin, P62, TOM20, ATG16L1, LC3I/II, Cytochrome-c, and Caspase-9 and cleaved caspase-3 from Control±Bafilomycin treated mice normalized to β-actin. Data expressed as means±SE of three independent experiments. *P<0.05, **P<0.01, ***P<0.001. #P<0.05, ##P<0.01. (TIFF)

S3 Fig. TEM image of B cell from Clec16a KO. Zoomed in TEM image of B-cell from Clec16a KO mice depicts mitochondria with abnormal morphology (white arrows). (TIFF)

S4 Fig. TEM image of T cell from Clec16a KO. Zoomed in TEM image of T-cell from Clec16a KO mice depicts mitochondria with abnormal morphology (white arrows). (TIFF)

S5 Fig. T cell subpopulation comparison of control and Clec16a KO mice. (A) The dot plot shows CD4 (top-panel) and CD8-T (bottom-panel) cell subpopulation between control (vehicle) and TAM-treated mice. (B) Graph depicts percent of CD4+ naïve, memory and effector T cells. (C) Graph depicts percent of CD8+ cells naïve, memory and effector T cells. Results for each group (Control n = 7; Clec16a KO n = 10) are presented as means±SE. **P<0.01, ***P<0.001 (unpaired two-tailed Student’s t-test). Splenocytes were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD62L, anti-CD44, and UV live/dead. Cells were gated on CD3^+CD4^+ or CD3^+CD8^+ cells. Numbers indicate the percentage of Naïve and effector cells. (TIFF)

S6 Fig. CD4 Cre Clec16a^loxp^ mice T-cell subpopulation analysis. CD4 Cre Clec16a^loxp^ mice T-cell subpopulation analysis from thymus (A) and spleen (B) showed no significant differences. CD4 Cre Clec16a^loxp^ mice. Clec16a^loxp^ mice were mated to B6.Cg-Tg (CD4-cre) 1Cwi/BfluJ mice (The Jackson Laboratory). CD4-Cre transgenic mice contain CD4 enhancer, promoter and silencer sequences driving the expression of a Cre recombinase gene. By crossing Clec16a^loxp^ mice to this strain we generated CD4 Cre Clec16a^loxp^ mice with Clec16a
conditional mutations in CD4-expressing tissues. Specifically, Cre recombinase expression is observed in CD4-expressing T cells during sequential stages of T cell development in lymphoid tissues. Mutation of the Clec16a gene was confirmed by PCR on DNAs and by RT-PCR on RNAs of T cells isolated from splenocytes of Clec16a CD4 T cell specific mutant and control littermate mice.

S7 Fig. Control±U0126 Immunoblot for mitophagy. (A) Representative immune blot images from splenocyte lysate depicting CLEC16A Nrdp1, PINK1, Parkin, P62, TOM20, ATG16L1, LC3I/II, Cytochrome-c, and Caspase-9 expression in Control±U0126 treated mice. (B) Quantitation graph depicting expression levels of CLEC16A, Nrdp1, PINK1, Parkin, P62, TOM20, ATG16L1, LC3I/II, Cytochrome-c, and Caspase-9 from Control±U0126 treated mice normalized to β-actin. Data is expressed as means±SE of three independent experiments.

S8 Fig. Mouse Cytokine Array. (A) Representative Array blot of plasma cytokine and chemokine from Control (Vehicle), Control (U0126), Clec16a KO, and U0126 treated Clec16a knockout mice. For each, 100 ul of plasma was run on the array. Data shown are from a two-hour exposure to X-ray film. The average signal (pixel density) of the pair of duplicate spots representing each cytokine or chemokine was analyzed using Image-J software (B). Table depicts cytokines, chemokines, adipokines, growth factors and immune related proteins coordinates on Mouse Cytokine Array Panel A.

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