Characteristics of Multi-Organ Lymphangiectasia Resulting from Temporal Deletion of Calcitonin Receptor-Like Receptor in Adult Mice

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Abstract

Adrenomedullin (AM) and its receptor complexes, calcitonin receptor-like receptor (Calcrl) and receptor activity modifying protein 2/3, are highly expressed in lymphatic endothelial cells and are required for embryonic lymphatic development. To determine the role of Calcrl in adulthood, we used an inducible Cre-loxP system to temporally and ubiquitously delete Calcrl in adult mice. Following tamoxifen injection, Calcrlfl/fl/CAGGCre-ERTM mice rapidly developed corneal edema and inflammation that was preceded by and persistently associated with dilated corneoscleral lymphatics. Lacteals and submucosal lymphatic capillaries of the intestine were also dilated, while mesenteric collecting lymphatics failed to properly transport chyle after an acute Western Diet, culminating in chronic failure of Calcrlfl/fl/CAGGCre-ERTM mice to gain weight. Dermal lymphatic capillaries were also dilated and chronic edema challenge confirmed significant and prolonged dermal lymphatic insufficiency. In vivo and in vitro imaging of lymphatics with either genetic or pharmacologic inhibition of AM signaling revealed markedly disorganized lymphatic functional proteins ZO-1 and VE-cadherin. The maintenance of AM signaling during adulthood is required for preserving normal lymphatic permeability and function. Collectively, these studies reveal a spectrum of lymphatic defects in adult Calcrlfl/fl/CAGGCre-ERTM mice that closely recapitulate the clinical symptoms of patients with corneal, intestinal and peripheral lymphangiectasia.

Introduction

The lymphatic vascular system is a complex vascular network that permeates nearly every organ of the body and plays a critical role in the maintenance of fluid homeostasis, the absorption of intestinal lipids and the trafficking and maturation of immune cells [1]. Despite its pervasive functions, it is surprising that very little is known about the genetic and molecular pathways that regulate lymphatic vascular function in adults [2]. Fortunately, the past dozen years has provided a relative explosion of new and sometimes unexpected genes involved in the development of the lymphatic vascular system, based largely on elegant and exciting embryonic phenotypes uncovered in gene knockout studies in mice [3]. Some of these discoveries have even paved the way toward the identification and better understanding of human genes in which mutations are causally associated with congenital, primary lymphedema such as FOXC2, FLT4, SOX18, GJC2 and CCBE1. Nevertheless, the development of additional genetic mouse models of lymphatic insufficiency during adulthood is still needed in order to identify novel candidate genes either for genetic testing in families with congenital forms of lymphedema or for therapeutic targeting of lymphatics in disease.

Failure of lymphatic vessels to function properly in adults can result in numerous types of clinical conditions, including primary and secondary lymphedema, which can have a broad range of clinical presentations and associated correlates [4,5]. Some congenital forms of primary lymphedema are associated with lymphangiectasia, which is typically characterized as dilation and enlargement of lymphatic vessels. Interestingly, there are a few organ systems, including the intestine [6], the conjunctiva of the eye [7] and the dermis [8], that are particularly prone to developing lymphangiectasia. While the pathophysiological mechanisms leading to lymphangiectasia are not well understood, it is likely that dilated lymphatic vessels are the result of lymphatic obstruction and improper drainage or lymph stasis. The consequences of persistent lymphangiectasia include, on a cellular level, increased permeability of dilated lymphatic vascular beds, and on a systemic level, protein-losing enteropathy, limb lymphedema and ocular irritation with dryness. Although lymphangiectasia can be associated with a variety primary, congenital lymphedema syndromes, there is currently no known genetic pathway that directly and predominantly contributes to lymphangiectasia.

Using gene targeting approaches in mice, we have previously discovered and characterized an essential role for adrenomedullin (gene = Adm, peptide = AM) peptide and its receptor complex in lymphatic vascular development. Adrenomedullin, a secreted,
multi-functional peptide that is highly expressed in endothelial cells, binds and signals through a G protein-coupled receptor, calcitonin receptor-like receptor (gene = Calcrl; protein = CLR), when the receptor is associated with receptor activity modifying proteins 2 or 3 (RAMP2/3). The complex formed by CLR and RAMP2 is referred to as the adenomedinulin 1 (AM1) receptor, while the CLR and RAMP3 complex is referred to as the AM2 receptor; both of which bind AM peptide, but differ in their relative binding affinities [9]. Gene knockout mice for Adm [10], Calcrl [11], and Ramp2 [12,13] all exhibit mid-gestational embryonic lethality characterized by hydrodrops fetalis, or marked edema, that is associated with arrested lymphatic vascular development. Conditional deletion of Calcrl in endothelial cells confirmed that AM signaling, and its downstream activation of the MAPK/ERK signaling cascade, is required for normal lymphatic endothelial cell proliferation during development. AM signaling through Calcrl/Ramp2 also has robust effects on endothelial cell permeability. For example, AM can abrogate the permeabilizing effects of hydrogen peroxide and thrombin on human umbilical vein endothelial cells [14] and it can retard the transport of molecules across the blood brain barrier by tightening the permeability of cerebral endothelial cells [15,16]. Similarly, we have shown that AM can impact the permeability and function of lymphatic endothelial cells (LECs). Treatment of cultured LECs with AM significantly and functionally reduced their permeability by causing a subcellular reorganization of the junctional proteins ZO-1 and VE-Cadherin [17]. Furthermore, in vivo tail microlymphography reinforced these findings since mice injected with AM showed reduced lymph velocity through dermal lymphatic capillaries, indicative of functionally reduced permeability [17].

The apparently biased effects of AM signaling on the embryonic development of lymphatic vessels, versus blood vessels, is likely attributable to the increased expression of Calcrl and Ramp2 in LECs, compared to blood endothelial cells [18–20]. Consistent with this notion, continuous administration of AM promoted lymphangiogenesis and ameliorated secondary tail lymphedema in a surgical injury mouse model [21]. Whether the maintained expression of Calcrl in adult animals is also required for appropriate lymphatic function remains unclear. To address this question, we used a ubiquitously expressed, tamoxifen-inducible expression of Calcrl in adult animals is critical for maintaining the proper function of lymphatic vessels in a wide variety of organs.

Methods

Animals

Mice used in these studies were generated from crossing Calcrl<sup>fl/fl</sup> [12] mice (N7-10 on C57BL/6 background) to CAGCre-ER<sup>TM</sup> mice [The Jackson Laboratory, Bar Harbor, ME 04845, B6.Cg-Tg(CAG-cre-Esr1)5Amc/J]. Male and female adult mice aged 3–4 months were administered tamoxifen (Sigma) consecutively for 5 days (5 mg/40 g body weight; IP). Mice were genotyped for the excised allele after tamoxifen injection. Primer sets (5'-3') P1: ggggagatccatggaacag, P2: gaaagaagttgcactgga, P3: gcgcgctctctaaaggt, and P4: gagaagaggtggtttgag were used ([P1/P2 for wildtype allele; P1/P3 for floxed allele; P1/P4 for excised allele]. Mice were routinely anesthetized using 0.2–0.4 ml/10 g body weight of avertin (2,2,2,-Tribromoethanol, 20 mg/ml, Sigma).

For Western Diet studies, mice were fed Teklad Adjusted Calories Diet (TD.08137; 42% from fat; Harlan Laboratories) for 1½ weeks and then housed in metabolic cages for 24 hours during which food intake, urine, and fecal samples were measured. Weights of mice were also recorded before tamoxifen injection, after tamoxifen injection, and after Western Diet.

All experimental procedures involving mice were approved by the Institutional Animal Care and Use Committee of The University of North Carolina Chapel Hill and all efforts were made to minimize suffering.

Cell Culture

Human adult dermal lymphatic endothelial cells (HMVEC-dLyAd-Der Lym Endo Cells, Lonza) of 8 passages or less were maintained using EGM-2MV media with bullet kit (Lonza). Cells were seeded in 6 well plates at 100,000 cells/well and grown on acid washed coverslips until monolayers formed. Treatment conditions included no treatment (control), 10 nM AM (American Peptide Co.,Inc.), 1 µM AM22-52 (AM antagonist; American Peptide Co.,Inc.) or AM+AM22-52. Cells were treated for 15 minutes and in the condition with AM+AM22-52, cells were pre-treated with AM22-52 for 30 minutes. Cells were rinsed with HBSS, fixed with 1% PFA, rinsed 3×5 minutes with PBS, and then blocked for 20 minutes with 2% normal donkey serum/0.1% Triton X in PBS. The cells were then incubated overnight at room temperature with primary antibodies (VE-Cadherin = 1:200, goat polyclonal; sc-6458, Santa Cruz Biotechnology, Santa Cruz, CA; ZO-1 = 1:200, monoclonal rat α mouse; clone R40.76, Millipore, Billerica, MA), rinsed 3×5 minutes with PBS, blocked with 2% normal donkey serum (NDS) for 10 minutes, followed by incubation with secondary antibody for 1 hour at room temperature, rinsed 3×5 minutes with PBS and then mounted on slides using Mowiol.

Immunohistochemistry and Immunofluorescence

Tissues were dissected, fixed with 4% PFA overnight and embedded in paraffin or protected in 30% sucrose and embedded in OCT (Tissue-Tek) for sectioning. Sections were permeabilized using 0.1% Triton X-100 (in 0.01 M PBS; pH 7.2; 15 minutes), blocked with 5% NDS (in 0.1% Triton X-100; 30 minutes), incubated overnight in primary antibodies, PBS rinsed (3×5 minutes), blocked with 5% NDS (30 minutes), incubated with secondary antibodies (2 hours), rinsed with PBS and coverslipped with Mowiol. Primary antibodies included: LYVE-1 (1:200; polyclonal rabbit α mouse; Fitzgerald, Acton, MA), podoplanin (1:200, Syrian hamster α mouse, Developmental Studies Hybridoma Bank, Univ. Iowa), ZO-1 (1:200; monoclonal rat α mouse; clone R40.76, Millipore, Billerica, MA) and VE-Cadherin (1:200, goat polyclonal; sc-6458, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies included Alexa Fluor 594 and Cy3 (1:200, Jackson Immunoresearch) and nuclear marker DAPI (1:1000, bisbenzimide 33258; Sigma, St. Louis, MO). TUNEL staining was performed using the ApopTag Fluorescent In Situ Apoptosis Detection Kit (S7110, Chemicon International) according to the manufacturer’s protocol.

Tonometry

Tonometry was performed in anesthetized adult mice using a TonoLab tonometer (Colonial Medical Supply) as described previously [22,23]. After avertin injection, a drop of tetracaine hydrochloride 0.5% (Alcon) was placed on the eye as a local anesthetic. Eyes were lubricated throughout testing with TEARS Naturale FORTE (Alcon). At least six readings were recorded per eye and averaged.
Tail Microlymphography and Vessel Diameter

Three to four months post tamoxifen injection, adult mice were used for tail microlymphography as described previously [17] with several modifications. FITC-conjugated dextran (200 kDa; 1 μl; Molecular Probes, Invitrogen Detection Technologies) was injected intradermally into the mouse tail using a 5 μl Hamilton syringe fitted with a 30 gauge needle. Images were taken every minute for 15 minutes and image analysis was performed using Adobe Photoshop 7.0 and Image J.

Lymphatic and Blood Permeability Assays

An ear lymphatic permeability assay was performed as previously described [24] with minor modification. Ears of anesthetized mice were injected intradermally with 2 μl of 0.5% Evan’s Blue dye (in saline) with a 10 ul Hamilton syringe. Images were taken immediately after injection and 5 minutes after injection. A blood permeability assay was performed as previously described with slight modifications to the protocol [25]. Anesthetized mice were retro-orbitally injected with 200 μl of 0.5% Evan’s Blue dye (in saline). After 30 minutes, the mice were perfused with saline and the liver, lung, adductor muscle, spleen, intestine, heart, and brain were harvested. Tissues were weighed and desiccated overnight at 55°C followed by formamide extraction (55°C, overnight) and 100 μl was used for absorbance reading at 600 nm.

Acid Steatocrit/Lipase/Triglyceride Measurements

Fecal samples collected after Western Diet were examined by testing for fecal steatocrit and fecal lipase as previously described [26] with recent modifications [27]. Fecal specimens were powdered and mixed with 1N perchloric acid and 0.5% oil red O and placed in a capillary tube and centrifuged. Steatocrit was calculated as 100 × [length of fatty layer/length of solid layer+length of fatty layer]. Fecal lipase and serum triglycerides were analyzed at the Animal Clinical Chemistry and Gene Expression Labs (UNC-CH).

Dot Blot Assay

A dot blot assay using digested fecal samples was performed as previously described [28] with slight modification. In short, mice were fed Western Diet for 1 week and fecal samples were collected and stored at −20°C. TBS with 5% nonfat dry milk (with protease inhibitors) was added to the fecal samples (20 μl/mg). The samples were vortexed and sonicated then centrifuged at 16,000 g for 10 minutes after which the supernatant was collected. Three microliters of each supernatant (1:2000 dilution) was dotted onto a nitrocellulose membrane. The membrane was blocked (TBS+3% nonfat dry milk) for 2 hours at room temperature, rinsed with TBST (3×5 minutes), then incubated overnight with primary antibody (mouse anti alpha-1 antitrypsin-1:500; Novus Expression Labs (UNC-CH)). We also found no significant histological differences in the optic nerve of Calcrlfl/fl/CAGGCre-ERTM mice compared to Calcrlfl/fl control mice when compared either before injection or after injection of tamoxifen (Figure S1A). We also found no significant histological differences in the optic nerve of Calcrlfl/fl/CAGGCre-ERTM mice compared to Calcrlfl/fl control mice (data not shown). Finally, we found no significant difference in the intraocular pressure of Calcrlfl/fl/CAGGCre-ERTM and Calcrlfl/fl control mice when compared either before injection or after injection of tamoxifen (Figure S1C) and all intraocular pressure measurements were within the normal range for C57BL/6 mice [22]. Taken together, these data rule out the possibility that the acute-onset eye phenotype in Calcrlfl/fl/CAGGCre-ERTM mice is associated with classical features of glaucoma. Based on the well-established role of Calcrl in smooth muscle of mice resulted in a phenotype similar to glaucoma [29]. To this end, TUNEL staining indicated no difference in retinal ganglion cell death between Calcrlfl/fl and Calcrlfl/fl/CAGGCre-ERTM control mice (Figure S1A and S1B). We also found no significant histological differences in the optic nerve of Calcrlfl/fl/CAGGCre-ERTM mice compared to Calcrlfl/fl control mice (data not shown). Finally, we found no significant difference in the intracorneal pressure of Calcrlfl/fl/CAGGCre-ERTM and Calcrlfl/fl control mice when compared either before injection or after injection of tamoxifen (Figure S1C and S1B). We also observed pronounced inflammation in the anterior chamber and cornea of Calcrlfl/fl/CAGGCre-ERTM mice (Figure 1E,F, arrowhead).

Analysis of integrated density was performed using Image J.

Edema Formation Assay

Anesthetized mice were injected with 10 μl of 4 μg/μl Complete Freund’s Adjuvant (CFA) in one hind paw. The other hind paw served as an internal control. Paw thickness was measured with calipers before injection of CFA and every other day after injection up to 21 days.

RNA and qRT-PCR

Lung and heart tissue were collected in RNAlater Solution (Ambion). RNA was extracted from tissue using TRIZOL (Ambion, Life Technologies) isolation followed by DNase treatment (Promega) and cDNA preparation. Quantitative RT-PCR was performed on a Stratagene Mx-3000p machine (La Jolla, CA) using TAQMAN GEX Master Mix (Applied Biosystems). Caelr expression was assessed using Assay-on-Demand for Caelr (Mm00516986_m1; Applied Biosystems). The comparative quantitation (ΔΔCT) method was used to determine the relative level of Caelr expression in the tissues compared to mouse embryo total RNA calibrator (Ambion). All assays were repeated at least three times and run in duplicate.

Statistical Analysis

All experiments were repeated at least 3 times and data are expressed as means with SEM values. Student t tests (tails = 2, type = 3) and two-way ANOVA were performed and P≤0.05 was considered significant.

Results

Temporal Deletion of Calcrl Results in Acute Onset Eye Phenotype with Enlarged Comeoscleral Lymphatic Vessels

Tamoxifen injection resulted in a significant reduction of Caelr gene expression in Caelrfl/fl/CAGGCre-ERTM animals compared to Caelrfl/fl animals and to Caelrfl/fl/CAGGCre-ERTM non-injected animals (control animals) as indicated by qRT-PCR of lung and heart tissue (Figure S2). Within 7 to 10 days of tamoxifen injection, the majority of Caelrfl/fl/CAGGCre-ERTM mice, but none of the control Caelrfl/fl tamoxifen-injected mice, developed distinct grafting of their eyes and the surface of their corneas appeared rough and coarse (Figure 1A,B). The rapid onset of this phenotype prompted us to determine whether it was associated with glaucoma, since a previous study by Ittner et al. indicated that overexpression of Caelr in smooth muscle of mice resulted in a phenotype similar to glaucoma [29]. To this end, TUNEL staining indicated no difference in retinal ganglion cell death between Caelrfl/fl/CAGGCre-ERTM and Caelrfl/fl control mice (Figure S1A and S1B). We also found no significant histological differences in the optic nerve of Caelrfl/fl/CAGGCre-ERTM mice compared to Caelrfl/fl control mice (data not shown). Finally, we found no significant difference in the intraocular pressure of Caelrfl/fl/CAGGCre-ERTM and Caelrfl/fl control mice when compared either before injection or after injection of tamoxifen (Figure S1C and S1B). We also observed pronounced inflammation in the anterior chamber and cornea of Caelrfl/fl/CAGGCre-ERTM mice (Figure 1E,F, arrowhead).

Based on the well-established role of Caelr in lymphatic vascular development [12], the edema and inflammation in the corneas of
Calcrlfl/fl/CAGGCre-ERTM mice suggested to us that there may be problems with the lymphatic vessels of the eyes, particularly those within the corneoscleral junction, which is analogous to the conjunctival lymphatics in humans. Specifically, we found podoplanin-positive and LYVE-1-positive staining in the ciliary body and in vessels at the corneoscleral junction (Figure 1G,H, arrow). Interestingly, the lymphatic vessels at the corneoscleral junction of Calcrlfl/fl/CAGGCre-ERTM mice were significantly dilated and twice the size of corneoscleral lymphatics in Calcrlfl/fl control mice (Figure 1I,J,K), similar to the phenotype observed in humans with conjunctival lymphangiectasia. More importantly, the eyes of Calcrlfl/fl/CAGGCre-ERTM mice that did not present with the overt corneal pathology (approximately 1/3rd of the mice), either because they failed to develop the phenotype or they were euthanized prior to the onset of the phenotype, still showed significantly dilated lymphatics at the corneoscleral junction. Taken together, these data demonstrate that an abnormal lymphatic vessel phenotype precedes the onset of acute corneal pathology in Calcrlfl/fl/CAGGCre-ERTM mice.

Calcrlfl/fl/CAGGCre-ERTM mice exhibited enlarged submucosal lymphatic vessels and lacteals in the intestine with dysfunctional mesenteric collecting lymphatic vessels.

Since dilated lymphatic vessels were observed at the corneoscleral junction in the Calcrlfl/fl/CAGGCre-ERTM mice, we wanted to assess the morphology and function of lymphatic vessels in other lymphatic vascular beds, for example, within the intestine. The overall histology of the intestines of Calcrlfl/fl/CAGGCre-ERTM mice was normal when compared to that of Calcrlfl/fl control mice under normal conditions (Figure 2A,B). Lymphatic vessels within the intestine were identified with LYVE-1 and podoplanin staining, showing co-localization within the lacteals and the submucosal lymphatics (Figure 2C–E). Interestingly, the submucosal lymphatics and lacteals of the jejunum were also markedly dilated in the Calcrlfl/fl/CAGGCre-ERTM mice (Figure 2F,G), as evidenced by the visibly larger diameter of the submucosal lymphatics and a greater proportion of villi sections revealing enlarged, LYVE-1-positive lacteals. Once again, these dilated lymphatics vessels are

Figure 1. Acute-onset eye phenotype, eye inflammation, edema, and enlarged lymphatic vessels in Calcrlfl/fl/CAGGCre-ERTM mice. A,B, Gross eye images indicating normal appearance of the control Calcrlfl/fl mice (A) and the distinct color change and disruption of the cornea of Calcrlfl/fl/CAGGCre-ERTM (B), (scale = 2 mm). C,D, Hematoxylin and eosin staining of mouse eyes indicating normal histology in Calcrlfl/fl (C) and disruption of the cornea in Calcrlfl/fl/CAGGCre-ERTM mice (D), (4x objective, scale = 500 μm). E,F, Higher magnification of histological sections of eyes from Calcrlfl/fl mice (E) as compared to Calcrlfl/fl/CAGGCre-ERTM mice (F) exhibiting corneal edema (arrow) and inflammation (arrowhead) (10x objective, scale = 200 μm). Gross anatomy and histology images are representative from Calcrlfl/fl mice (n = 8) and Calcrlfl/fl/CAGGCre-ERTM mice (n = 9). G, Eye diagram indicating the location of components of the eye (l = lens, c = cornea, ac = anterior chamber, cb = ciliary body, i = iris). H, Lymphatic markers expressed in the eye shown by podoplanin(red) and Lyve-1(green) staining in a control mouse eye (20x objective, scale = 100 μm). I,J, Visualization of lymphatic vessels at the corneoscleral junction in the Calcrlfl/fl (I) and Calcrlfl/fl/CAGGCre-ERTM mice (J) indicating enlarged lymphatic vessels in Calcrlfl/fl/CAGGCre-ERTM mice (Lyve-1 = green; DAPI = blue; 20x objective, scale = 100 μm). K, Graph representing increased lymphatic vessel area at the corneoscleral junction in Calcrlfl/fl/CAGGCre-ERTM mice compared to control mice calculated using Image J software (*p < 0.015). Mice used were 3–4 months of age. doi:10.1371/journal.pone.0045261.g001

Temporal Loss of Calcitonin Receptor-Like Receptor
reminiscent of the dilated lymphatics observed in human patients with intestinal lymphangiectasia.

Intestinal lymphatics are required for normal lipid absorption, and patients with intestinal lymphangiectasia often present with weight loss as a result of lipid malabsorption [30]. Therefore, the function of these vessels was evaluated by placing Calcr<sup>fl/fl</sup>/CAGGCre-ERTM and Calcr<sup>fl/fl</sup> control mice on a short term Western Diet following an overnight fast. After 1½ hours of Western Diet, the Calcr<sup>fl/fl</sup>/CAGGCre-ERTM mice exhibited chyle-filled mesenteric lymphatic vessels which were not visible in the Calcr<sup>fl/fl</sup> control mice (Figure 2H,I). Chyle-filled submucosal lymphatic vessels were also visibly distinguishable in Calcr<sup>fl/fl</sup>/CAGGCre-ERTM mice (arrows; inset refers to enlarged image of valve; scale = 3 mm; n = 4 per genotype). Mice used were 6–8 months of age.

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Figure 2. Dilated lacteals and submucosal lymphatics in Calcr<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice and chyle-filled lymphatics after short-term Western diet. A,B, Hematoxylin and eosin staining of mouse intestine showing normal histology in both Calcr<sup>fl/fl</sup>(A) and Calcr<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice(B) (6.3x objective, scale = 500 μm). C,D,E, Lymphatic marker expression in the lacteals and submucosal lymphatic vessels in wildtype mouse. Image was obtained from the jejunum of the intestine. Lyve-1 (C,green) and podoplanin(D,red) colocalize in the lymphatic vessels as seen in the merged image(E) (20x objective; scale = 100 μm). F,G, Lyve-1(green) and DAPI(blue) staining in Calcr<sup>fl/fl</sup>(F) and Calcr<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> (G) mice indicating dilated lacteals and submucosal lymphatic vessels with temporal deletion of Calcr in the jejunum of the intestine (4x objective, scale = 500 μm). Histology and immunofluorescent images are representative from Calcr<sup>fl/fl</sup> mice (n = 7) and Calcr<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice (n = 6). H,I, Chyle-filled mesenteric collecting lymphatic vessels in Calcr<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice (I) relative to non-chyle filled vessels in control animals (H). Valves are distinctly visible in Calcr<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice (arrows; inset refers to enlarged image of valve; scale = 3 mm; n = 4 per genotype). Mice used were 6–8 months of age.

CAGCre-ERT™ mice and contributed to the whitened appearance of the intestine (Figure 2I), and some animals additionally exhibited chyle leakage into the mesenteric space. Importantly, the lymphatic valves of Calcrlfl/fl/CAGCre-ERT™ mice appeared normal and were present at regular intervals along the mesenteric collecting lymphatics (Figure 2I, arrows and inset). Therefore, although the intestinal lymphatic vessels of Calcrlfl/fl/CAGCre-ERT™ mice were present and appeared overtly normal, the collecting vessels were significantly dysfunctional in their transport of chyle as compared to control mice.

Reduced Body Weight and Impaired Lipid Absorption with Protein-losing Enteropathy in Calcrlfl/fl/CAGCre-ERT™ Mice

We next wanted to assess the impact of a longer term high fat diet on intestinal lipid absorption in the Calcrlfl/fl/CAGCre-ERT™ mice. There were no significant differences in body weights between 3–4 month old, male or female Calcrlfl/fl/CAGCre-ERT™ mice and Calcrlfl/fl control mice before the injection of tamoxifen (Figure 3A,B). However, 3–4 months after the injection of tamoxifen, we found that the Calcrlfl/fl/CAGCre-ERT™ mice weighed significantly less than their control counterparts (Figure 3A,B), indicating that the tamoxifen-induced loss of Calcrl contributes to a failure of Calcrlfl/fl/CAGCre-ERT™ mice to gain weight and thrive. The failure of Calcrlfl/fl/CAGCre-ERT™ mice to gain weight and thrive was significantly exacerbated (Figure 3A,B) and visibly apparent (Figure 3C) when the mice were fed a Western Diet for 1½ weeks. Moreover, fecal acid steotcrit levels, representative of lipid excretion levels, were significantly elevated in Calcrlfl/fl/CAGCre-ERT™ animals maintained on a Western Diet for 1½ weeks compared to similarly fed Calcrlfl/fl control animals (Figure 3D), demonstrating reduced lipid absorption. Consistently, fecal pancreatic lipase levels were also increased in Calcrlfl/fl/CAGCre-ERT™ mice.
Figure 4. Dilated dermal lymphatic capillaries with exacerbated and prolonged edema. A,B, Images of dermal lymphatic capillaries in the tail of \textit{Calcrl}^{fl/fl} (A) and \textit{Calcrl}^{fl/fl}/CAGGCre-ERTM (B) mice indicating increased diameter of these lymphatic vessels in \textit{Calcrl}^{fl/fl}/CAGGCre-ERTM mice (scale = 0.5 mm). C, Graphic representation of the increase in vessel diameter in the \textit{Calcrl}^{fl/fl}/CAGGCre-ERTM mice with respect to \textit{Calcrl}^{fl/fl} mice (*p\leq0.05). D, Edema formation assay using hindpaw injections of CFA (4 μg/μl on Day 0). Assessment of paw thickness over 3 weeks (n = 5 for \textit{Calcrl}^{fl/fl} and n = 4 for \textit{Calcrl}^{fl/fl}/CAGGCre-ERTM mice) indicated enhanced and prolonged edema in \textit{Calcrl}^{fl/fl}/CAGGCre-ERTM mice relative to control mice (***p\leq0.05, **p\leq0.01, *p\leq0.001). Representative images of CFA-injected hindpaws at Day 11 for \textit{Calcrl}^{fl/fl} and \textit{Calcrl}^{fl/fl}/CAGGCre-ERTM mice (scale = 3 mm). Mice used were 6–8 months of age.

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Figure 5. Increased lymphatic vascular permeability without change to blood vascular permeability in Calcr^{fl/fl}/CAGGCre-ERTM mice. A,B,C,D, In vivo lymphatic permeability assay assessing the leakage of Evan’s blue dye from the dermal lymphatic vessels in the ear. Images represent Evan’s blue dye location directly after injection of the dye and 5 minutes post injection. There is an increase in leakage of the dye from the
ER\textsuperscript{TM} animals, likely due to the compensatory effects of pancreatic lipase conversion of triglycerides into monoglycerides and free fatty acids during periods of reduced lipid absorption (Figure 3E). Importantly, levels of circulating triglycerides were unchanged in the Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice compared to Calcr\textsuperscript{fl/fl} control animals (Figure 3F), indicating that overall metabolism is not compromised in Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice and supporting the conclusion that their failure to gain weight is due to abnormal lipid absorption in the intestine. Finally, fecal samples of Western Diet-fed Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice contained a significantly elevated level of alpha-1 antiprotein-a—clinical diagnostic marker for protein-losing enteropathy—compared to similarly fed Calcr\textsuperscript{fl/fl} control mice (Figure 3G,H).

**Temporal Deletion of Calcr Results in Increased Dermal Lymphatic Capillaries with Exacerbated and Prolonged Edema**

The dermal lymphatic capillaries of Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice also exhibited significant dilation and dysfunction. Specifically, intradermal injection of a large molecular weight (200 kD) FITC-dextran into the subdermal area of the tail tip revealed significantly enlarged dermal capillaries in Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice compared to Calcr\textsuperscript{fl/fl} control mice (Figure 4A,B,C). Despite this dermal lymphangiectasia, we noticed that at the basal or quiescent state, Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice did not exhibit pronounced edema in their extremities (Figure 5F, day 0). Thus, we injected the hindpaws of Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} and Calcr\textsuperscript{fl/fl} control mice with CFA in order to challenge the lymphatic vascular system with localized edema. Both Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice and Calcr\textsuperscript{fl/fl} control mice exhibited a rapid and significant increase in hindpaw thickness within 1 day of CFA injection (Figure 4D). However, unlike the Calcr\textsuperscript{fl/fl} control mice which immediately began to resolve their edema by day 3, the Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice developed exacerbated and prolonged edema that peaked between days 9–11, and only began to show slight improvement after two weeks following CFA injection (Figure 4D). These data demonstrate that the expression of Calcr is required for maintaining highly effective lymphatic function under conditions of edema and inflammation.

**Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} Mice Exhibit Increased Lymphatic Capillary Permeability with No Apparent Disruption of Blood Vascular Permeability**

To better evaluate the permeability of lymphatic and blood vasculatures in Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} and Calcr\textsuperscript{fl/fl} mice, we used the small molecular weight Evan’s blue dye which can freely penetrate in and out of dermal capillaries. Injection of 0.5% Evan’s blue dye intradermally in the ear showed rapid uptake of the dye by dermal lymphatics in both Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} and Calcr\textsuperscript{fl/fl} mice (Figure 5A,B). However after 5 minutes, Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice exhibited increased leakage of the dye from the lymphatic vessels, as evidenced by the diffuse spreading of the dye and poorly demarcated lymphatics throughout the ear region compared to the Calcr\textsuperscript{fl/fl} control mice (Figure 5C,D). To determine whether this lymphatic permeability defect was impacted or perhaps confounded by a permeability defect in the blood vasculature, we also measured relative blood vascular permeability in mice receiving a venous injection of Evan’s blue dye. Absorbance readings of Evan’s blue dye showed no statistically significant differences between Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice and Calcr\textsuperscript{fl/fl} control mice for multiple tissues including liver, lung, adductor muscle, spleen, intestine, heart and brain (Figure 5E). Based on these data, we conclude that temporal deletion of Calcr in adult animals results in increased lymphatic capillary permeability with no overt or functional changes in blood vascular permeability.

**Inhibition of AM Signaling Results in Disorganization of Lymphatic Endothelial Cell Junctions**

To elucidate the molecular mechanisms contributing to the lymphatic dysfunction in Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice, we evaluated VE-Cadherin expression and localization in mesenteric lymphatic vessels of Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} and Calcr\textsuperscript{fl/fl} control mice that had been fed a high fat diet for 1.5 hours. VE-Cadherin expression was visibly disrupted in lymphatic vessels of Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice (Figure 6B,D) compared to control mice (Figure 6A,C). More specifically, while the relative expression levels of VE-cadherin appeared similar between genotypes, the VE-cadherin in mesenteric lymphatic vessels of Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} appeared as punctate lobules throughout the cells and was not localized to well-defined cell boundaries, as seen in the Calcr\textsuperscript{fl/fl} control mice.

To better characterize the effects of inhibiting Calcr-mediated signaling in cultured lymphatic endothelial cells (LEC) we used an adrenomedullin-specific peptide inhibitor, AM22-52. As we have previously described, treatment of LECs with AM peptide resulted in a marked reorganization of junctional proteins, VE-Cadherin and ZO-1, from a jagged, zipper-like configuration to a cohesive and stabilized cell-cell barrier that is functionally associated with reduced permeability [6E,F and [17]]. In contrast, treatment with the Calcr antagonist, AM22-52, either alone or in combination with AM, abolished the effects of AM peptide and resulted in highly disorganized and jagged junctional protein configurations (Figure 6G,H). Taken together, these results demonstrate that in vitro inhibition of Calcr signaling, either by antagonist treatment or by genetic deletion, results in a profound loss of junctional protein organization, likely resulting in increased permeability of lymphatic endothelial cell barriers.

**Discussion**

These studies demonstrate that temporal loss of murine Calcr in adulthood causes lymphatic insufficiency in a wide range of organs, representing functional similarities to the sequelae observed in patients with a variety of lymphangiectasia conditions. Consistently, the lymphatic vessels in the eye, intestine and skin of Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice were dilated, had irregular junctional protein organization and were dysfunctional when challenged with either fat absorption or edema and inflammation. Taken together, these data identify an important new role for AM signaling as a potent regulator of lymphatic vascular drainage and permeability in adult animals.

The rapid-onset eye phenotype in Calcr\textsuperscript{fl/fl}/CAGGCre-ER\textsuperscript{TM} mice provides novel and clinically relevant insights to the potential role of lymphatic vessels in the eye. Several recent studies have shown that lymphatic markers are expressed in the human eye [31,32], but it is still unclear whether and how these lymphatic vessels contribute to fluid homeostasis of the eye. Our staining of
Figure 6. Inhibition of AM signaling disrupts lymphatic endothelial cell-cell junctions. A,B,C,D, Confocal images of VE-Cadherin (red) and Lyve-1(green) expression in mesenteric lymphatic vessels of Calcr$^{fl/fl}$ (A,C) and Calcr$^{fl/fl}$/CAGGCre-ER$^{TM}$ mice (B,D) (scale = 10 μm). Boxed region depicted in C and D. Junctional protein, VE-cadherin, is disorganized in Calcr$^{fl/fl}$/CAGGCre-ER$^{TM}$ mice relative to Calcr$^{fl/fl}$ mice (representative images from n = 4 per genotype, age 6–8 months). E,F,G,H, Lymphatic endothelial cells stained with VE-Cadherin (green), ZO-1 (red) and DAPI (blue) after various treatments including a no treatment control (A), 10 nm AM (B), 1 μm AM22-52 (C), AM+AM22-52 (D) (arrow refers to inset region). Disorganization of cell-cell junctions occurs with inhibitor treatment (AM22-52) as compared to AM treatment. (VE-cadherin = red, Lyve-1 = green, DAPI = blue, 40x objective, scale = 100 μm; representative images from 3 independent experiments). doi:10.1371/journal.pone.0045261.g006
lymphatic markers in the eye correlates well with these previous studies, since we showed robust LYVE-1 and podoplanin staining in the ciliary body and distinct LYVE-1-positive lymphatic vessels in the corneoscleral junction. Most importantly, we found that temporal deletion of Calcrl resulted in dilated corneoscleral lymphatic vessels that preceded and were associated with the formation of corneal edema and inflammation. Therefore, it is likely that appropriate fluid homeostasis and hydration of the cornea, which is an important physiological feature to consider in terms of dry eye disease, corneal surgeries or conjunctival lymphangiectasia, is modulated by lymphatic vessels. Since AM peptide can be clinically administered [33] and the Calcrl/Ramp interface is pharmacologically tractable [34,35], the potential of harnessing these targets for the therapeutic modulation of fluid homeostasis in the eye may prove to be an exciting avenue.

The intestinal lymphatic phenotype of Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice also correlates well with the clinical presentation of intestinal lymphangiectasia in humans. Under a short-term Western Diet, Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice showed signs of lymphatic insufficiency because their intestinal mesenteric lymphatic vessels failed to transport chyle as effectively as similarly fed control mice. Because Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice are significantly leaner than age-matched control mice 3–4 months post tamoxifen injection, it is likely that the collecting mesenteric lymphatics of these animals function at a consistently reduced capacity. In this regard, it is important to note that weight loss is often associated with lymphangiectasia in the form of protein-losing enteropathy [5] and lipid malabsorption. Consistently, the Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice also exhibit elevated alpha-1 antitrypsin in fecal samples after Western diet, which is indicative of protein-losing enteropathy, similar to the clinical phenotype that is frequently observed in humans with intestinal lymphangiectasia. While the mechanism of lipid absorption through lymphatic lacteals is not completely understood, it is thought to involve both active and passive transport mechanisms through lymphatic endothelial cells [36]. Our data demonstrate that AM signaling through Calcrl/Ramp2 is required for normal intestinal lipid uptake and junctional protein organization in intestinal lymphatic capillaries. Whether the maintenance of the lymphatic permeability barrier and loss of Calcrl is connected with the active and/or passive transport mechanism of lipid absorption within the lacteal will be an important future area of study that may have bearing on better understanding the functional underpinnings of intestinal lymphangiectasia.

It is notable that lymphangiectasia is commonly associated with limb edema. When placed under challenge, we found that the hindpaw of Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice had significantly exacerbated edema that resolved over a longer time period than similarly challenged wildtype animals. These results correlate with the results from the high fat diet in that the lymphatic system of the Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice do not respond effectively as that of control mice to different stresses indicating that there are dysfunctional lymphatic vessels in the Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice.

Importantly, the Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice do not exhibit overt edema in the basal state and we found no significant effects of Calcrl loss on blood vascular permeability. Studies by T. Shindo and colleagues using Ramp2 gene targeted mice suggested that loss of Ramp2 led to a reduction in the expression of junctional proteins and a loss of blood vascular integrity [37]. Using an independent line of Ramp2 gene targeted mice, we have demonstrated that Ramp2<sup>−/−</sup> mice also have arrested lymphangiogenesis [12]. Because Ramp2 associates with multiple G protein-coupled receptors beyond Calcrl, it is likely that the expanded vascular phenotypes of Ramp2<sup>−/−</sup> mice can be attributed to additional signaling pathways, and this notion is further supported by the extensive endocri ne phenotypes of Ramp2<sup>−/+</sup> mice compared to Calcrl<sup>−/+</sup> mice [38]. Taken together, these data continue to support a predominant and preferential role for Calcrl in the lymphatic vasculature compared to the blood vasculature [39], which may be partially explained by the fact that Calcrl and Ramp2 are expressed at higher levels in lymphatic endothelial cells compared to blood endothelial cells [19–20].

Calcrl also serves as a receptor component for the neuropeptide, calcitonin gene related peptide (CGRP), when the receptor is associated with RAMP1. Therefore, we cannot formally exclude the possibility that the phenotypes from temporal loss of Calcrl are not partially attributable to loss of CGRP signaling. For example, other studies have indicated that adult αCGRP knockout mice fed a high fat diet do not gain as much weight as control mice. However, in contrast to the Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice, the αCGRP knockout mice eat more and have higher levels of energy expenditure compared to controls [43]. Also, in our colony, αCGRP knockout mice and RAMP2 knockout mice have never exhibited the visible eye phenotype that is hallmark of the Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice. Finally, the αCGRP knockout mice [40–42] are not embryonic lethal and no vascular defects have been reported in these mice. In contrast, many similarities exist between the phenotypes of Calcrl knockout mice and those of AM and Ramp2 knockout mice, with a primary defects in the vasculature. Therefore, while some implication of CGRP signaling cannot be excluded in the Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice, the phenotypes revealed are more consistent with a predominant attribution to AM signaling. Nevertheless, additional characterization of Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice for phenotypes more closely associated with the physiological functions of CGRP, like pain perception, will be an interesting future direction.

Ultimately these studies indicate functional similarities between temporal loss of Calcrl in adult mice and human lymphangiectasia, but the mechanistic relationship remains elusive and will be an interesting focus for future studies. The underlying cause of lymphatic insufficiency in Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice is likely attributable to various mechanisms including insufficient lymph transport and disrupted lymphatic vessel permeability. The Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice do not respond sufficiently to stress on the lymphatic system indicating the lymphatic network is dysfunctional. Moreover, our permeability findings are consistent with previous studies showing that addition of AM both in vitro and in vivo results in decreased permeability of LECs and lymphatic vessels through reorganization of the junctional proteins VE-cadherin and ZO-1 [17]. In the blood vasculature, Rap1 (Ras-related protein 1), a small GTPase, plays a predominant role in regulating cell adhesion and cell junction organization in response to cAMP/Epac/ERK signaling pathways [44]. Since the major downstream effectors of AM signaling in LECs are cAMP/Epac/ERK, it will be interesting in future studies to determine whether similar or identical Rap1 mechanisms contribute to the lymphatic permeability phenotypes of Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice.

Supporting Information

Figure S1  The acute onset eye phenotype with temporal deletion of Calcrl is not associated with glaucoma-like characteristics. A, B, TUNEL staining of retinal ganglion cells (arrows) in Calcrl<sup>fl/fl</sup> (A) and Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice (B) (DAPI = red; TUNEL = green; scale = 100 μm) C, Tonometry measurements of intraocular pressure in Calcrl<sup>fl/fl</sup> and Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup> mice before tamoxifen injection and one month post-injection. (TIF)

Figure S2  Calcrl gene expression in lung and heart tissue of Calcrl<sup>fl/fl</sup> and Calcrl<sup>fl/fl</sup>/CAGGCre-ERT<sup>TM</sup>. A, qRT-PCR quantitation of relative expression of Calcrl normalized
to mouse elongation factor in \textit{Calcrl}−/− and \textit{Calcrl}−/−/\textit{CAGGCre-ER}ΔM non-injected control mice relative to \textit{Calcrl}−/−/\textit{CAGGCre-ER}ΔM mice. There is a significant reduction in \textit{Calcrl} expression in both lung and heart tissue of \textit{Calcrl}−/−/\textit{CAGGCre-ER}ΔM mice relative to control mice (\textit{p}<0.04, **\textit{p}<0.02).

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\section*{Author Contributions}

Conceived and designed the experiments: SLH KMC. Performed the experiments: SLH HHW. Analyzed the data: SLH KMC. Contributed reagents/materials/analysis tools: SLH HHW KMC. Wrote the paper: SLH KMC.

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