Fungal-derived cues promote ocular autoimmunity through a Dectin-2/Card9-mediated mechanism

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Complete List of Authors:	Brown, Brieanna; Oregon Health & Sciences University, School of Medicine Lee, Ellen; Oregon Health & Sciences University, Molecular Microbiology & Immunology Snow, Paige; Oregon Health & Sciences University, Molecular Microbiology & Immunology Vance, Emily; Oregon Health & Sciences University, VA Portland Health Care System Iwakura, Yoichiro; Tokyo University of Science, Division of Laboratory Animal, Research Institute for Biomedical Sciences Ohno, Naohito; Tokyo University of Pharmacy and Life Sciences, Laboratory for Immunopharmacology of Microbial Products Miura, Noriko; Tokyo University of Pharmacy and Life Sciences, Laboratory for Immunopharmacology of Microbial Products Lin, Xin; University of Texas, MD Anderson Cancer Center, Houston, Department of Molecular and Cellular Oncology Brown, Gordon; University of Aberdeen, Aberdeen Fungal Group. MRC Centre for Medical Mycology, Institute of Medical Sciences Wells, Christine; University of Melbourne, Centre for Stem Cell Systems Smith, J R; Flinders University, Eye and Vision Health, Flinders University School of Medicine Caspi, Rachel; National Eye Institute, National Institutes of Health, Laboratory of Immunology Rosenzweig, Holly; Oregon Health & Sciences University, Molecular Microbiology & Immunology
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Fungal-derived cues promote ocular autoimmunity through a Dectin-2/Card9-mediated mechanism.

Short title: Dectin-2/Card9 pathway in fungal-promoted uveitis

Brieanna R. Brown*[1,2], Ellen J. Lee*[1,3], Paige E. Snow[1], Emily E. Vance[1,3], Yoichiro Iwakura[4], Naohito Ohno[5], Noriko Miura[5], Xin Lin[6], Gordon D. Brown[7], Christine A. Wells[8], Justine R. Smith[9], Rachel R. Caspi[10], Holly L. Rosenzweig[1,3]

*these authors equally co-authored this work

[1]VA Portland Health Care System, Portland, OR, USA

[2]School of Medicine, Oregon Health & Science University, Portland, OR, USA

[3]Department of Molecular Microbiology & Immunology, Oregon Health & Science University, Portland, OR, USA

[4]Research Institute for Biomedical Sciences, Tokyo University of Science, Tokyo, Japan

[5]Tokyo University of Pharmacy and Life Science, Tokyo, Japan

[6]Department of Molecular and Cellular Oncology, University of Texas, MD Anderson Cancer Center, Houston, TX, USA

[7]Aberdeen Fungal Group. MRC Centre for Medical Mycology, Institute of Medical Sciences, University of Aberdeen, Aberdeen, Scotland, UK

[8]The University of Melbourne Centre for Stem Cell Systems, University of Melbourne, Parkville, Victoria, Australia.

[9]Eye and Vision Health, Flinders University School of Medicine, Adelaide, Australia

[10]Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, MD, USA

Please address correspondence to:

Holly L. Rosenzweig, PhD

Oregon Health & Science University

VA Portland Health Care System

3710 SW US Veterans Hospital Rd.

Bldg 103, Room E-221A

Mail stop: VA R&D-14

Portland, OR 97239

Phone: 503-220-8262 extension 52256

Fax: 503-402-2816

Email: rosenzwh@ohsu.edu

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Key words: Rodent, Autoimmunity, Eye, Uveitis, Inflammation

SUMMARY

Uveitis (intraocular inflammation) is a leading cause of vision loss. Although its etiology is largely speculative, it is thought to arise from complex genetic-environmental interactions that break immune tolerance to generate eye-specific autoreactive T cells. Experimental autoimmune uveitis (EAU), induced by immunization with the ocular antigen, interphotoceptor retinoid binding protein (IRBP), in combination with mycobacteria-containing CFA, has many clinical and histopathological features of human posterior uveitis. Studies in EAU have focused on defining pathogenic $CD4^+$ T cell effector responses, such as those of Th17 cells, but the innate receptor pathways precipitating development of autoreactive, eye-specific T cells remain poorly defined. In this study, we found that fungal-derived antigens possess autoimmune uveitis-promoting function akin to CFA in conventional EAU. The capacity of commensal fungi such as *C. albicans* or *S. cerevisae* to promote IRBP-triggered EAU was mediated by Card9. Since Card9 is an essential signaling molecule of a subgroup of C-type lectin receptors (CLRs) important in host defense, we further evaluated the proximal Card9-activating CLRs. Using single receptor-deficient mice, we identified Dectin-2, but not Mincle or Dectin-1, as a predominant mediator of fungal-promoted uveitis. Conversely, Dectin-2 activation by α-mannan sufficiently reproduced the uveitic phenotype of EAU, in a process mediated by the Card9-coupled signaling axis and IL-17 production. Taken together, this report relates the potential of the Dectin-2/Card9-coupled pathway in ocular autoimmunity. Not only does it contribute to understanding of how innate immune receptors orchestrate T cell-mediated autoimmunity, it also reveals a previously unappreciated ability of fungal-derived signals to promote autoimmunity.

INTRODUCTION

Uveitis (intraocular inflammation), a leading cause of vision loss in the Western world [1, 2], exists as an isolated condition or can occur as a manifestation of some systemic autoimmune diseases. Approximately 10% of cases of severe vision loss are thought to be attributed to autoimmune uveitis [3]. While the importance of autoreactive T cells is well-accepted in autoimmune uveitis, it is also known that innate signals, e.g. initiated by microbial detection, are required to generate pathogenic cellular responses. In classical experimental autoimmune uveitis (EAU), mice immunized with the retinal antigen, interphotoreceptor retinoid binding protein (IRBP), in the presence of CFA containing *M. tuberculosis,* develop T cell-mediated intraocular inflammation that clinically and pathologically resembles human uveitis [4]. Th1 and Th17 responses are known immunopathological mechanisms in EAU [4], yet little is known about the innate receptors that initiate this disease. To date, research on innate receptors in the eye has focused primarily on the contribution of Toll-like receptors (TLRs) to a wide range of ocular diseases. In ocular autoimmunity, as modeled in EAU, TLRs have been reported to play redundant roles, as mice deficient for TLRs 2, 3, 4 or 9 (as well as double-knockout (KO) mice) develop EAU comparable to their WT counterparts [5, 6]. Studies should therefore be broadened to include consideration of other innate receptor families, such as C-type lectin receptors (CLRs) or NOD-like receptors (NLRs), which have emerged as essential factors in host defense against microbes, but whose dysregulation has also been linked with inflammatory disease [7]. In the proposed paradigm where autoimmune disease develops as a result of complex interactions between individual genes and the environment (e.g. microbes, toxins, etc.), further exploration of innate receptor function in autoimmune uveitis is warranted.

The CLR family of immune sensors constitute an integral part of host defense against fungal infection via their ability to recognize components of the carbohydrate-rich cell walls of fungi such as *Candida* spp. or mycobacteria [8-10]. Several well-studied CLRs including Dectin-1, Dectin-2 and Mincle are known to elicit inflammatory responses through the adaptor molecule caspase-associated recruitment domain adaptor 9 (Card9) [11]. Intriguingly, Mincle and its associated Card9-coupled signaling axis were recently identified as a key pathway in the development of EAU [12]. Dectin-1 has also been implicated in EAU [13], thereby supporting an autoimmunity-promoting function for CLR family members in events that underlie break in tolerance and development of uveitis.

Aside from Dectin-1, which directly activates the protein tyrosine kinase Syk, other CLRs (e.g. Mincle, Dectin-2, Dectin-3) associate with FcRγ in order to activate Syk and subsequent signal transduction mediated by the adaptor molecule Card9. Assembly of a multi-protein complex involving Card9, Bcl10 and Malt1 is required for activation of MAPK- and NF-κB-mediated transcription of a network of genes important for generation of Th1 and Th17 responses [14]. While the Card9-activating CLRs may have differential roles in infection, Card9 expression appears absolutely critical for host defense against fungal and mycobacterial infection [15, 16]. Indeed, patients with mutations in *CARD9* succumb to candidemia [17, 18], an observation which has been investigated at a functional level in *Card9*-deficient mice infected with *C. albicans* [16]. On the other hand, dysregulation of *CARD9* also appears to be related to immune dysfunction, as it has been identified as a major genetic susceptibility determinant in systemic autoimmune diseases [19, 20]. It is thus intriguing to consider a central Card9-pathway that

exists at a pivotal intersection between microbial sensing and autoimmune disease. These collective observations led us to postulate that fungi could provide sufficient innate cues to elicit autoimmune uveitis via Card9 activation.

C. albicans is a commensal organism found in harmless association with humans, but can be pathogenic in immunocompromised individuals. Yet, here we report that innate cues provided solely by *C. albicans* or another commensal fungus (*S. cerevisiae*) are capable of promoting IRBP-triggered autoimmune uveitis in immunocompetent mice akin to classical EAU through a Card9-dependent mechanism. Conceptually, this observation is intriguing because it uncovers a previously unconsidered capacity for commensal fungi to promote autoimmunity akin to that of mycobacteria-containing CFA, a commonly used adjuvant for induction of EAU as well as other experimental autoimmune disease models. Moreover, the fungal-promoted form of uveitis is initiated by activation of Dectin-2, rather than Mincle or Dectin-1, and α-mannan sufficiently replicates the uveitis phenotype induced by *C. albicans* via a Dectin-2/Card9-coupled mechanism. This report extends our understanding of innate receptors in ocular autoimmunity beyond TLRs to include CLRs, thereby revealing the potential for fungal-derived components as environmental triggers of autoimmune uveitis. Our data, along with those of others, point to the Card9 pathway as an important mechanism in the control of immune tolerance and autoimmunity, a concept that could influence how we approach the study of autoimmune diseases.

MATERIALS AND METHODS

Mice: B10.RIII and C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice lacking expression of Card9, Mincle, Dectin-2, and Dectin-1 (encoded by *Card9*, *Clec4e*, *Clec4n*, *Clec7a*, respectively) have been described [8-10, 12, 21, 22]. Littermate controls were used to generate wild type (WT) and homozygous null mice, and all animals were bred in-house under specific pathogen-free (SPF) conditions at the VA Portland Health Care System. Mice aged between 8-12 weeks were used in experiments. All studies were carried out in accordance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals, and were performed under institutional protocols approved at the VA Portland Health Care System.

Induction of autoimmune uveitis: Induction of ocular autoimmunity was modeled after the classical model of EAU which employs CFA [12, 23]. Here, mice were immunized subcutaneously (s.c.) with 200 µl of an emulsion (1:1 v/v) of IRBP (125 µg purified IRBP + 300 µg $IRBP_{1–20}$ peptide (Anaspec, Fremont, CA)) and IFA containing 2.5 mg/ml (or ~1.5 $x10^7$ cells/mouse) of the fungal agonists: heat-killed *C. albicans* (HKCA; ATCC 10231, Invivogen), heat-killed *S. cerevisiae* (HKSC; ATCC 32119, Invivogen), α-mannan from *C. albicans* as described [24, 25] or purchased (Sigma). In the case of the highly susceptible B10.RIII strain, mice were immunized with 25 µg $IRBP_{161-180}$ peptide emulsified in fungal-supplemented IFA without pertussis toxin. For comparison, some studies included induction of EAU as per standard methods using CFA (Sigma) and 2.5 mg/ml *M. tuberculosis* (H37RA, Difco) [12]; or with IFA reconstituted with the mycobacterial cell-wall derived products: trehalose-6,6'-dibehenate (TDB, a synthetic analogue of mycobacterial cord factor; InvivoGen), peptidoglycan (PGN; InvivoGen) and muramyl dipeptide (MDP; Bachem, Torrance, CA).

Evaluation of clinical and histopathology of uveitis: Clinical fundus (posterior eye) images were acquired from live, anesthetized mice by topical endoscopic fundus imaging (TEFI) and the severity of inflammation was graded on a scale of 0 to 4 [12] as detailed [12]. At the termination of studies, eyes were harvested, and ocular histopathological changes were scored for severity on a scale of 0 to 4 [12]. The numbers of leukocytes infiltrating the anterior chamber and vitreous cavity were counted from histological sections. All scoring and enumeration were conducted in masked fashion. Representative images acquired (at 200X original magnification).

Multiplex quantitative real-time PCR: Dissected retinas from both eyes were pooled (n=6 mice/condition). Total RNA was extracted (RNeasy kit, Qiagen), and cDNA was synthesized (Reverse Transcription Kit, Applied Biosystems). Transcripts were analyzed using multiplex quantitative real-time PCR (RT^2Profiler™ PCR Gene Expression Assay kit, SABiosciences) and the PRISM® Sequence Detection system (Applied Biosystems) as described [12]. Array results are representative of four independently performed experiments, with a total of 24 mice/condition.

Antigen-recall assay of Th17 response-related cytokine production: To evaluate the antigen-specific Th17 cellular response, single cell suspensions prepared from spleens harvested 14d post-immunization were stimulated as previously reported [12]. Briefly, splenocytes were seeded in round-bottomed, 96-well tissue culture plates ($1.5x10^6$ cells/200 µl) and cultured overnight in the presence or absence of 20 µg/ml $IRBP_{1–20}$ peptide (Anaspec). Supernatants

were then collected and the concentration of IL-17A was measured as per manufacturer's instructions (Mouse IL-17 DuoSet ELISA; R&D Systems).

Immunoblotting: Ocular tissues were dissected and pooled (n=3 mice/tissue), and homogenized in lysis buffer. Immunoblotting was performed using antibodies to: Mincle (clone 4A9, MBL International), Dectin-1 (clone 2A11, AbD Serotec), Dectin-2 (polyclonal, Abcam), and β-actin (clone AC-15, Sigma), which were then detected with IRDye®-conjugated secondary antibodies and visualized with an Odyssey® scanner and software (LI-COR Biosciences). Results are representative of three independently performed experiments.

Statistical analysis: For quantitative real-time-PCR, conditions were compared using ANOVA with Tukey-Kramer post-hoc (Prism, GraphPad Software). For non-parametric data (i.e., clinical and histopathology scores), the Mann–Whitney *U* test (two-tailed) was used for comparison testing. All experiments were independently repeated. Results are presented as mean ± SEM and $p<0.05$ was considered significant.

RESULTS

C. albicans-derived signals promote autoimmune uveitis.

In light of the recently identified importance for the Card9-signaling pathway in induction of classical EAU [12], we hypothesized that fungi recognized by CLRs could similarly elicit autoimmune uveitis via a Card9-mediated mechanism. To explore this postulate, we first tested

whether substitution of mycobacteria with fungi could elicit ocular autoimmunity. Mice were immunized with IRBP in IFA (devoid of mycobacteria) that was supplemented with *C. albicans* instead. Clinical monitoring by fundus imaging revealed uveitis onset 7-14 days following immunization that continued to worsen up to 21d (Fig. 1A). This IRBP-dependent uveitis, which first appeared at and around the optic nerve head (arrow head; Fig. 1B), progressed along the blood vessels (i.e. retinal vasculitis), and encompassed larger areas of retinal lesions over time (Fig. 1B). Histopathology corroborated the clinical findings (Fig. 1C,D), and revealed pronounced pathological features including retinal structural damage (i.e. folding), vasculitis (green asterisk, Fig. 1D) and perivascular exudates in the ganglion cell layer (GCL). Granulomatous-like inflammation in the neural retina was consistently observed within the inner and outer nuclear layers (INL and ONL) as well as the retinal pigmented epithelial (RPE) layer. Extensive chorioretinal infiltrates were also frequently observed with subretinal hemorrhage. Leukocyte infiltration into the intraocular cavities, an additional parameter of uveitis, was significantly increased in the vitreous of the posterior segment (PS) and aqueous humor of the anterior segment (AS) (Fig. 1E). Importantly, control animals immunized with adjuvant alone (*C. albicans*-containing emulsion without IRBP) did not show any clinical or histopathological evidence of uveitis (Fig. 1); thereby underscoring the combinatorial requirement for innate signals provided by fungi and autoantigen in eliciting uveitis.

Fungal-promoted uveitis requires Card9 but is independent of Mincle.

Sensing of cord factor (or trehalose-6,6-dimycolate, TDM), a constituent of *M. tuberculosis* cell wall and immunostimulatory component of CFA [26], by the CLR Mincle is an important factor

in host defense against *M. tuberculosis* infection [27]. Mincle is also reported to be involved in detection of *C. albicans* [10], although the identity of the *C. albicans* ligand(s) remain(s) unknown. Since Mincle was previously identified as a CLR responsible in activation of a Card9-mediated mechanism in classical EAU [12] and the *C. albicans*-promoted uveitis observed here has pathological features and kinetics similar to classical EAU, we theorized that a similar mechanism, involving Mincle/Card9-coupled signaling, would be functionally important in fungal-promoted uveitis. Contrary to our postulate, Mincle-deficiency had a negligible effect on fungal-promoted uveitis, as evaluated both clinically and histopathologically (Fig. 2A, C). The protective effect of Mincle-deficiency as reported [12], however, was reproduced with IRBP-immunization using CFA (Supplemental Figure 1A).

Nonetheless, we found Card9 to be a requirement for induction of fungal-promoted uveitis, as disease severity was dramatically reduced in the absence of *Card9* expression (Fig. 2B, C). Most clinical and histological disease features were attenuated in Card9-deficient mice relative to WT; albeit we noted some mild inflammation surrounding optic nerve and blood vessels remained (Fig. 2C). These results suggest either a redundancy amongst CLRs and/or involvement of additional CLR(s) apart from Mincle in *C. albicans*-promoted uveitis. Intriguingly, the development of fungal-derived uveitis was not limited to *C. albicans*, because similar results were obtained in studies using another commonly encountered fungus, *S. cerevisiae* (Supplemental Fig. 1B, C). Card9-deficiency also ameliorated *S. cerevisiae*-induced uveitis, demonstrating the central role for Card9 in promotion of ocular autoimmunity in the context of fungi.

The Dectin-2/Card9 coupled signaling axis promotes autoimmune uveitis.

The above data underscore the central importance of the Card9 pathway in induction of ocular autoimmunity, be it promoted by fungi or mycobacterial triggers. They also suggest that signal activation may originate from unique CLRs apart from Mincle. The Card9-coupled CLRs Dectin-1 and Dectin-2 recognize β-1-3-glucan-containing and high mannose-based polysaccharides, respectively, both of which comprise a large portion of the cell wall of *C. albicans* and many other fungi [9, 28]. We have observed unique baseline protein expression profiles amongst the CLRs in naïve murine ocular tissues (Fig. 3A), with Dectin-2 expressed at considerable higher levels than Dectin-1 or Mincle within the retina (the primary tissue target in IRBP-induced uveitis). To address whether either of Dectin-1 or Dectin-2 might be involved in triggering fungal-uveitis, we evaluated uveitis severity in single receptor KO mice. Our data indicate that *C. albicans*-promoted uveitis occurs independent of Dectin-1 expression, as evaluated both clinically and histopathologically (Fig. 3B, C), suggesting either a redundant or non-essential role for Dectin-1 in *C. albicans*-promoted uveitis. However, uveitis was significantly reduced in Dectin-2 KO mice based on both clinical and histopathological assessment (Fig. 3D, E), indicating a novel role for Dectin-2 in the promotion of uveitis. In contrast to fungal-derived uveitis in WT mice, Dectin-2 KO mice exhibited marked reduction in retinal folding, photoreceptor destruction and granulomatous inflammation within the ONL and RPE layers. Clinical fundus imaging of these Dectin-2-deficient mice revealed residual inflammation seen primarily along the retinal vasculature (indicated by green asterisk in fundus image; Fig. 3E), which is a consistent pathological feature observed in fungal-derived uveitis in WT mice (green star, H&E-stained histology image; Fig. 3E). This indicates a residual Dectin-2

independent mechanism in the pathogenesis of fungal-derived uveitis. Adjuvant-injected control mice of either genotype showed no detectable histopathological or clinical signs of uveitis (n=6 mice/genotype; data not shown).

Collectively, the above data indicate that despite a shared ability amongst these CLRs to recognize fungal cell components and signal by way of Card9, Dectin-2, but not Dectin-1 or Mincle, plays a hitherto unrecognized role in *C. albicans*-dependent uveitis. To further investigate the ability of Dectin-2 in promotion of ocular autoimmunity, we explored whether uveitis could be reproduced using the recently identified Dectin-2 agonist, α-mannan [9]. Initially, the ability of α-mannan to elicit IRBP-induced uveitis was screened in B10.RIII mice, a strain that is known to be highly susceptible to EAU. Mice immunized with IRBP in conjunction with purified α-mannan (in lieu of intact *C. albicans*) developed robust uveitis (Fig. 4A), which was comparable to that promoted by *C. albicans*. Interestingly, we noted that the capacity of α-mannan to elicit ocular autoimmunity was similar to that of CFA (used in classical EAU), and was significantly more potent than mycobacterial agonists TDB, peptidoglycan (PGN) or muramyl dipeptide (MDP), all of which have been previously regarded as the responsible immunostimulants of CFA adjuvant. Negative control mice immunized with α-mannan in IFA alone (in the absence of IRBP) did not develop clinical or histopathological signs of uveitis, thereby demonstrating requirement for antigen.

The interaction between α-mannan and Dectin-2 in induction of uveitis was further evaluated using Dectin-2 KO mice (Fig. 4B, C). In contrast to uveitis promoted by α-mannan in WT mice, uveitis was significantly attenuated in Dectin-2 KO mice, as determined by clinical and

histopathological scoring (Fig. 4B, C), thereby signifying the importance of α-mannan in activating Dectin-2 in *C. albicans*-promoted uveitis. Finally, we determined whether the uveitis promoted by α-mannan/Dectin-2 interaction also required Card9. Indeed, Card9 deficiency significantly ameliorated uveitis, by both clinical and histopathological measures (Fig. 4B, C). Interestingly, the residual vessel damage and vasculitis was again noted in Dectin-2 KO mice (depicted in fundus and histology images; Fig. 4C, green asterisks), indicating a potential for α-mannan to elicit vasculitis via a Dectin-2-*independent* mechanism. Collectively, these data support the ability of α-mannan to promote development of autoimmune uveitis, which involves a Dectin-2/Card9-coupled mechanism.

The Th17 response is a key aspect of host defense against fungal infection in which the Card9-pathway mediates transcription of cytokines and chemokines that promote Th17 differentiation [14]. The flip side is that the Th17 response has also been established as a pathogenic mechanism that drives autoimmune uveitis [4]. To gain further insight into the extent to which polarizing factors associated with the Th17 response might be induced in fungal-promoted uveitis, we examined the transcriptional response in the retina, focusing particularly on a group of previously identified Card9-controlled signatures [12]. Examination of retinas dissected from mice with fungal-promoted uveitis revealed substantial induction of a number of cytokines and chemokines related to promotion of Th17 responses (Fig. 4D), as well as factors related to microbial detection such as: complement components C3 and C5aR, TLRs, and the Nod-like receptor (NLR) family member Nlrp3. Notably, genes encoding CLRs such as Mincle (*Clec4e*), Dectin-1 (*Clec7a*), and Dectin-2 (*Clec4n*) were all highly upregulated in retinas of immunized mice. Using antigen-recall assays, we next examined the extent to which activation of the

Dectin-2/Card9-coupled axis elicits an IRBP-specific Th17-associated response (Fig. 4E). Antigen-stimulated splenocytes showed significant increase in IL-17A production compared to media controls. Moreover, consistent with the marked reduction in uveitis, Card9-deficiency resulted in significantly reduced IRBP-specific induction of IL-17. This data supports a role for the Dectin-2/Card9-coupled pathway in promotion of a Th17 cellular response in uveitis.

DISCUSSION

The discovery of innate receptors such as TLRs has revolutionized our understanding of the mechanisms underlying microbial sensing and host defense. However, how innate receptor systems might be involved in autoimmunity remains poorly understood. Here, we present data that: 1) reveals a previously unappreciated capacity for fungi to promote development of autoimmune uveitis, 2) identifies the CLR Dectin-2 as a critical receptor, and 3) affirms Card9 as a central signaling pathway for promoting uveitis and Th17 responses. Together, this report sheds light onto microbial-host interactions in autoimmunity and provides a novel conceptual paradigm within which we can approach future exploration that could lead to the development of alternative therapies for autoimmune disease of the eye or other organs.

These data reveal a novel role for Dectin-2 in its ability to promote autoimmune uveitis. We noted a marked upregulation in genes encoding Card9-activating CLRs (Dectin-1, Dectin-2 and Mincle) within the retina in fungal-promoted uveitis. While the baseline protein expression of CLRs in retinas of naïve mice appeared to vary, Dectin-2 expression was constitutively

expressed (Fig. 3A). The cellular source(s) of CLRs such as Dectin-2 which function in uveitis remain to be determined, but our observations support consideration of Dectin-2 in neural tissues such as the eye or brain. Retinal expression of Dectin-2 could be attributable to resident immune-related cells (e.g. microglia, astrocytes), similar to what has been observed for Dectin-2 expression in microglia in the brain [29]. It is also worthwhile to consider expression and function of Dectin-2 in non-myeloid cells, as has recently been reported for Mincle in the case of ischemic brain injury [30]. Since CLRs are known to be predominantly expressed on myeloid cells [14], ocular function of Dectin-2 during uveitis could be further mediated by peripheral dendritic cells during priming or by phagocytes such as neutrophils and macrophages that infiltrate the eye during uveitis.

Few studies have examined the biological function of CLRs such as Dectin-2 in the context of autoimmunity. In experimental autoimmune encephalomyelitis (EAE), a model for CNS autoimmunity that shares immunological mechanisms with EAU, it was recently reported that Dectin-2 promoted autoimmunity [31], which is in line with our present observation in uveitis. Collectively, their report and ours support Dectin-2 as a CLR important to human health and further studies are warranted in non-CNS related autoimmune models such as experimental arthritis, particularly given the association between polymorphisms in *Clec4n* (gene encoding Dectin-2) and rheumatoid arthritis [32]. It is also interesting to consider how distinct microbial cues could elicit differential CLR activation. For example, our prior work in classical EAU (using CFA) demonstrated a role for Mincle, but not Dectin-2 [12]. This contrasts the present study on fungal-promoted uveitis, wherein Mincle was not involved. In terms of other experimental models of autoimmunity, Dectin-1 has been identified as an important inducer of

arthritis in the genetically predisposed SKG strain of mice [33]. The differential roles of individual Card9-associated CLRs, be they activated by mycobacterial and/or fungal-derived signals, in distinct autoimmune-mechanisms will be an interesting avenue of future research.

While the specific cellular and molecular mechanisms underlying the autoimmunity-promoting effects of the Dectin-2/Card9-pathway or of α-mannan were not explored in detail here, *in vitro* studies by others have demonstrated the requirement for Dectin-2 in cytokine responses (e.g. increased TNF, IL-6, IL-1β, IL-23 and IL-10) triggered by α-mannan [9]. In the case of Dectin-2, the Card9 signaling pathway, which is initiated by FcRγ-coupled Syk and mediated by the multi-protein complex of Bcl10 and Malt1, leads to activation of MAPK- and NK-κB-mediated transcription, activation of the Nlrp3 inflammasome, and production of reactive oxygen species [14], all of which are thought to be important in optimization of the Th17-mediated anti-fungal immunity. Our data support a central role for a Card9-mediated mechanism, as fungal-promoted uveitis as well as α-mannan-initiated uveitis were abrogated by Card9-deficiency. Indeed, retinas of fungal-immunized mice showed induction of similar sets of cytokine and chemokine genes that are involved in promotion of Th17 responses, an important aspect of the Card9-coupled signaling axis for controlling fungal infections [14]. Microbial detection genes such as complement components C3 and C5aR were also highly upregulated, which could be of functional relevance since anaphylatoxins C3a and C5a have been identified as mediators in classical EAU [34]. Also of note, the enhanced transcription of IL-1-related genes supports the requirement of the IL-1R/MyD88-signaling pathway reported in classical EAU [6], suggesting that the Nrlp3-inflammasome and downstream biological responses of IL-1 are a principal pathway for induction of ocular autoimmunity in both scenarios. Thus, it seems conceivable that

CLR signaling, which has even been shown to regulate IL-17 in a feed forward autocrine fashion in both innate myeloid cells [35] and Th17 cells, could be an important mechanisms involved in the breakdown of immunological tolerance that leads to ocular autoimmunity through the Card9-pathway.

Another intriguing observation from this study was that α-mannan appeared inherently more potent than the individual mycobacterial components TDB, PGN, and MDP and equivalent to that of whole *M. tuberculosis* in CFA in inducing autoimmune uveitis (Fig 4A). It would thus be intriguing and important to better understand the properties of α-mannan as it could have ramifications for adjuvant development. As a recently identified Dectin-2 agonist, the biological responses to α-mannan exposure are still being defined, but the observation that injection of α-mannan causes vasculitis in mice is especially interesting [36] given that α-mannan injection, even in Dectin-2 KO mice, have pronounced retinal vasculitis. One interpretation of this would be that α-mannan-mediated effects on endothelial cells are involved in breakdown of the blood-ocular barrier in uveitis. The residual disease in α-mannan-immunized Dectin-2 KO mice would suggest involvement of an additional immune receptor(s) besides Dectin-2 that signals by way of Card9 in the sensing of *C. albicans* mannoproteins. One putative candidate responsible for these attributes is the newly discovered CLR, Dectin-3, which is capable of forming a heterodimeric complex with Dectin-2 and acting synergistically to promote TNF, IL-6, and IL-17 production to *C. albicans* [37]. Alternatively, contaminants within the α-mannan preparation could conceivably activate CLRs-independent of Dectin-2 as well. Further studies examining Dectin-3 amongst in promotion of ocular autoimmunity would be of significant interest.

In conclusion, this study demonstrates that irrespective of upstream CLRs, the Card9 pathway is likely positioned at a crossroad between microbial response and autoimmune disease. Different classes of microbes that activate the Card9 pathway, including *Mycobacteria* or fungi, such as *C. albicans* or *S. cerevisiae* (as demonstrated here; supplementary data), have the capacity to provide the innate signals that drive retina-specific T cells in uveitis. Aberrant dysregulation of this pathway could be envisioned in genetically predisposed individuals, such as patients with *CARD9* polymorphisms, who develop ankylosing spondylitis [19], a condition that is strongly linked to uveitis. Given the ubiquity of *C. albicans* in our environment, demonstration of its relevance to disease may be complicated, but a number of observations in patients lend support to the concept of fungi as initiating and/or perpetuating factors in autoimmune disease. Elevated levels of anti-*S. cerevisiae* antibodies (ASCA) have been documented in systemic inflammatory disorders including Crohn's disease (another *CARD9*-linked condition) [20] and Behçet's disease [38, 39]. Notably, in Behçet's disease, uveitis is a diagnostic criterion [40], as up to 90% of patients with this condition develop uveitis [41]. The presence of anti-*S. cerevisiae* antibodies, and by extension T cells reactive to *S. cerevisiae,* in inflammatory disorders strongly linked with uveitis suggests that fungal elements may serve as important and previously unappreciated antigens capable of causing breaks in ocular-immune tolerance and predisposing individuals to uveitis. Certainly, observations made in autoimmune polyendocrinopathy, candidiasis, ectodermal dystrophy (APECED), argue for a more direct role for *C. albicans* in uveitis. This rare autosomal recessive syndrome is caused by a mutation in *AIRE* [42] and characterized by organ-specific autoimmunity, including uveitis [43, 44], as well as chronic mucocutaneous candidiasis [42], suggesting an interaction between fungal-induced pathways and autoimmunity beyond candidiasis.

Remarkable advances have been made in the understanding of immune recognition processes by CLRs during *C. albicans* infection, but have been relatively limited in the areas of autoimmunity. However, because EAU shares essential immunological mechanisms with other tissue-specific autoimmune disorders, including experimental autoimmune encephalomyelitis, neuritis, arthritis, orchitis, and others, these findings expand our understanding of the importance of CLRs in autoimmune disease, whether they are activated by fungi or mycobacteria, and of the central importance of the Card9 pathway in driving retina-specific T cells. Moreover, these findings could influence future basic science exploration of mechanisms of autoimmunity using experimental models that have historically relied on CFA.

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CONFLICT OF INTEREST: None

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Figure 1. ***C. albicans-derived signals promote autoimmune uveitis.*** Uveitis was evaluated in mice immunized with IRBP by both clinical examination (A,B) and histopathology scoring (C, D). (B) Representative fundus images depicting uveitis; black arrow denotes the optic nerve head. (D) Representative histological images depicting pathological features; green asterisk indicates vasculitis. L: lens; V: vitreous; R: retina. Ganglion cell layer (GCL), inner nuclear layer (INL), outer nuclear layer (ONL), retinal pigment epithelium (RPE). (E) Enumeration of cells within the aqueous of anterior segment (AS) or vitreous cavity of posterior segment (PS). An asterisk (*) indicates significance in IRBP-immunized mice compared to adjuvant-immunized (n = 10-20 mice/group/time from 2-3 experiments).

Figure 2. Fungal-derived uveitis requires Card9 but is independent of Mincle. To assess the role of Mincle (A, C) or Card9 (B, C) in fungal-promoted uveitis, WT, Card9 KO and Mincle KO mice were immunized with IRBP in an emulsion containing *C. albicans* and uveitis was evaluated by clinical examination and histology on day 21. (C) Clinical and histological images of the fundus and posterior eye are shown. An asterisk (*) indicates significance compared to WT mice (n=12 mice/group combined from 2 independently performed experiments).

Figure 3. Dectin-2 contributes to pathogenesis of fungal-promoted uveitis. Representative immunoblotting of indicated ocular tissues (pooled from both eyes of n=3 mice) from naïve WT mice showing CLR expression (A). Uveitis was induced in WT or Dectin-1 KO (B, C) vs. Dectin-2 KO (D, E) mice by immunization with IRBP in an emulsion containing *C. albicans* and evaluated by clinical and histological examination. Fundus and histological images of the posterior eye d21 post-immunization (green asterisk indicates vasculitis). No significant

differences between WT and Dectin-1 KO mice were observed (n=10-12 mice/group combined from 2 independently performed experiments). An asterisk (*) indicates significance compared to WT mice (n=12 mice/group combined from 2 independently performed experiments).

Figure 4. Activation of the Dectin-2/Card9 coupled signaling axis promotes autoimmune uveitis. B10.RIII mice were immunized with IRBP emulsified in IFA supplemented with α-mannan and uveitis was scored from histological sections (A). For comparison, mice were also immunized with heat-killed *C. albicans* (HKCA), CFA, or the mycobacterial-derived products TDB, PGN or MDP. An asterisk (*) indicates significance compared to CFA-immunized mice n=10 mice/group; combined from 2 independent experiments). To determine the specific role(s) for Dectin-2 or Card9 in α-mannan-induced uveitis, disease severity was evaluated clinically and histopathologically in KO mice (B, C). An asterisk (*) indicates significance compared to WT mice while # indicates significance between Dectin-2 and Card9 KO mice (n=24 mice/group combined from 3 independently performed experiments). (C) Fundus and histological images of the posterior eye d21 post-immunization. (D) Heat map showing retinal gene expression at d10 post-immunization as evaluated by multiplex quantitative PCR analysis. Fold-change in gene expression is relative to naïve control animals (n=6 mice pooled/group; array results are representative of 4 independently performed experiments). (E) Cytokine concentration in culture supernatants was determined at 24 h post IRBP-stimulation by ELISA (n=5 mice/group, performed in triplicate). An asterisk (*) indicates significance compared to media-stimulated control cells.

Figure 1.

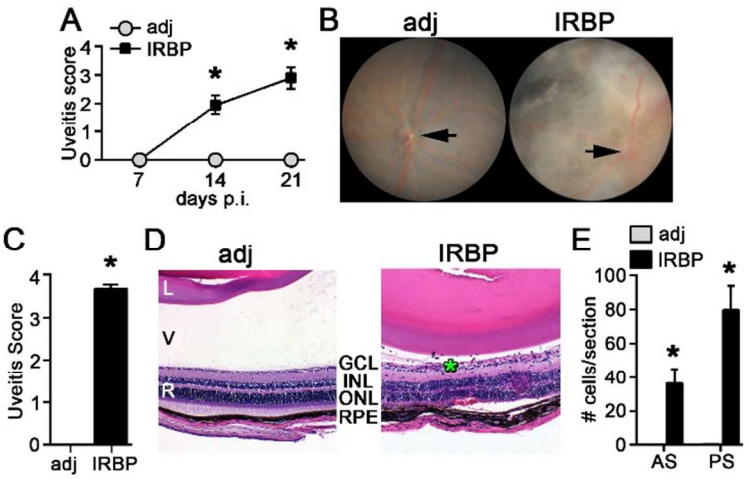


Figure 2

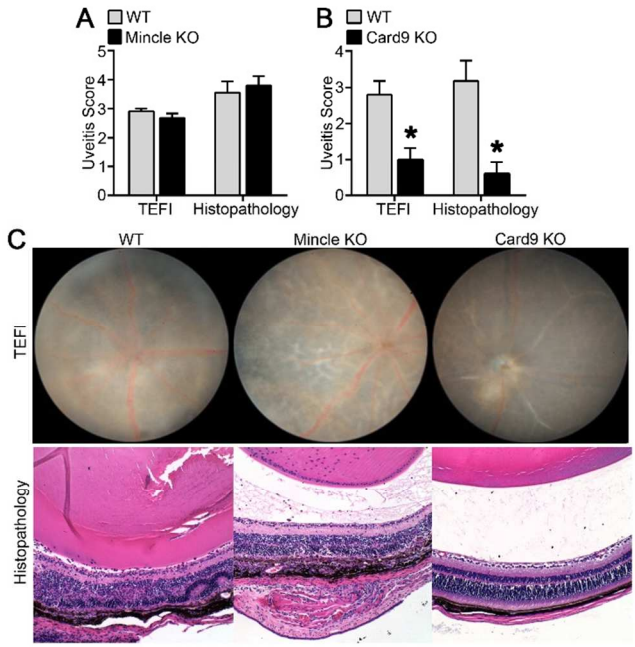


Figure 3

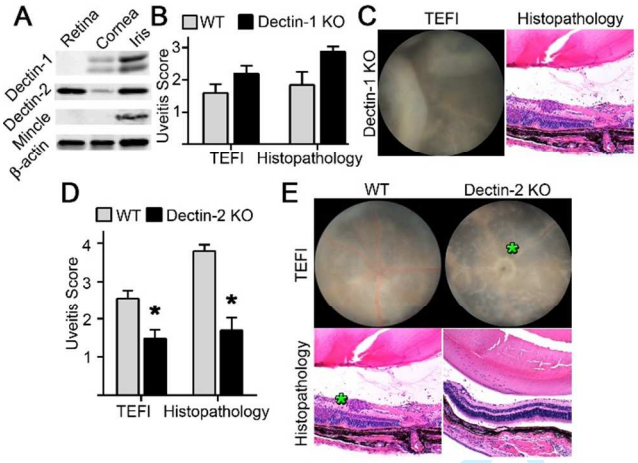
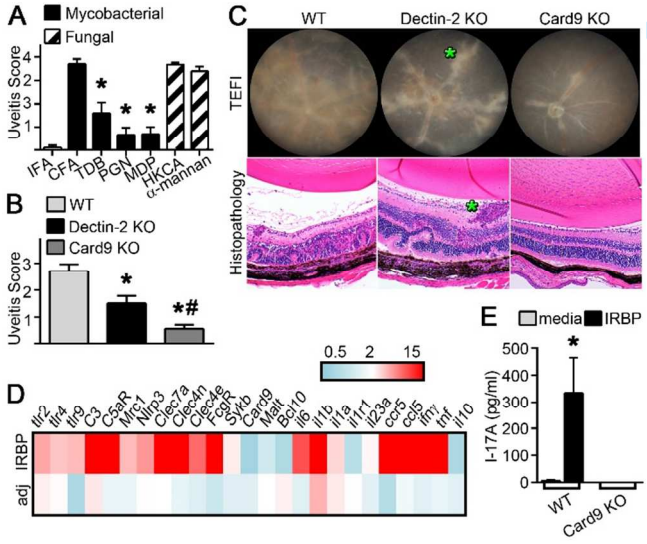
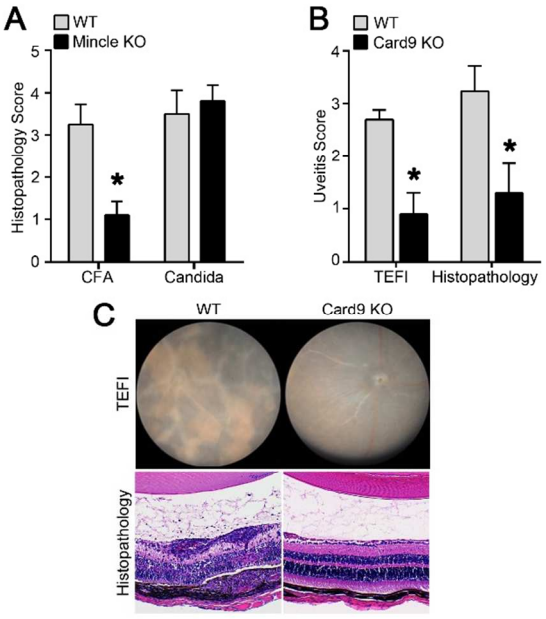
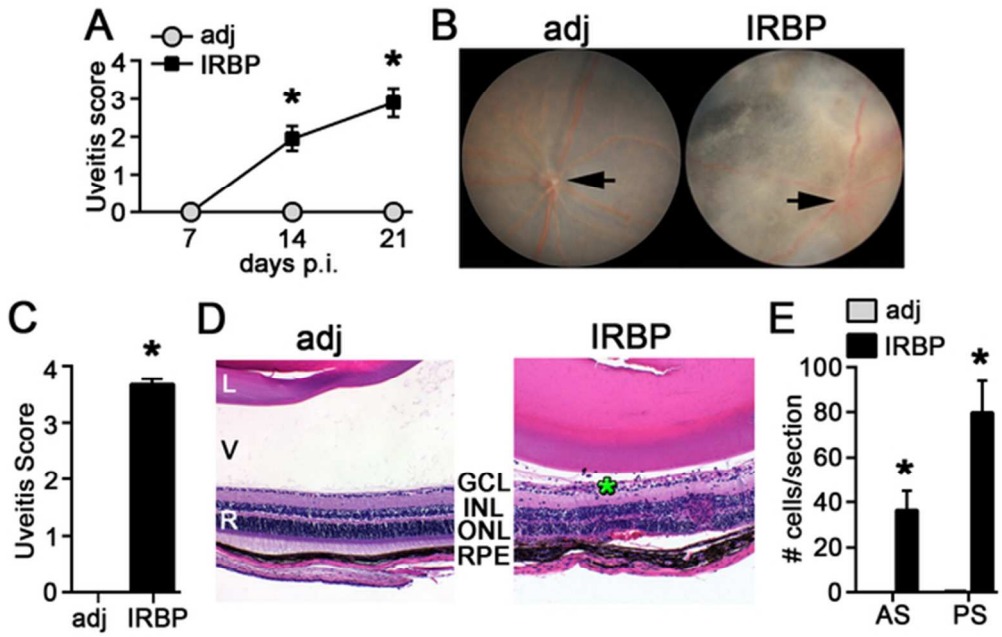


Figure 4





Supplemental Figure 1. EAU triggered by the yeast *S. cerevisiae* is mediated by Card9. For comparison of the role of Mincle in the different modes of uveitis-induction, uveitis severity was evaluated in WT and Mincle KO mice immunized with standard CFA vs. *C. albicans*-containing adjuvant (A). WT and Card9 KO mice were immunized with IRBP emulsified with heat-killed *S. cerevisiae* and uveitis was assessed clinically and histologically on d21 post-immunization (B, C). (C) Examples of clinical and histological images of the posterior eye. An asterisk (*) indicates significance compared to WT mice (n=10-12 mice/group combined from 2 independently performed experiments).



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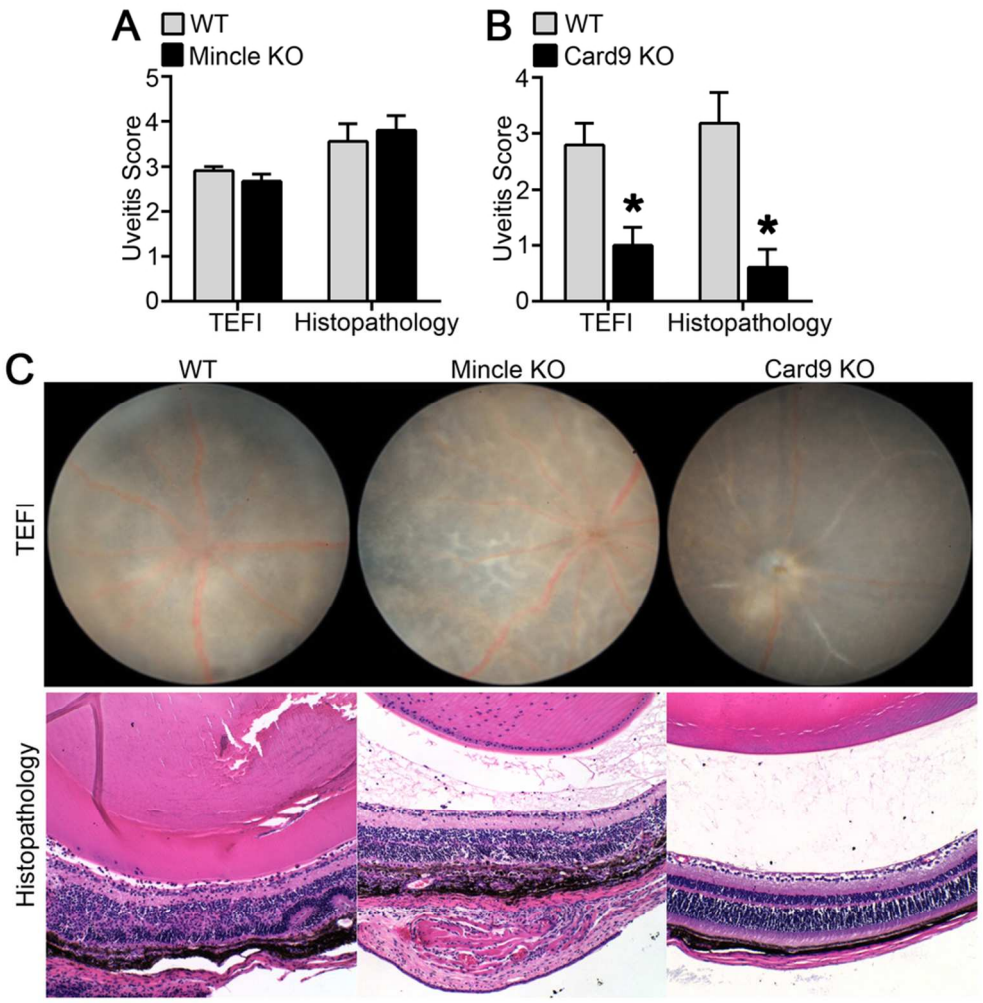

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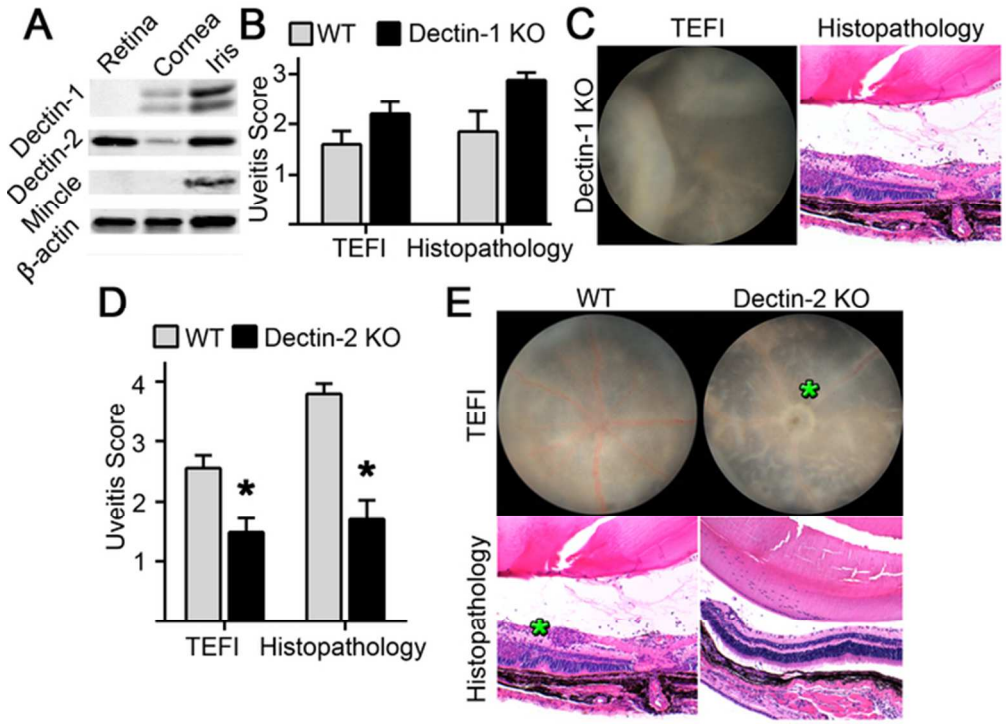

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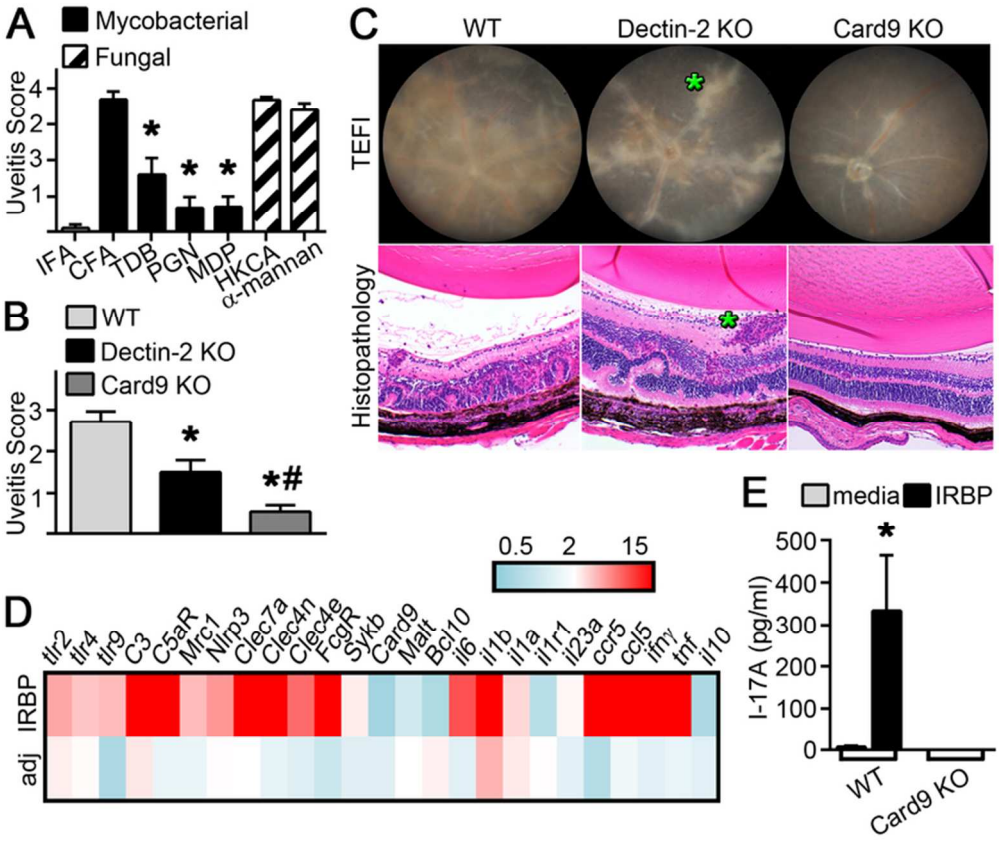

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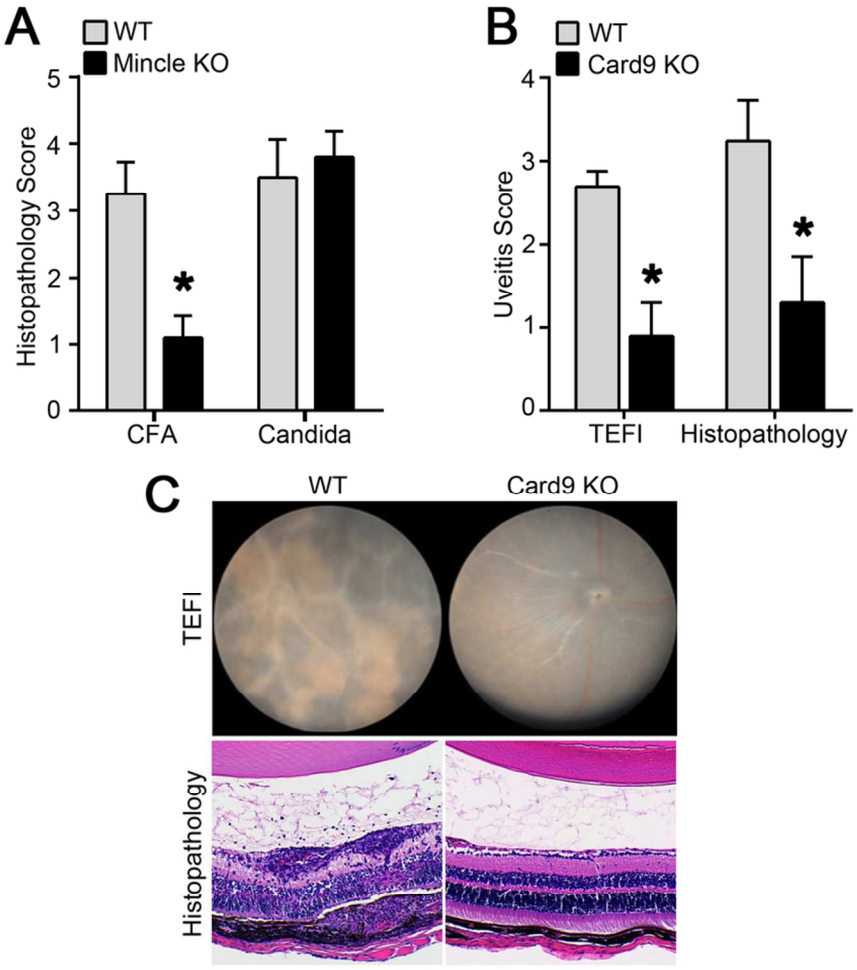

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