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Innate immune recognition of molds and homology to the inner ear protein, cochlin, in patients with autoimmune inner ear disease

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Abstract

Autoimmune Inner Ear Disease (AIED) is characterized by bilateral, fluctuating sensorineural hearing loss with periods of hearing decline triggered by unknown stimuli. Here we examined whether an environmental exposure to mold in these AIED patients is sufficient to generate a pro-inflammatory response that may, in part, explain periods of acute exacerbation of disease. We hypothesized that molds may stimulate an aberrant immune response in these patients as both several *Aspergillus* species and *penicillium* share homology with the LCCL domain of the inner ear protein, cochlin. We showed the presence of higher levels of anti-mold IgG in plasma of AIED patients at dilution of 1:256 ($p=0.032$) and anti-cochlin IgG 1:256 ($p=0.0094$ and at 1:512 $p=0.024$) as compared with controls. Exposure of peripheral blood mononuclear cells (PBMC) of AIED patients to mold resulted in an up-regulation of IL-1 mRNA expression, enhanced IL-1 and IL-6 secretion, and generation of IL-17 expressing cells in mold-sensitive AIED patients, suggesting mold acts as a PAMP in a subset of these patients. Naïve B cells secreted IgM when stimulated with conditioned supernatant from AIED patients' monocytes treated with mold extract. In conclusion, the present studies indicate that fungal exposure can trigger autoimmunity in a subset of susceptible AIED patients.

Keywords

mold; cochlin; Interleukin-1 beta (IL-1 β); LCCL domain; Autoimmune Inner Ear Disease (AIED)

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Disclosures

Dr. Vambutas and the Feinstein Institute for Medical Research hold a patent for the use of IL-1 receptor antagonists for the treatment of AIED and related diseases.

1. Introduction

Autoimmune inner ear disease (AIED) is an inflammatory disease of the inner ear characterized by rapidly progressive bilateral sensorineural hearing loss [1]. Thirty percent of AIED patients have systemic autoimmune diseases [2]. Notably, first degree relatives are not afflicted, arguing against a heritable disease, although HLA predispositions have been reported [3]. AIED may be associated with the release of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF α) [4, 5], and Interleukin-1 beta (IL-1 β) [6]. These cytokines may be released by macrophages, or fibrocytes which are macrophage-like cells resident in the cochlea [7]. COCH5B2 (the “coagulation factor C homolog” encoded by the COCH gene), is known as cochlin, and is one of the most predominant proteins found in the cochlea [8]. Cochlin is a highly conserved protein, and contains a LCCL domain that has been identified in many species [9, 10], including several mold species to which some AIED patients anecdotally report sensitivities. Anti-cochlin antibodies have been identified in the sera of patients with AIED [11, 12] and cochlin-specific CD4⁺ T cells have also been identified in AIED patients [12].

AIED, Meniere's disease and sudden sensorineural hearing loss are three conditions that are characterized by a sudden decline in hearing that may be recoverable with corticosteroids in approximately 60% of cases [13], suggesting these three disorders may be related and share similar immunologic responses [14, 15]. Patients with AIED and Meniere's disease exhibit enrichment of concurrent systemic autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and ankylosing spondylitis [16]. Factors that trigger a decline in hearing are largely unknown. Patients report a variety of triggers, including environmental allergen exposure. The prevalence of inhalant allergy is considerably higher in patients with Meniere's disease compared to controls [17, 18], and changes in electrocochleography, an objective measure of cochlear fluid retention, pathognomonic for Meniere's Disease, have been observed following an inhalant allergy challenge in atopic Meniere's patients [19]. Elevated IgE levels in response to mold are present in patients with idiopathic sudden sensorineural hearing loss [20], although we have not identified similar IgE elevations by CAPRAST testing in a subset of our patients (not shown).

The role of IL-1 β has become evident in the pathogenesis of immune mediated hearing loss, including AIED. Expression IL-1 β has been shown to be up-regulated in the early events following acoustic trauma in animals [21]. Increased IL-1 β expression was observed in an animal model of immune mediated hearing loss [22]. We have been able to segregate corticosteroid responsive and resistant AIED patients based on their expression of an IL-1 decoy receptor, (IL1R2) [23], and recently identified enhanced IL-1 β expression in corticosteroid-resistant AIED patients [6], although corticosteroid-sensitive AIED patients expressed greater IL-1 β than unaffected individuals [6]. We hypothesized that in a subset of AIED patients, mold exposure initiated IL-1 β release in these patients, and thus contributed to exacerbation of their disease.

Aspergillus species have been shown to upregulate pro-IL-1 β expression, and the induction of IL-1 β secretion in human monocytes [24]. IL-1 β is induced *in vitro* by alveolar macrophages and in peripheral blood monocytes in response to *Aspergillus* antigens [25, 26]. IL-1 β is also induced in the lungs of mice with an X linked Chronic Granulomatous Disease-like disease that have invasive aspergillosis [27]. Presence of IL-1 β is essential for propagation of inflammation [28,29]. Several groups have established that IL-1 β has a crucial role in inducing the differentiation of naive human T-cells to IL-17-producing T-cells [30, 31], and IL-17 secretion [32-34]. In IL-1 receptor antagonist-deficient mice inflammatory arthritis spontaneously develops due to unopposed excess IL-1 β signaling driven by IL-17-producing T cells [35]. IL-17 has been associated with many inflammatory

diseases, such as rheumatoid arthritis, lupus and other autoimmune diseases [36-38]. Lasigliè *et al.* recently showed that exaggerated IL-1 secretion due to NLRP3 mutations affected the IL-23/IL-17 axis in Cryopyrin Associated Periodic Syndrome (CAPS) patients, providing further evidence of the role of IL-1 in T_H17 differentiation [39].

Material and methods

Human subjects

These studies were approved by the Institutional Review Board at the North Shore-LIJ Health System, and after obtaining informed consent, patients with AIED were recruited by two Neurologists and had their blood drawn for PBMC isolation. Inclusion criteria were as previously published, and are shown in Table I [6]. Patients were not excluded if they exhibited systemic autoimmune diseases. The demographic information on the 20 AIED patients is shown in table II.

Human PBMC culture and stimulation

Peripheral blood mononuclear cells (PBMC) were isolated as previously described [4] and cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals, Norcross, GA), 100 units/ml penicillin, 100 µg/ml streptomycin and 4.1 mM glutamine and plated at 1×10^6 cells/ml in 24-well plate. In some experiments, mold extract was added (Allergenic Extract-Mix Molds contains 4 equal parts of Mix *Aspergillus* (*oryzae*, *repens*, *niger* and *terreus*) and 2 equal parts mix *Penicillium* (*notatum*, *chrysogenum*), *Alternaria* (*cladosporium*, *sphaerospermum*) (ALK Abelló, Round Rock, TX). The mold mix was dialyzed to remove phenol using Slide-A-Lyzer dialysis cassette (Thermo Scientific, Rockford, IL) against deionized distilled water. The mold protein concentration was analyzed by bicinchoninic assay (Pierce, Rockford, IL). One $\times 10^6$ PBMC per ml was stimulated with 5µg/ml dialyzed, mixed mold or 2.5 µg/ml cochlin (US Biological, Swampscott, MA) for 16 h at 37°C in 5% CO₂ and compared with cultured, unstimulated PBMC. The optimal concentrations of these reagents were previously identified by culturing PBMC with increasing concentrations of these reagents to identify the maximal concentration (mold mix: 5µg/ml, cochlin: 2.5 µg/ml) that could be used without affecting cell viability. At the end of all incubations, PBMC were centrifuged, RNA harvested and supernatants were collected and stored in -20 °C.

BLAST search and sequence alignments

Protein sequences were obtained from the National Center for Biotechnology Information (NCBI) World Wide Web site. Amino acid sequence alignments were performed on the BLAST NCBI site. NCBI protein accession numbers for Cochlin, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus oryzae* and *Penicillium chrysogenum* are NP 001128530.1, GI 134056528, GI 1114191847, GI 83773484 and GI 255946413 respectively. Homology between LCCL domain of cochlin and LCCL domain of *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus oryzae* and *Penicillium chrysogenum* was identified by BLAST search (supplementary figure 1). We designed 5 overlapping peptides within this LCCL domain, approximately 25 amino-acids in length purchased from Mimotopes (Victoria, Australia), optimized for class II presentation (supplementary figure 2).

Direct ELISA for cochlin or mold mix antibody titers

Plasma from AIED patients and control subjects were tested by direct ELISA for cochlin and mold mix IgG levels. Recombinant human cochlin was coated at concentration of 10 µg/ml in a 100 µl volume or dialyzed Allergenic Extract-Mix was coated at 6 µg/ml in a 100 µl final volume on 96 well ELISA plate (Immulon 2B) incubated overnight (4°C), washed 3x

in 0.05% Tween 20 in Phosphate buffered Saline (PBST), blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO), and washed in 0.05% PBST. Plasma was titrated from 1:64-1:2048 in the case of molds and 1:128-1:1024 in the case of cochlin in normal healthy controls and AIED patients. Plasma was tested by direct ELISA for binding to either mixed mold or recombinant human cochlin at all plasma dilutions, and the presence of bound antibody was determined using a peroxidase conjugated-goat anti-human IgG heavy and light chain antibodies (Zymax Grade Invitrogen, Carlsbad, CA). Detection was performed by treatment with H₂O₂ in ABTS substrate (Sigma-Aldrich St. Louis, MO), and stopped after 30 min by adding ABTS Peroxidase Stop Solution (KPL, Gaithersburg, MD) containing 5% SDS. Change in absorbance was read at 405 nm in an ELISA reader equipped with Biolinx 2.2 software (Dynatech laboratories, Chantilly, VA). IgG Concentrations in plasma were determined by coating purified Human IgG (Invitrogen, Carlsbad, CA)

Quantitative Real Time PCR (Q-RT-PCR)

Quantitative Real-Time (Q-RT-PCR) PCR was performed using the ABI 7900HT Fast Real-time PCR System (Applied Biosystems), Primer sequences, nucleotide position number, gene bank accession numbers for IL-1 , and actin and Q-RT-PCR conditions were as described previously [6]. Relative quantification of the PCR signals was calculated by comparing the cycle threshold value (C_t), in duplicate, of the gene of interest of each sample with the C_t values of the housekeeping gene actin. Q-RT-PCR analysis for each sample was performed in duplicate.

IL-1 β ELISA

Plasma and conditioned supernatants were collected and stored at -20°C until a sufficient number of samples were acquired. Frozen samples were thawed immediately prior to analysis and none of the samples underwent repetitive freeze-thawing cycles prior to analysis. IL-1 levels in conditioned supernatant were quantified using a sandwich ELISA (R&D Systems, Minneapolis, MN) as previously described [6]. All samples were run in duplicate, and the mean variance was 0.02%. Several duplicates samples of previously run plates were repeated to ensure reproducibility.

IL-17 ELISPOT assay

PBMC from AIED patients and control subjects (2.5×10^5 PBMC/well) were plated in 10% FBS RPMI 1640 medium in flat-bottom 96-well anti-IL-17 coated ELISPOT PVDF plates (R&D Systems, Minneapolis, MN) in the presence of medium alone, or 5 μ g/ml mold mix, or 2.5 μ g/ml cochlin or 2.5 μ g/ml overlapping peptides or 0.5% DMSO for 16 hours. Overlapping peptides of approximately 25 amino-acids length generated from the LCCL domain of the cochlin (P1-P5) were purchased from Mimotopes (Victoria, Australia) (see supplemental figure 2 for peptide sequences) in lyophilized state. In brief peptides were rehydrated in 100% DMSO keeping stock concentration 10 mg/ml. Several concentrations of peptides in DMSO were tried ranging from 0.005, 0.05, 0.5 and 2.5 μ g/ml before using 2.5 μ g/ml as final optimized concentration for all peptides. After 16 hours, wells were washed, and detection of IL-17 was performed as per manufacturer's protocol. Results are shown as mean antigen-specific spot forming cells (SFC) after background subtraction of control wells containing no antigen. All peptide treated wells were subtracted from DMSO control wells. Each antigen condition was performed in duplicate or triplicate. Spots were scanned and enumerated with Phoretix Expression software (Non Linear Dynamics).

Generation of antigen-specific enriched T-cell cultures

PBMC from 2 AIED patients (1 clinically corticosteroid-sensitive and mold sensitive by skin testing) and 2 normal healthy controls were cultured in 1 ml RPMI 1640 supplemented with 10% human AB serum (Invitrogen, Carlsbad, CA), 4.1 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at a density of 1×10^6 cells/ml in 24-well plates (Costar). Cultures were stimulated with either mold extract (5 µg/ml), cochlin (2.5 µg/ml) or Tetanus toxoid TT (1.5 µg/ml) (Massachusetts Biological Labs, Boston, MA) to induce proliferation over a 17 day period [40]. On day 3, 1/5th of the media was removed and replaced with fresh antigen. On day 7, 1×10^6 irradiated (4000 Rad) feeder cells (cryopreserved, pooled PBMC from 12 healthy controls) were added to each well and cultures were incubated without additional antigen (resting phase). On day 14, feeder cells were replenished and cells were re-stimulated with antigen for an additional 3-4 days. On day 17, cells were harvested, washed and then resuspended in 2.5% BSA/PBS with 1mM EDTA and sorted on a BD FACSAria II flow cytometer using a blast gate (based on the increased forward and side scatter characteristics of blast cells as compared to resting lymphocytes). Antigen-stimulated blast cells (n=100) per well were deposited onto an IL-17 ELISPOT plate, and cross-stimulated for 16 hours with either cochlin, or mixed mold or tetanus toxoid.

Monocyte isolation and stimulation with mold extract

Monocytes were isolated from buffy coats by negative selection using MACS monocyte isolation kit II (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions as described previously [6]. Granulocytes were removed by positive magnetic selection by adding CD15 microbeads (Miltenyi Biotec, Auburn, CA). Purified monocytes were plated at 0.25×10^5 per 0.25 ml in RPMI 1640, supplemented with 10% FBS and penicillin/streptomycin, incubated in a 5% CO₂ incubator at 37°C. Monocytes were exposed for 16 h at 37°C to Mold extract (5 µg/ml). Supernatants from the stimulated monocytes were collected and frozen at -70°C until used in the B cell cultures or analyzed further. The purity of negatively selected monocytes, as determined by flow cytometry (FACS Canto II; BD Biosciences), was 87.1% stained with CD14-PE mAb (BD Immunocytometry Systems, San Jose, CA).

B cell cultures

Naïve B cells were isolated from PBMCs by negative selection using Naïve B Cell Isolation Kit II (Miltenyi Biotec, Auburn, CA). The purity of naïve B cells was determined by staining with anti-CD19/PE (SJ25-C1, BD Pharmingen, San Jose, CA), CD27-APC (BD Biosciences San Jose, CA) and anti-human IgD FITC (Life Technologies Corporation) based on their expression of CD19 and lack of CD27 (purity exceeded 82 %). Isolated cells expressed surface IgD. Total CD4⁺ T cells were isolated using a CD4⁺ T cell enrichment cocktail (Stem Cell Technologies). The purity of CD4⁺ T cells was determined by staining for anti-human CD4-APC (purity exceeded 96 %). (eBioscience). Immediately before use, CD4⁺ T cells were -irradiated at 50 Gy using Gamma cell 1000 (Best Theratronics), washed, and pulsed with the superantigen TSST-1 (5 ng/ml; Sigma-Aldrich). Purified, naïve B cells were cultured for 6 days at 37°C in polystyrene round-bottom 96-well plates (Costar) at 5×10^5 cells/ml in complete medium. Naïve B cells were cultured alone or together with irradiated CD4⁺ T cells (B cell: T cell ratio, 1:4). In addition, 50µg/ml recombinant IL-2 (Roche) was added. All cultures were carried out in the presence of BCR ligand, F(ab)₂ anti-human IgM/IgG/IgA (2.5 µg/ml; Jackson ImmunoResearch, West Grove, PA, USA). B and B&T cell cultures were exposed to supernatants (at 1:10 or neat) from untreated, Mix mold (5 µg/ml) or LPS (1µg/ml) overnight treated autologous monocytes at a 1:4 ratio of supernatant: culture volume. These B & B/T cell cultures were exposed to monocyte supernatant for 3 days, and then replenished with another aliquot of the same monocyte

supernatant (stored in -70°C) for an additional 3 days. The B cell and B/T cultures were then analyzed for the presence of IgM in the supernatants.

Evaluation of antibody production

The presence of IgM in the supernatants from B cells in the culture conditions was detected by an anti-IgM (Human) ELISA Kit, a sandwich enzyme immunoassay (Abnova). All samples were run in duplicate and were done as per manufacturer's protocol. Substrate reactions were terminated with 0.5 N HCl and the Optical densities were read at 450 nm.

Statistical analysis

Data analysis was carried out using the graph pad statistical package, Version 5.00 for Windows, Graph Pad Software, San Diego, California USA, www.graphpad.com. In all figures, the mean and standard error of the mean (SEM) are shown. Data groups were analyzed either by the Mann–Whitney U test, or by one-way analysis of variance (ANOVA). Post hoc testing was performed using a Bonferroni's Multiple Comparison Test on selected columns. Statistical significance was achieved at p less than, or equal to 0.05.

Results

AIED patients have higher levels of anti-mold and anti-cochlin IgG when compared to normal healthy controls

Plasma from 20 AIED patients and 10 normal healthy control subjects were tested by direct ELISA for IgG levels to mixed molds and recombinant human cochlin at serial dilutions from 1:256 through 1:1024. AIED patients showed presence of greater IgG levels to mold and cochlin in comparison to normal healthy controls. This difference was statistically significant at dilution 1:256 ($p=0.032$) in case of mold and at 1:256 ($p=0.0094$) and at 1:512 ($p=0.024$) in case of cochlin when compared to normal healthy controls (Figure 1) by the Mann–Whitney U test with 95% CI. Sixteen out of 20 patients (80%) at dilutions of 1:256 and 1:512, and 12 out of 20 patients (60%) at a dilution of 1:1024, have higher titers of anti-cochlin antibodies compared to normal healthy controls. Interestingly, there was no correlation between anti-cochlin antibodies, or anti-mold antibodies and IL-1 β expression or in the average anti-mold or anti-cochlin titers when we stratified the AIED patients into groups based on the ability to respond to corticosteroids (not shown).

Mold induces IL-1 β expression

In order to determine whether mold induces IL-1 β expression, Q-RT-PCR was used to determine the expression of the IL-1 β mRNA by mold-stimulated PBMC compared to unstimulated PBMC in patients (N=24) and controls. Incubation of PBMC with mold extract resulted in a dramatic increase in IL-1 β mRNA expression by AIED patients, as compared with a low level of mold induced IL-1 β mRNA expressed by controls (Figure 2A). Consistent with enhanced IL-1 β mRNA expression in response to mold, mold-stimulated PBMC released IL-1 β into the culture supernatant of AIED patients (Figure 2B). Interestingly, we observed that samples from corticosteroid-sensitive AIED patients who have higher basal expression of IL-1 β by their PBMC mounted a less robust induction of IL-1 β in response to mold (1.9 fold vs. 128 fold induction), likely due to their enhanced basal expression (Figure 2C). These results demonstrate that mold extracts stimulate IL-1 β transcription and release in PBMC from AIED patients.

Mold acts as a PAMP (Pathogen Associated Molecular Pattern)

Given that mold is capable of inducing IL-1 β in PBMC, we asked whether mold alone was sufficient to induce IL-1 β release from monocytes without the addition of LPS. Indeed,

monocyte cultures from AIED patients stimulated with mold was capable of inducing IL-1 release (Figure 2D). Notably, IL-1 release from monocytes from AIED patients was statistically significantly different from either monocytes of control subjects or the non-monocyte fraction of AIED patients at $p < 0.05$. Mold was also capable of inducing IL-6 in the PBMCs of AIED patients and control subjects, and was comparable to the effect seen with LPS. Notably, IL-1 stimulation resulted in enhanced IL-6 release with PBMC from AIED patients ($p < 0.05$) as compared with controls, suggesting PBMC of AIED patients are more exquisitely responsive to pro-inflammatory signals, and thus serve to perpetuate inflammation.

Mold or cochlin stimulus induces IL-17 production in AIED patients

Given that IL-1 is known to induce expression of IL-17, we asked whether mold or cochlin stimulus would also induce IL-17 production in AIED patients. PBMCs from AIED patients and control subjects were stimulated with either cochlin (2.5 $\mu\text{g/ml}$), or mold (5 $\mu\text{g/ml}$) for 16 hours. These stimulated PBMCs were compared to unstimulated controls by IL-17 secretion by ELISPOT (Figure 4). Indeed, PBMC of AIED patients showed much higher numbers of IL-17 secreting cells in response to mold and cochlin compared to control subjects. PBMC from AIED patients showed 3.7 and 10.6 fold higher expression of IL-17 in response to mold and cochlin respectively, compared to mold and cochlin stimulated PBMC from normal controls (figure 4). In order to identify whether the LCCL domain of cochlin and mold was responsible for the observed IL-17 secretion, 5 overlapping peptides from the LCCL domain (supplemental figure 2) of cochlin that shared homology with three *Aspergillus* species (*A. niger*, *A. terreus*, *A. oryzae*) and *Penicillium chrysogenum* were used (figure 4, supplementary figure 2). Use of individual peptides from the LCCL domain also were able to initiate IL-17 release in 2 AIED patients, most notably by peptide 3, and to a lesser extent, peptide 2 which overlaps with peptide 3 by 9 amino acids, although comparisons between the peptides did not reach statistical significance. These experiments suggest the LCCL domain appears to constitute a minimal epitope necessary for IL-17 release, and shared homology exists between the environmental molds tested and cochlin.

To further investigate whether these were indeed cross-reactive epitopes, isolation of antigen specific T cell blasts to mold from a corticosteroid-sensitive, mold-sensitive AIED patient were capable of producing IL-17 when exposed to cochlin (Figure 5), highlighting the similar immunologic responses to disparate antigens. The ratio of antigen specific IL-17 secreting cells to unstimulated IL-17 secreting cells for each condition is shown. A five-fold difference in IL-17 release was observed in the mold enriched T cell cultures from AIED patients stimulated with cochlin as compared to mold cultures stimulated with tetanus, or tetanus enriched cultures.

AIED patients secrete IgM in response to mold challenge

To determine whether mold could induce IgM synthesis in naïve B cells of AIED patients, purified B cells alone or in combination with T cells, were stimulated for 6 days with supernatants of monocytes treated with mold, LPS or untreated, were analyzed for IgM production. The assay was performed on 4 AIED patients and 2 normal healthy control subjects. Addition of supernatants from mold-stimulated-monocytes to B cells resulted in enhancement of IgM production when compared to B cells cultured with supernatant from unstimulated monocytes or diluted mold stimulated supernatant (1:10) (Figure 6). Mold stimulated B cells alone resulted in a markedly higher level of IgM release when compared to B cells cultured in presence of CD4^+ T cells, suggesting T cell help was not necessary for IgM production (not shown).

Discussion

The most common theory of development of AIED is existence of cross-reactive epitope(s) between environmental antigens and inner ear proteins [41]. We hypothesized that a sequence similarity in the LCCL domain between mold and the inner ear protein cochlin may permit mold to act as a pathogen associated molecular pattern (PAMP) to trigger an innate immune response upon exposure to mold in susceptible AIED patients. Cochlin is one of the predominant proteins of the inner ear, and mold reactivity has been previously reported in patients with two conditions related to AIED: Menieres' Disease and Sudden Sensorineural Hearing Loss [41]. Cochlin's LCCL domain shares homology with the LCCL domain of several *Aspergillus* species (*A. niger*, *A. terreus*, *A. oryzae*) as well as *Penicillium chrysogenum* as identified by protein sequence by BLAST protein sequence alignment (supplementary Figure 1). Although alignment of the sequences of the LCCL domain of 4 fungal species with the LCCL domain of cochlin demonstrate common residues Tyr⁶⁷, Ser⁷¹, Ala⁷⁵ and Ala⁷⁷, peptides 5 demonstrated the least reactivity, suggesting these residues are not critical (supplementary figures 1, 2). However, common residues, His⁷⁸, Gly⁸¹, Iso⁸³, Gly⁸⁸, Gly⁹⁷ were tightly clustered and conserved between the LCCL domains of cochlin, *Aspergillus oryzae* and *terreus*, and peptide 3. Peptide 3 demonstrated the greatest IL-17 production suggesting these residues were critical for IL-17 production. These common residues may permit mold and cochlin reactivity, whereas inherent gaps in the peptide sequence would result in low affinity binding, and thus would avoid negative selection in the thymus [42] as T-cells that cross-react with self and microbial antigens should be eliminated in the thymus under normal circumstances. Cao et al, demonstrated a HLA enrichment of HLA-DRB1*0301 in patients with idiopathic progressive SNHL [43], a related condition to AIED. Interestingly, using the IEDB [Immune Epitope Database Analysis] Resource analysis tool, amino acids VHRGIVSNSGGPVRV of peptide 3 is a high affinity binding peptide for HLA-DRB1*0301, with a score of 2.28 [44,45], suggesting a possible HLA enrichment in this patient cohort, although HLA typing on our patients has not yet been performed.

In these studies, we have determined that mold is capable of acting as a PAMP in patients with Autoimmune Inner Ear Disease (AIED) that are sensitive to environmental molds, and can alter the subsequent adaptive immune responses. These patients have low titer IgG antibodies to mixed molds and cochlin, and can be shown to release IgM from naïve B cells when stimulated with conditioned supernatants from monocytes treated with mixed molds. Furthermore, mold stimuli of PBMC induce IL-1 transcription and release. The increased IL-1 release also biases these patients to produce IL-17 in response to molds, and further skew their immunopathology.

Development of Th17 responses to molds have been observed in other systems, and calls into question why these exaggerated immune responses have not been silenced in these otherwise immunocompetent patients. Th17 activation by *aspergillus* is known to result in increased immunopathology rather than protection [46]. We have identified that mold enriched PBMC cultures from a mold-sensitive AIED patient were capable of cross-reacting with cochlin to elicit IL-17 production. Mold-enriched cultures showed 5 fold greater induction of IL-17 producing cells in response to cochlin, whereas IL-17 was not induced in tetanus toxoid enriched T cell cultures challenged with mold or cochlin, arguing that this cross-reactivity between mold and cochlin was specific. Regulatory T cells (Treg) should ordinarily provide a counter-balance to these Th17 cells. Investigation is ongoing in our laboratory as to Treg function in these patients, as our cursory inspection has not revealed a defect in Treg number in these patients (not shown).

Our observation of a cross reactivity between mold and cochlin adds to other observations where fungal proteins have been shown to cross-react with other known allergens [47], or self proteins. In genetically predisposed individuals, exposure to fungal infections can trigger autoimmunity in systemic lupus erythematosus through molecular mimicry between mold and the Ku autoantigen [48]. Molecular mimicry between fungal proteins and the U1-70kD protein associated with mixed connective tissue disease has also been described [49]. In all cases, the basis of this reactivity was peptide homology between the exogenous antigen and self-proteins.

Our prior studies have shown that elevated plasma levels of IL-1 are found in AIED and correlate with the inability to respond to corticosteroids [6]. Thus, mold exposure in this patient group may serve to worsen their hearing loss by enhancing IL-1 expression. In the present study, we observed both a transcriptional up-regulation of IL-1 and enhanced secretion of IL-1 by PBMC from some AIED patients exposed to mold extract. Interestingly, in patients with a high basal expression of IL-1, a very modest augmentation was observed with the addition of mold to the cultures, suggesting that these high basal IL-1 expressers may be close to maximal stimulation. To further investigate this possibility, AIED patients were divided into high (>15pg/ml IL-1) and low (<15pg/ml) basal IL-1 secreting groups. The low secretion group augmented IL-1 release by 128 fold over basal secretion in response to mold, whereas the high basal expressers only increased their IL-1 release by only 1.9 fold in response to the same mixed molds (Figure 2C). Phenotypically, these patients do not appear to have Muckle-Wells disease, despite similar biologic responses. These findings suggest that the mold stimulus may be a weak antigenic stimulus in patients with high basal IL-1 expression, or, more likely, these patients are already maximally stimulated and cannot respond to additional exogenous antigen. Interestingly, the IgG antibody titers observed to mold and cochlin are similar in both corticosteroid responsive and resistant AIED patients and are considered to be relatively low titer. These findings are consistent with previous publications demonstrating rare and inconsistent presence of autoantibodies in this disease [50]. Taken together, we hypothesize that IL-1 is a central mediator of AIED, however, the mechanisms by which IL-1 is expressed in these patients clearly vary. For the subset of AIED patients that demonstrate high IL-1 expression and IL-6 release in response to mold antigen, mold is a PAMP and results in robust inflammatory responses to extrinsic pathogenic stimuli, IgM release from naïve B cells cultured with mold stimulated monocytes, and enhanced, albeit low titer anti-mold IgG antibodies. This observation is consistent with the work of Hirano et. al. which showed that IL-6 (BSF-2) induce the final maturation of B cells into immunoglobulin-secreting cells [51]. In our patients, B cells alone were more potent in inducing IgM synthesis compared to B and T cells (1:4 ratio), indicating no need of additional PRR signals or T cell-mediated help for optimal B cell activation. This subset of patients is similar to Muckle-Wells disease, which is a genetically inherited syndrome of IL-1 dysregulation that is manifest by sensorineural hearing loss and mutations in the NALP3 inflammasome, where microbial stimuli result in massive IL-1 release [52]. AIED patients do not possess the clinical stigmata of Muckle-Wells, as they do not describe fevers, joint pain or skin rashes, nor do they have afflicted relatives. Moreover, some corticosteroid resistant AIED patients have been tested for NLRP3 exon 3 mutations and have been found to be sequence negative (not shown).

Genetic predispositions augment the susceptibility for autoimmunity. Environmental factors may then be sufficient to induce disease symptoms. TLR signaling by exposure to extrinsic PAMPs has been known to be a prerequisite for the development of various autoimmune diseases. TLR9 involvement in the development of SLE has been well documented by many researchers [53]. Compared to mice, human TLR-4 is highly polymorphic. Genetic polymorphism in TLRs, specifically TLR-4, may have lead to altered immune responses

between individuals. The high level of variability in the TLR4 extracellular domain is the result of selective evolutionary pressure exerted by microbes and may explain the variability in the response to LPS within a species. We believe the highly polymorphic nucleotide sequence of human TLR4 (as sequence conservation between TLR-4 extracellular domain of human and mouse is 61.9%) [54-56] may explain why other species do not develop AIED, and in fact, human polymorphisms in TLR4 are extensive and may explain why AIED is a rare disease. Fungal PAMPs are recognized by several TLRs in addition to TLR4, such as TLR2/1, TLR3, TLR2/6, TLR7 and TLR9 [57]. It is possible that the observed IL-1 expression may be a result of signaling through a different TLR, rather than TLR4. In support of such a possibility, a recent study showed that allelic variance in TLR10 was associated with bilateral sensorineural hearing loss and worse progression in hearing loss in patients with Meniere's disease [58]. In order to see the alignment between human TLR4 transcript 1(longest transcript) with TLR 10 transcript 1(longest transcript), we ran EMBOSS (European Molecular Biology Open Software Suite) Needle for nucleotide sequences which creates an optimal global alignment of two sequences using the Needleman-Wunsch algorithm [59]. This showed similarity of about 40.1 % indicating that conserved residues between TLR4 and TLR10 may be implicated in the pathogenesis of immune mediated hearing loss. We will investigate this possibility further in future studies.

One of the hallmarks of development of autoimmune disease is the expression of IL-17. It has been previously shown that high basal IL-1 levels skew T-cell development toward Th17 cell development as shown in human cord blood cells [30]. Similarly, it has been shown that IL-1 receptor antagonist-deficient mice exhibit unrestricted IL-1 activity and develop a rheumatoid arthritis-like disease [60], whereas IL-17-deficient mice do not [34]. In our studies, mold induced generation of IL-1 and the subsequent generation of IL-17 expressing cells in AIED patients, compared with controls, likely occurs through an IL-1 mediated pathway [30]. Generation of these IL-17 producing cells, although propagating autoimmunity, may also serve to confer resistance to pathogens, as has been postulated in models of inflammatory bowel disease [61]. Further investigations, however, actually demonstrate IL-17 expression results in failure to clear fungal infections [62, 63].

In conclusion, the present studies provide insight into the molecular mechanisms that are active in AIED suggesting that some patients are at risk for fungal exposure triggering autoimmunity. Further studies are necessary to define which AIED patients are at risk, and what interventions are available to counteract this unopposed Th17 development in response to mold. Finally, these studies may assist in providing new insights in some of the underlying mechanisms in patients with immune mediated hearing loss reporting environmental sensitivities to molds, where a type I hypersensitivity cannot be detected.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AIED	autoimmune inner ear disease
BLAST	basic local alignment search tool
CAPS	cypropyrin associated periodic syndrome
COCH	cochlin
C_t	cycle threshold value
CTRL	control
GI	genInfo identifier
LCCL	limulus factor C, Coch-5b2
MWS	Muckle-Wells syndrome
NCBI	national center for biotechnology information
NLRP3	NON-RESP, corticosteroid unresponsive patient
Q-RT-PCR	quantitative-real time polymerase chain reaction
RESP	corticosteroid-responsive patient
SFC	spot forming cells
SSNHL	sudden sensorineural hearing loss
TT	tetanus toxoid
UNTX	untreated

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Figure 1. AIED patients have greater anti-mold and anti-cochlin (COCH) IgG antibody titers compared with controls

A: Direct ELISA done on the Plasma of AIED (N=20) patients showed higher levels of mold-specific IgG compared to control subjects (N=10) at serial dilutions of 1:256 to 1:1024. SEM is shown for all dilutions. The comparison between IgG levels of AIED patients and normal healthy controls at dilution 1:256 was significant ($p=0.032$) by the Mann–Whitney U test with 95% CI.

B: Direct ELISA done on the Plasma of AIED (N=20) patients showed higher levels of cochlin-specific IgG compared to control subjects (N=10) at serial dilutions of 1:256 to 1:1024. SEM is shown for all dilutions. The comparison between IgG levels of AIED patients and normal healthy controls at dilution 1:256 ($p=0.0094$) and 1:512 ($p=0.025$) was significant by the Mann–Whitney U test with 95% CI.

Figure 2. Mold extract induces IL-1 mRNA expression and release in AIED patients

A: Mold exposure induces IL-1 mRNA expression from PBMC of AIED patients. PBMC from AIED patients and controls subjects were treated with either mold extract (MOLD) or cochlin (COCH) for overnight and compared with unstimulated cultures (UNT) to determine whether stimulation with either would induce expression of IL-1 mRNA. IL-1 mRNA expression was measured by Q-RT-PCR. Fold change is shown relative to the unstimulated condition. Error bars show $\pm$ SEM.

B: Mold stimulus induces IL-1 release from PBMC of AIED patients. Release of IL-1 into conditioned media was determined by ELISA in AIED patients and control subjects when treated with mold (MOLD) (5 μ g/ml) and cochlin (COCH) (2.5 μ g/ml) for 16h. Mold exposure clearly induces IL-1 release. Error bars show $\pm$ SEM. The difference between stimulated and unstimulated (UNT) conditions was significant in PBMC from AIED patients ($p=0.007$) by the Mann-Whitney U test with 95% CI. The difference between mold-stimulated and unstimulated PBMC was not significant in controls (N.S.).

C: IL-1 release in response to mold is dependent on basal IL-1 expression. The ratio of IL-1 release in mold stimulated PBMC to unstimulated PBMC was calculated for AIED patients based on high (>15pg/ml IL-1) and low (<15pg/ml) basal IL-1 secreting groups. The unstimulated condition was set at 1. The low secretion group augmented IL-1

release by 128 fold over basal secretion in response to mold, whereas the high basal expressers only increased their IL-1 release by only 1.9 fold.

D. Mold stimulated monocytes of AIED patients release significantly higher level of IL-1 when compared to non-monocyte fraction or with monocytes from healthy controls. Release of IL-1 into conditioned media was determined by ELISA in monocytes (MONO) and non-monocyte (NM) fractions of AIED patients (N=4) and control subjects (CTRL) (N=2) when treated for overnight with mold as compared to untreated monocytes (UNTX). Mold stimulated monocytes of AIED patients release a significantly higher level of IL-1 when compared to non-monocyte fraction (NM) or with monocytes from healthy controls ($p<0.05$) done by ANOVA followed by a Bonferroni's Multiple Comparison Test on selected columns. Error bars show $\pm$ SEM.

Figure 3. Mold stimulus induces IL-6 release from PBMC of AIED patients

Release of IL-6 from PBMC into conditioned media was determined by ELISA in AIED patients (N=3) and control subjects (N=2) when treated with mold (MOLD) (5 µg/ml), cochlin (COCH) (2.5 µg/ml), IL-1 (20ng/ml) and LPS (1 µg/ml) for 16h. Mold exposure clearly induces IL-6 release. Error bars show ±SEM. A statistical analysis was done by means of a one-way analysis of variance (ANOVA) which was significant was followed by a Bonferroni's Multiple Comparison Test on selected columns. IL-1 preferentially induces IL-6 release from PBMC of AIED patients as compared with controls (p<0.05).

Figure 4. Mold, cochlin, and overlapping peptides to the LCCL domain of cochlin with shared homology to mold augment IL-17 production in PBMC in AIED patients

2.5×10^5 PBMC from AIED patients and healthy donors were plated on an IL-17 ELISPOT plate, treated with either mold extract (5 $\mu\text{g/ml}$) (MOLD), purified recombinant cochlin (2.5 $\mu\text{g/ml}$) (COCH), overlapping peptides (P1-P5, see supplemental figure 2 for peptide sequences) (2.5 $\mu\text{g/ml}$) generated from the LCCL domain of cochlin, or kept untreated (UNTX) for 16 hours and analyzed for IL-17 producing cells. The mean antigen-specific spot forming cells, SFC (after background subtraction of DMSO controls) is plotted for both groups. Error bars show $\pm$ SEM. A statistical analysis was done by means of a one-way analysis of variance (ANOVA) which was significant ($p < 0.05$) followed a Bonferroni's Multiple Comparison Test. Comparison between selected columns did not reached statistical significance.

Figure 5. Mold-antigen specific T cells produce IL-17 in response to cochlin

Antigen-specific blast cells to mold (MOLD) or tetanus (TT) from a corticosteroid-sensitive, mold sensitive AIED patient, and controls were deposited on an IL-17 ELISPOT plate and cross-stimulated with cochlin (COCH), tetanus, or mold for 16 hours. Spot forming cells were counted, and the ratio of antigen stimulated IL-17 producing T-cells to unstimulated IL-17 producing T cells was calculated for patients and controls.

Figure 6. Supernatants from mold-treated monocytes enhance naïve B cell differentiation into IgM secreting plasmablasts

Naïve B cells from AIED patients and controls were stimulated with supernatant (neat or at a 1:10 dilution) from monocytes cultured with either no stimulus (UT), mold extract (ME) or LPS for 6 days. IgM production was measured in the culture supernatants using ELISA. Error bars show $\pm$ SEM.

Table I**AIED Patient Enrollment Criteria**

Inclusion	Exclusion
Bilateral hearing loss, with progression of hearing loss in at least one ear (loss of 15dB or greater at 1 frequency or 10dB at 2+ frequencies), evolving in >3 days but <90 days	Presence of retrocochlear pathology
May have concurrent, systemic autoimmune disease	If the progression of hearing loss coincided with severe, disabling vertigo

Table II

Demographics on the 20 patients are shown. Concurrent, systemic autoimmune disease, reported environmental allergies, IgG titer to mold, presence of vertigo and corticosteroid responsiveness are shown.

Age	Gender	Other Autoimmune Disease	Family History	Allergy History	Mold IgG (ng/ml at 1:256)	Vertigo	Steroid Responsive?
51	F	No	No	No	25	Yes	No
51	F	Hashimotos	Unk	No	31	Yes	Yes
50	F	No	No	No	35	Unk	Unk
69	F	No	Unk	No	55	No	Unk
69	F	No	No	No	63	Yes	No
59	M	No	Meniere's	No	74	No	Yes
60	F	No	Addison's	No	74	No	No
				AVG	51		
39	F	No	No	Unk	55	Yes	Yes
55	F	No	No	Unk	62	Yes	Yes
35	F	Hashimotos	No	Yes	28	Unk	Yes
61	F	No	Meniere's	Yes	33	Yes	Yes
32	F	No	no	Yes	42	Yes	Yes
66	F	No	No	Yes	54	No	Yes
57	F	Hashimotos	No	Yes	67	Yes	Yes
54	F	No	No	Yes	72	Yes	Yes
42	M	No	No	Yes	73	Yes	No
44	F	No	No	Yes	103	Yes	No
46	F	Alopecia Areata	MS	Yes	104	Rare	Unk
58	M	No	No	Yes	115	No	Yes
46	F	PMOF, Psoriasis, Hashimotos	No	Yes	130	No	No
				AVG	75		

UNK=unknown; MS=Multiple Sclerosis, PMOF= Premature Ovarian Failure, AVG= average. In patients that report environmental allergies, anti-mold IgG levels were higher than in patients that did not report environmental allergies (75ng/ml vs. 51ng/ml).