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IL-1β Is Overexpressed and Aberrantly Regulated in Corticosteroid Nonresponders with Autoimmune Inner Ear Disease

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Abstract

Autoimmune inner ear disease is an enigmatic disorder characterized by recurring episodes of sudden or progressive sensorineural hearing loss. Hearing loss can be improved by timely corticosteroid administration, but only half of those treated respond, and for many responders, that response is lost over time. The mechanisms that control corticosteroid responsiveness in this disorder are largely uncharacterized. We have previously identified that the induction by dexamethasone of IL-1R type II (IL-1R2) expression in PBMC predicts corticosteroid responsiveness in this disorder. In this study, we asked whether IL-1β was overexpressed, and whether clinical corticosteroid responders differentially regulated IL-1β expression or release in response to dexamethasone, as compared with nonresponders. IL-1β has been reported to induce matrix metalloproteinase-9 (MMP-9) expression. Given that metalloproteinases can cleave IL-1R2, we also asked whether MMP-9 expression was altered in this disorder. In this study, we demonstrate that corticosteroid nonresponders have elevated plasma levels of IL-1β and MMP-9 as compared with clinically responsive patients ($p = 0.0008$ and $p = 0.037$, respectively). Increasing MMP-9 expression correlated with increasing IL-1β concentration, suggesting that IL-1β expression regulates MMP-9 expression. As expected, monocytes were the predominant producers of IL-1β. In vitro exposure of PBMC to dexamethasone from clinical corticosteroid responders suppressed IL-1β release. PBMC of corticosteroid nonresponders have substantially higher release of IL-1β into the conditioned media, and when exposed to dexamethasone, failed to repress IL-1β release ($p = 0.05$). Treatment of PBMC from clinical corticosteroid non-responders with anakinra resulted in repression of IL-1β release, suggesting that IL-1β blockade may be a viable therapy for these patients.

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Disclosures

A.V. and the Feinstein Institute for Medical Research hold a patent for the use of IL-1R antagonists for the treatment of AIED, SSNHL, and Meniere's Disease and for IL-1R2 expression as a diagnostic marker of corticosteroid responsiveness.

The mechanisms that control corticosteroid-responsive sensorineural hearing loss (SNHL) remain enigmatic. For patients who experience an acute, sensorineural decline in hearing, timely corticosteroid administration may result in preservation of some or all of the hearing. Potentially reversible SNHL can be divided into two subgroups: autoimmune inner ear disease (AIED) and sudden SNHL (SSNHL). SSNHL is usually a unilateral, isolated event. Patients with AIED usually experience multiple episodes of rapid hearing loss either concurrently or sequentially in both ears. Of those with AIED, up to 30% may have a systemic autoimmune disease (1). Some patients with SSNHL are considered to have an autoimmune etiology for their disease (2), although the majority of these patients have a viral trigger of their disease. Nonetheless, the immunologic responses may be similar, regardless of whether the Ag is a viral Ag or a self-Ag. Although a number of Abs to autoantigens have been found in patients with AIED (3), no single diagnostic biomarker has been identified (4). Interestingly, the influence of cytokine microenvironment has not been investigated to any great degree in this disorder, largely because the events in the cochlea may not be reflected in the peripheral blood immune cells (PBMC), and access to the human cochlea is limited. Some physicians have used initial responsiveness to glucocorticoids as a hallmark of this poorly defined clinical disorder (1,5–7), although of the 70% of patients who are initially steroid responsive, only 14% remain so after 34 mo (8). The mechanism(s) that governs development of steroid resistance in these patients is unknown.

In recent years, the critical role of the IL-1 family as regulators of inflammation and immunity has become apparent (9). Early immune system reactions to perceived pathogens dictate many of the later adaptive T cell responses that perpetuate disease. Expression of IL-1β and IL-1R type I (IL-1R1) is critical to the development of Th17 cells (10,11) and the subsequent expression of IL-17. Absence of IL-1R antagonist (IL-1RA) expression during an immune response, or other molecules that oppose the IL-1β inflammatory cascade, can promote the development of autoimmune disease, possibly including AIED. The role of IL-1β in hearing disorders is largely unknown; however, examples exist both in animal models of AIED and in clinical autoinflammatory disorders with associated SNHL. In an animal model of AIED, priming with systemic LPS and intrathecal Ag was necessary to observe IL-1β expression and subsequent adaptive immune responses in the cochlea, although the adaptive immune responses were not attributed to IL-1β expression (12). SNHL has been observed as a component of clinical diseases of IL-1β dysregulation, such as neonatal onset multisystem(ic) inflammatory disease syndrome (13) and Muckle Wells syndrome (14,15). Furthermore, amelioration of SNHL has been observed in response to treatment with the soluble IL-1RA, anakinra, in Muckle-Wells syndrome (15).

IL-1R type II (IL-1R2) is known to be a molecular decoy expressed on monocytes/macrophages that sequesters IL-1β, but fails to initiate downstream signaling, thereby preventing inflammation (as reviewed in Refs. 16,17). Glucocorticoids enhance IL-1R2 expression (17) and control IL-1β expression by increasing mRNA instability (18), possibly explaining the ability of steroids to reverse some sensorineural hearing declines. Other cytokines can affect expression of IL-1R2. IFN-γ expression has been observed in AIED patients (19). Notably, IFN-γ inhibits expression of IL-1R2 (20). Expression of IL-1R2 is an important mechanism that prevents IL-1β–mediated inflammation at sites where inflammation is poorly tolerated. In the brain, introduction of IL-1β results in the preferential expression of the IL-1R2 decoy receptor as compared with IL-1R1, the cognate IL-1Rβ (21).

To identify molecules that may regulate immunologic responses in AIED, we previously performed microarrays of RNA expression from peripheral blood immune cells (PBMC) stimulated with autologous perilymph from cochlear implant patients with AIED and compared these with RNA expression in PBMC from control patients undergoing cochlear

implantation. Autologous perilymph-stimulated PBMC from cochlear implant patients with AIED failed to exhibit full-length, membrane-bound IL-1R2 (mIL-1R2) by quantitative RT-PCR (Q-RT-PCR) at 45 cycles, whereas PBMC from patients undergoing cochlear implantation for non-immunologic causes of hearing loss were strongly induced to express mIL-1R2 in response to autologous perilymph ($p < 0.05$) (22). On the basis of this differential expression of mIL-1R2, we hypothesized that increased expression of mIL-1R2 in response to dexamethasone would have a protective clinical effect.

Indeed, we identified that in vitro expression of mIL-1R2 in PBMC exposed to dexamethasone from prospectively enrolled patients who experienced an acute decline in hearing correlated with clinical steroid responsiveness, $p < 0.0001$ (22). In steroid-responsive patients, the long mIL-1R2 transcript could not be detected in unstimulated PBMC prior to clinical treatment. Following treatment, basal expression of mIL-1R2 increased dramatically, thus further strengthening the likelihood of involvement of IL-1R2 in the pathophysiology of this response. Clinical non-responders demonstrated high basal (unstimulated) levels of mIL-1R2 in their cultured PBMC prior to clinical corticosteroid therapy, and introduction of dexamethasone to the cultured PBMC only minimally augmented their mIL-1R2 expression prior to treatment.

Levels of IL-1R2 may be controlled by matrix metalloproteinases (MMPs), as they have been shown to induce cleavage of mIL-1R2 (23) and release soluble IL-1R2 (sIL-1R2), the less inhibitory form of IL-1R2 (24). IL-1β induces MMP-9 transcription (25); thus, one would expect patients with elevated IL-1β levels to also demonstrate elevated MMP-9 levels. MMP-9 overexpression is poorly controlled by glucocorticoids in steroid-resistant asthma, and therefore, MMP-9 overexpression may be integral to a steroid-resistant phenotype (26). MMP-9 overexpression has been shown to be relevant in a number of autoimmune diseases, most recently in multiple sclerosis (27,28). MMP-9 has been shown to be involved in opening the blood-brain barrier (29), and therefore, may exert a similar effect at the blood-labyrinthine barrier. Minocycline, a nonspecific inhibitor of MMP-9, has also been demonstrated to reverse cochlear damage in an ototoxicity model, suggesting a potential role of MMP-9 in the pathophysiology of hearing loss (30).

We hypothesized that steroid-resistant AIED patients have unopposed endogenous IL-1β expression and subsequent MMP-9 overexpression, which is associated with irreversible SNHL. To begin to establish a causal role among IL-1β, MMP-9, and IL-1R2, we have studied expression of these molecules in plasma and in cultured PBMC.

Materials and Methods

Patient recruitment

This study was approved by the North Shore-Long Island Jewish Institutional Review Board. Patients were identified by several neurotologists who clinically care for patients with AIED and SSNHL. Adult male and female patients were prospectively recruited for this Institutional Review Board-approved study. Additionally, some minor subjects were also recruited who had clinical manifestations of AIED. Inclusion criteria was similar to that of the AIED study group (5). Inclusion criteria was development of SNHL of >30 decibel (dB) at one or more frequencies in both ears with evidence of active deterioration (elevated threshold) in at least one ear of 15 dB at one frequency (excluding 250 or 8 kHz as a sole indicator), or 10 dB at 2^+ frequencies developing in >3 d, but <90 d (5). We additionally included a small subset of SSNHL, especially if the demonstrated features are suspicious for autoimmune disease (if they have a systemic autoimmune disorder, family history of autoimmune disease, or if they had previous episodes of sudden SNHL in the same or contralateral ear). The clinical characteristics of these patients, including concurrent use of

other immunosuppressants, are shown in Table I. Notably, the majority of the patients enrolled were not using other immunosuppressive agents. Patients were excluded from study if they had evidence of retrocochlear pathology on their magnetic resonance imaging scan.

All patients must have been treated with 60 mg oral prednisone for a minimum of 7 d with a variable taper thereafter. A pure tone average (PTA; in this study to include 250, 500, 1000, 2000, and 4000 Hz) and speech discrimination score (SDS) were recorded pre- and posttreatment. Clinical responsiveness was defined as either an average of 5 dB or greater PTA improvement (as the average of five frequencies was recorded) in their posttreatment audiogram, or >24% improvement in the speech discrimination scores (SDS). In the event that a pretreatment SDS was 0%, clinical improvement would require improvement of the SDS, regardless of a positive change in the PTA.

Culture and stimulation of PBMC

PBMCs were isolated by density centrifugation of heparinized blood (Ficoll-Paque Plus; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 20 min at 400 × *g*. After two washings in 1× RPMI 1640 (Life Technologies), cells were counted in a Beckman Counter, and trypan blue exclusion of dead cells was performed using Cellometer Auto T4 cell counter. The PBMCs were then diluted in RPMI 1640 enriched with 4.1 mM L-glutamine,100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 10% FBS (Atlanta Biologicals, Atlanta, GA), and plated at 1×10^6 cells/ml in 1-ml wells of a 24-well plate (Costar). A single lot of FBS was used for all experiments, discounting the possibility of varying cortisol content between different FBS lots. A fraction of PBMC was exposed to 4 μg/ml dexamethasone (APP Pharmaceuticals, Schaumburg, IL), or 100 ng/ml anakinra (Kineret; Amgen, Thousand Oaks, CA), or human rIL-1β (PeproTech, Rocky Hill, NJ) for 16 h at 37°C in 5% CO_2 and compared with cultured, unstimulated PBMC. Optimal concentrations of these drugs were previously identified by culture of PBMC, as follows: the maximal drug concentration that could be used without affecting cell viability was used for all experiments. Cell viability was measured after 16 h, and in all cases viability exceeded 80%. The concentration of dexamethasone used was based on previous experiments of the optimal concentration used to induce IL-1R2 expression (22), and it has previously been shown to be the optimal concentration for induction of IL-1β expression (31). Results of control PBMCs cultured in FBS with and without dexamethasone were compared with results achieved from these same cells cultured in charcoal-stripped serum with and without dexamethasone at 2, 4, 8, and 16 h. IL-1β expression was measured by Q-RT-PCR and normalized to actin. Variability in IL-1β expression between FBS and charcoal-stripped FBS was <4% between the two types of FBS used, thereby discounting endogenous cortisol in FBS as mediating the effects observed. Only at the 16-h time point was a 6% variance noted; however, the cell viability of cell cultured in charcoal-stripped serum dropped over 10% during the 16-h culture period, suggesting use of FBS as an optimal culture condition for these experiments, and most likely accounted for the increase in variability. At the end of all incubations, samples were centrifuged and supernatants were collected and stored in −20°C.

To determine whether MMP-9 influenced expression of IL-1β transcription or sIL-1R2 transcription or release, PBMCs were incubated for 2 h with the MMP-9 catalytic domain (Enzo Life Sciences International/formerly BIOMOL International) at a concentration of 100 and 500 ng/ml, washed, and then incubated for 16 h with or without added dexamethasone.

Q-RT-PCR

Q-RT-PCR was performed, as previously described (22). Briefly, the Eurogentec RTqPCR Mastermix (Eurogentec, Belgium) PCR mix contained 1× Mastermix and 0.125 μl

Euroscript+RT and RNase inhibitor (reverse transcription, 0.125 U/μl and RNase inhibitor, 0.05 U/μl). PCR was then performed using the forward and reverse primers at a final concentration of 12.5 μM in a sample volume of 25 μl. Intron-spanning primers were designed using the Universal Probe Library Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) from mRNA sequences submitted to GenBank. Table II lists the primer sequences, nucleotide position number, and GenBank accession numbers for IL-1β, MMP-9, and actin (Table I). Primers for the sIL-1R2 and mIL-1R2 were previously described (22). Q-RT-PCR was conducted using an ABI PRISM 7900 HT machine (Applied Biosystems) under the following cycling conditions: 30 min at 48°C (1 cycle), 10 min at 95°C (1 cycle), 15 s at 95°C, and 1 min at 60°C (45 cycles). For each gene (performed in duplicate or triplicate for each sample), cycle threshold (*Ct*) values were determined from the linear region of the amplification plot and normalized by subtraction of the *Ct* value for actin (generating a Δ*Ct* value). The response to the experimental gene of interest was determined by subtraction of the *Ct* value for the time-matched control from the *Ct* value for the experimental gene (*Ct* value). Fold change was subsequently calculated using the Eq. 2^{Ct} (where *Ct* was converted to an absolute value), and downregulated genes were represented as a value <1.

ELISA

Plasma and conditioned supernantants were collected and stored at −20°C until a sufficient number of samples could be acquired. Frozen samples were thawed immediately prior to analysis and no samples underwent repetitive freeze-thaws prior to analysis. All samples were run in duplicate: the maximal variance between replicate samples in all experiments was 0.2%, and the mean variance was 0.002%. Additionally, several replicate samples from previously run plates were included to ensure reproducibility.

IL-1β

IL-1β levels in plasma were quantified using a sandwich ELISA (R&D Systems, Minneapolis, MN), as per the manufacturer's instructions. The sensitivity of the assay was <1 pg/ml. An 8-point standard curve was constructed for each assay using a quadratic fit, and data were interpolated using BioLinx 2.2 software.

MMP-9

Levels of MMP-9 in plasma and conditioned supernatant were determined using the BIOTRAK MMP-9 activity assay kit (Amersham Biosciences [a division of GE Healthcare]), according to the manufacturer's protocol. In brief, total MMP-9 was measured in these samples by the addition of 4-aminophenylmercuric acetate, which converted free pro–MMP-9 to an active form MMP-9. The detection reagent (50 μl) was then added to each well and incubated at 37°C for 2 h. The concentration of active MMP-9 in a sample was determined by interpolation from a standard curve using a straight-line fit, and data were interpolated using BioLinx 2.2 software.

sIL-1R2

Plasma levels of sIL-1R2 were quantified by ELISA (Quantikine colorimetric sandwich ELISA; sensitivity <10 pg/ml; R&D Systems, Minneapolis, MN), as per manufacturer's instructions. An 8-point standard curve was constructed for each assay using a quadratic fit, and data were interpolated using BioLinx 2.2 software.

Monocyte isolation

Negative selection for monocytes was performed by MACS using the Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA), according to the manufacturer's instructions. In brief,

washed PBMC were suspended in 70 μl MACS buffer (PBS [pH 7.2], with 0.5% BSA and 2 mM EDTA) per 10^6 PBMC, mixed with a mixture of biotinylated Abs against nonmonocytic cells bound to magnetically labeled antibiotin microbeads (Miltenyi Biotec) to isolate monocytes. The CD15-expressing cells (granulocytes) were removed by positive magnetic selection by the addition of Abs to human CD15 conjugated to microbeads (Miltenyi Biotec).

The purity of negatively selected monocytes was determined by flow cytometry (FACS Canto II; BD Biosciences) using anti-CD14PE Abs (BD Immunocytometry Systems, San Jose, CA). Purity for these experiments exceeded 80%.

Statistical analysis

Data analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego, CA; http://www.graphpad.com). In all figures, the mean and SEM are shown. Statistically significant comparisons are labeled with their calculated *p* value.

Results

Clinical corticosteroid nonresponders express high levels of IL-1β and MMP-9

We examined a cohort of 47 patients (29 responders, 18 non-responders) with either AIED or SSNHL of likely immunologic origin, treated with corticosteroids. Plasma was collected at the time of recruitment. For most of these patients, recruitment occurred at the time of active hearing decline, prior to corticosteroid therapy. For some patients, recruitment occurred shortly after corticosteroid therapy commenced, and rarely during disease quiescence. Patients on immunosuppressants, including corticosteroids, at the time of blood collection for plasma and PBMC are shown in Table I. Plasma cytokine expression did not vary with time of recruitment (data not shown). Patients were divided into steroid-responsive or unresponsive groups based on clinical hearing recovery with oral corticosteroid therapy. Plasma cytokine levels for IL-1β, MMP-9, sIL-1R2, and IL-17 were measured (Fig. 1*A*–*D*). Strikingly, steroid nonresponders demonstrated a significantly higher level of IL-1β than corticosteroid responders (Fig. 1*A*, 67.6 versus 7.2 pg/ml [$p = 0.0008$, Mann–Whitney *U* test]). Similarly, for MMP-9, corticosteroid nonresponders also demonstrated statistically greater MMP-9 levels when compared with responders (Fig. 1*B*, 783.6 versus 468.5 ng/ml [$p = 0.037$, Mann–Whitney *U* test]). In comparison, IL-1β and MMP-9 plasma levels were measured in five healthy, disease-free, age-matched adults without hearing loss. The mean plasma values for IL-1β and MMP-9 were 0.43 pg/ml ± 0.27 SEM, and 120.3 ng/ml ± 20.2 SEM, respectively.

Given the overexpression of IL-1β and MMP-9 proteins in clinical nonresponders, we queried whether alterations in sIL-1R2 were present. IL-1R2 exists in two forms, as follows: a full-length, membrane-bound form (mIL-1R2) and a short soluble form (sIL-1R2). The sIL-1R2 can be made by alternate splicing (32,33), or by proteolytic cleavage by a MMP (23); however, the full-length IL-1R2 is considered to have the major inhibitory function (24). Notably, we previously found similar baseline expression levels of sIL-1R2 in cochlear implant patients and control cochlear implant subjects (22). Similarly, in this study, no difference in plasma sIL-1R2 levels was observed between responders and nonresponders (Fig. 1*C*), consistent with previously reported results (22).

Finally, given that IL-1β is capable of inducing IL-17, we asked whether IL-17 plasma levels were different between corticosteroid responders and nonresponders. No difference was observed (Fig. 1*D*), although the role of IL-17 is still under investigation in these patients.

IL-1β induces expression of MMP-9

Given the elevated levels of both IL-1β and MMP-9 in plasma, we asked whether IL-1β expression drove the increase in MMP-9. To be able to study this effect in the absence of high levels of endogenous IL-1β, MMP-9, or other confounding inflammatory proteins, we used PBMC from several healthy control subjects without hearing loss ($n = 3$) and exposed them to increasing amounts of IL-1β. MMP-9 mRNA expression was measured by Q-RT-PCR in these PBMC. Indeed, IL-1β exposure resulted in increased MMP-9 mRNA expression in a concentration-dependent manner (Fig. 2*A*). We then postulated that MMP-9 might similarly increase IL-1β expression, as in rheumatoid arthritis, microparticles from synovial fluid induced MMP-9 synthesis in synovial fibroblasts that could not be abrogated by blocking IL-1β (34). Again, PBMC from several control subjects ($n = 3$) were examined. These PBMC were treated with the active catalytic domain of MMP-9 to determine whether IL-1β mRNA expression was altered in the presence of MMP-9. Notably, MMP-9 alone had no effect on IL-1β expression; however, when the catalytic domain of MMP-9 was used in combination with dexamethasone, a dramatic increase in IL-1β expression was observed (Fig. 2*B*). This suggests both an indirect effect of MMP-9 on IL-1β expression and that endogenous MMP-9 levels can skew responses toward a proinflammatory state in the presence of dexamethasone. Similarly, the MMP-9 catalytic domain used in combination with dexamethasone also resulted in increased sIL-1R2 release (Fig. 2*C*). This additive effect of dexamethasone in combination with MMP-9 to induce IL-1β and sIL-1R2 may explain the poor ability of corticosteroids to repress inflammation where MMP-9 overexpression exists (26).

Dexamethasone fails to prevent IL-1β release in clinical nonresponders

Given the correlation of plasma IL-1β and MMP-9 expression with clinical steroid response, we asked whether the PBMC from steroid responders and nonresponders have altered cytokine production or release in response to anakinra (a 153-aa synthetic IL-1RA that functions as a competitive inhibitor for IL-1β by binding IL-1R1 and IL-1R2). PBMC from seven pretreatment clinical corticosteroid responders and seven pretreatment nonresponders were cultured with these putative inhibitors, and mRNA from these PBMC was assessed for IL-1β and MMP-9 expression by Q-RT-PCR (Fig. 3*A*, 3*C*). In all responders, dexamethasone inhibited mRNA expression of IL-1β, whereas in nonresponders, the effect of dexamethasone on IL-1β was variable, with no clear inhibitory pattern seen (Fig. 3*A*). The difference in dexamethasone induction of IL-1β mRNA expression between responders and nonresponders was significantly different ($p = 0.05$, Mann–Whitney *U* test). When the culture supernatant was examined, IL-1β was reduced, as expected, in the dexamethasone-treated PBMC of corticosteroid responders. Notably, however, in corticosteroid nonresponders, dexamethasone treatment caused a paradoxical increase in IL-1β mRNA transcription (Fig. 3*A*), and dexamethasone also failed to prevent IL-1β release (Fig. 3*B*), with high levels of IL-1β detected in the conditioned media ($p = 0.05$, Mann–Whitney *U* test). Identification of increased IL-1β release also argues that dexamethasone did not induce IL-1β mRNA instability as expected (35). This failure to repress IL-1β production and release would permit the initiation of a proinflammatory microenvironment that may preclude steroid response. Interestingly, one patient that was initially steroid responsive became unresponsive to steroids over a period of 6 mo. PBMC had been obtained at two time points, as follows: one while the patient was still steroid responsive, and one when the patient was no longer responsive. The basal expression of IL-1β mRNA from PBMC at the time the patient lost steroid responsiveness was dramatically increased (IL-1β detected at 16.7 cycles) from the time when the patient was still responsive (IL-1β detected at 26.1 cycles), strongly suggesting the increase in IL-1β may be preventing corticosteroid response (data not shown).

As anticipated, dexamethasone inhibited MMP-9 mRNA expression in responders, and to a lesser degree, nonresponders (Fig. 3*C*). It did not repress release of MMP-9 in nonresponders (Fig. 3*D*); however, differences between corticosteroid responders and nonresponders were not statistically significant.

Anakinra represses IL-1β release in clinical nonresponders

We also examined the effect of anakinra on transcription of IL-1β (Fig. 3*A*) and in the prevention of IL-1β release in responders and nonresponders as compared with dexamethasone (Fig. 3*B*). In clinical responders, anakinra reduced IL-1β transcription (Fig. 3*A*). In clinical responders and nonresponders, anakinra effectively prevented IL-1β release (Fig. 3*B*). Taken together with the observed overexpression of IL-1β in the plasma and conditioned supernatants of nonresponders, anakinra therapy may be beneficial to clinical corticosteroid nonresponders to restore hearing thresholds. Furthermore, the observation that anakinra is capable of inhibiting both transcription and release of IL-1β is suggestive that a positive feedback loop exists for perpetuation of IL-1β expression, as IL-1β is known to induce PBMC to synthesize IL-1β (36).

Anakinra demonstrated a greater capacity to reduce MMP-9 expression in nonresponders than responders (Fig. 3*C*). We hypothesize that a reduction of circulating IL-1β indirectly mediated this response.

Monocytes are the primary producers of IL-1β

Given the non aberrant expression of IL-1β in the PBMC of corticosteroid nonresponders, we hypothesized that monocytes were producing the increased IL-1β observed in these steroid-resistant AIED patients. Monocytes were separated from non-monocytes by negative selection, and the two fractions obtained were cultured with dexamethasone, anakinra, or minocycline and compared with unstimulated, cultured cells. RNA from monocyte and nonmonocyte fractions was analyzed by Q-RT-PCR (Fig. 4*A*–*D*), and culture supernatants were analyzed by ELISA (Fig. 5*A*–*D*). Given that monocytes constitute <20% of the PBMC population, we also queried whether a dilutional effect was observed by studying the whole PBMC pool. Three AIED patients (two steroid sensitive and one steroid resistant who had been previously steroid sensitive) and two healthy controls were studied. Basal (unstimulated) mRNA expression of IL-1β in the monocyte fraction of patients was the predominant fraction to express IL-1β as expression was detected at an average of 19.4 cycles, as compared with 25.9 cycles in controls (data not shown). Furthermore, monocytes from the steroid-resistant patient demonstrated the greatest expression, with detection occurring at 15.1 cycles. The nonmonocyte fraction of both patients and controls demonstrated minimal expression, with detection of IL-1β mRNA expression occurring, on average, at 24 cycles in patients and 26.2 in controls (data not shown). Although the enriched monocyte fraction (>80% pure) produces more IL-1β than the nonmonocyte fraction, it is unknown whether the monocyte production of IL-1β requires T cell contact, as 100% purity was not achieved. Future experiments will attempt to answer this question. Consistent with the mechanism of action of anakinra, anakinra reduced IL-1β mRNA expression in control monocytes (37); however, no effect was noted in monocytes from AIED patients (Fig. 4*A*, 4*C*). As expected, monocyte culture supernatants from AIED patients demonstrated significantly higher IL-1β levels (mean 122.7 pg/ml) than supernatants from either control monocyte cultures (mean 15.1 pg/ml) or nonmonocyte cultures (AIED patients [mean 60 pg/ml], and controls [mean 1.2 pg/ml]), suggesting monocytes are the primary producers of IL-1β (Fig. 5*A*, 5*B*), although differences from both the monocyte fraction and nonmonocyte fraction from AIED patients were statistically significantly different from controls, as follows: $p = 0.05$ and $p = 0.003$, respectively. The steroid-resistant patient demonstrated the highest levels of IL-1β released, consistent with

our observations in PBMC (data not shown). Anakinra was almost comparable to dexamethasone in reducing IL-1β release from monocytes in patients (Fig. 5*A*), again suggesting this may be a viable therapeutic option for AIED patients. Although analysis of these monocytes confirmed IL-1β to be largely produced in this fraction, the analysis was performed in a limited data set. Nonetheless, these proof-of-principle experiments provide insight for future studies in this disease.

MMP-9 RNA expression was reduced in both monocyte and nonmonocyte fractions with treated dexamethasone (Fig. 4*B*, 4*D*), but MMP-9 release into the supernatant was not altered in either fraction (Fig. 5*C*, 5*D*). Although patients' monocytes treated with anakinra increased MMP-9 RNA expression (Fig. 4*B*), this did not result in increased release of MMP-9 into the culture supernatant (Fig. 5*C*).

Discussion

Precedent for the causal role of IL-1β and macrophage involvement in hearing loss exists. Macrophage ingress into the cochlea has been demonstrated in animal models of acoustic-induced trauma (38). Moreover, in this model, proinflammatory cytokines, including IL-1β, are expressed (39). Administration of methyl-prednisolone in the acoustic injury trauma model was capable of preventing cochlear hair cell loss, however, only if administered prior to, or immediately following injury (40). IL-1β expression was also observed in an animal model of AIED. In this model, LPS was required in addition to Ag re-exposure to initiate cochlear IL-1β expression, leukocyte ingress into the cochlea, and hearing loss (12). SNHL has been observed in the autoinflammatory syndromes neonatal onset multisystem(ic) inflammatory disease and Muckle-Wells, whose hallmarks are IL-1β dysregulation. IL-1β blockade may represent an alternative method to clinically restore hearing in patients who do not respond to corticosteroid therapy. IL-1β blockade with anakinra has already been shown to reverse SNHL in Muckle-Wells syndrome (15). Unlike these autoinflammatory disorders, AIED is likely a true autoimmune disease in which cochlin has been identified as a good candidate Ag, an animal model based on cochlin reactivity has been established (41), and cochlin-specific T cells have been identified in patients with clinical disease (42).

In this study, we have demonstrated that corticosteroid non-responders have higher circulating plasma levels of IL-1β as compared with corticosteroid-responsive individuals in a group of 47 patients. Dexamethasone had greater ability to repress IL-1β transcription in clinical corticosteroid responders than in non-responders. Dexamethasone was anticipated to reduce IL-1β mRNA transcription and decrease mRNA message stability (35). Paradoxically, dexamethasone treatment of PBMC from clinical nonresponders augmented IL1β transcription and failed to prevent IL-1β release. Furthermore, dexamethasone only inhibited IL-1β release in corticosteroid responders. This lack of dexamethasone effectiveness has been previously observed in monocytes exposed to LPS (43), and suggests that endogenous LPS may similarly preclude dexamethasone-mediated IL-1β transcriptional repression in nonresponders. Treatment of PBMC of these non-responders with anakinra demonstrated effective inhibition of IL-1β release, comparable to that seen in corticosteroid responders (n = 14 patients total). A modest reduction in IL-1β mRNA expression and greater inhibition of IL-1β release were noted in response by anakinra, consistent with prior observations that IL-1RA can inhibit IL-1β production (37). Taken together, IL-1β can be inhibited with anakinra in corticosteroid nonresponders, whereas dexamethasone fails to exert similar control over IL-1β.

IL-1β has been previously reported to induce MMP-9 expression. MMP-9 expression is elevated in corticosteroid non-responders as compared with responders. Additionally, in the two corticosteroid-dependent individuals, significantly higher MMP-9 levels were observed,

suggesting that failure to reduce circulating MMP-9 may correlate with the inability to taper corticosteroid treatment in these individuals. Elevated MMP-9 levels have also been identified in bronchoalveolar lavage fluid of patients with steroid-resistant asthma (44), suggesting the elevation of MMP-9 may be a consistent marker of steroid resistance and/or dependence. Given that MMPs and other proteases induce cleavage of IL-1R2, elevated levels of MMP-9 protein serve to perpetuate the overexpression of IL-1β by inactivating molecules that control its expression.

In this study, we show that increased IL-1β expression is associated with corticosteroid resistance. Preliminary evidence in three AIED patients suggests that monocytes are the primary producer of IL-1β. Future studies will determine whether monocyte–T cell contact is necessary for IL-1β production in these patients. IL-1β expression and markers of classical macrophage activation have been observed in bronchoalveolar fluid lavage of patients with steroid-resistant asthma (45). IL-1β production in response to prednisolone, in part, may be modulated by IL-1β genotype, as follows: patients with certain IL-1β genotypes experience reduced responses to prednisolone (46). Genetic studies of single-nucleotide polymorphisms in IL-1β and IL-1β family members are in progress. However, corticosteroid resistance has been attributed to several nongenetic mechanisms, including altered histone deacetylase-2 (HDAC2) transcription and p-glycoprotein expression (47). Hypoxia in macrophages appears to result in reduced transcription of HDAC2, which in combination with IL-1β results in corticosteroid-resistant inflammation (48). Furthermore, knockdown of HDAC2 also inhibits the association of NF-κB with the glucocorticoid receptor, which resulted in reduced sensitivity to dexamethasone suppression of IL-1β–induced granulocyte/M-CSF production (49). Alternatively, presence of IL-1β may induce accumulation of the dominant-negative β isoform of the glucocorticoid receptor, leading to steroid insensitivity (50). IL-1β expression also results in decreased expression of the ATP-dependent ABC efflux transport protein, p-glycoprotein, which serves to maintain the blood-brain barrier (51). P-glycoprotein has been suggested to function as an extrusion pump at the blood-cochlear barrier (52). Overactivity of p-glycoprotein efflux function has been observed in monocytes of patients with systemic lupus erythematosus treated with long-term corticosteroids (53). Moreover, p-glycoprotein activity was reduced in lymphocytes in those patients with clinical remission as compared with active disease, suggesting a critical role for p-glycoprotein in those patients with chronic inflammatory disease (54). Whether similar mechanism(s) of corticosteroid-resistant SNHL exists is under investigation.

To date, no effective treatment has been identified for patients with either AIED or SSNHL who do not respond to steroids. Our data suggest that the PBMC of clinical corticosteroid-resistant AIED patients both overexpress IL-1β and MMP-9 in PBMC and fail to regulate expression of these proteins in response to dexamethasone. Anakinra proved effective in repressing both IL-1β transcription and release in these nonresponders. Taken together, these findings may suggest a role for IL-1β inhibition in clinical corticosteroid nonresponders. By using medications that are in current clinical use, we may be able to rapidly identify a potential treatment for these nonresponders, which will be explored in a future clinical trial.

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Abbreviations used in this article

AIED	autoimmune inner ear disease
Ct	cycle threshold
dB	decibel
HDAC2	histone deacetylase-2
IL-1R1	IL-1R type 1
IL-1RA	IL-1R antagonist
mIL-1R2	membrane-bound IL-1R2
MMP	matrix metalloproteinase
PTA	pure tone average
Q-RT-PCR	quantitative RT-PCR
SDS	speech discrimination score
sIL-1R2	soluble IL-1R2
SNHL	sensorineural hearing loss
SSNHL	sudden SNHL

References

1. Ruckenstein MJ. Autoimmune inner ear disease. Curr Opin Otolaryngol Head Neck Surg 2004;12:426–430. [PubMed: 15377956]
2. Berrocal JR, Ramírez-Camacho R. Sudden sensorineural hearing loss: supporting the immunologic theory. Ann Otol Rhinol Laryngol 2002;111:989–997. [PubMed: 12450172]
3. Agrup C, Luxon LM. Immune-mediated inner-ear disorders in neuro-otology. Curr Opin Neurol 2006;19:26–32. [PubMed: 16415674]
4. Ryan AF, Harris JP, Keithley EM. Immune-mediated hearing loss: basic mechanisms and options for therapy. Acta Otolaryngol 2002;(Suppl 548):38–43.
5. Niparko JK, Wang NY, Rauch SD, Russell GB, Espeland MA, Pierce JJ, Bowditch S, Masuda A, Gulya AJ, Gantz BJ, et al. AIED Study Group. Serial audiometry in a clinical trial of AIED treatment. Otol Neurotol 2005;26:908–917. [PubMed: 16151337]
6. McCabe BF. Autoimmune sensorineural hearing loss. Ann Otol Rhinol Laryngol 1979;88:585–589. [PubMed: 496191]
7. Harris JP, Weisman MH, Derebery JM, Espeland MA, Gantz BJ, Gulya AJ, Hammerschlag PE, Hannley M, Hughes GB, Moscicki R, et al. Treatment of corticosteroid-responsive autoimmune inner ear disease with methotrexate: a randomized controlled trial. JAMA 2003;290:1875–1883. [PubMed: 14532316]
8. Broughton SS, Meyerhoff WE, Cohen SB. Immune-mediated inner ear disease: 10-year experience. Semin Arthritis Rheum 2004;34:544–548. [PubMed: 15505770]
9. Sims JE, Smith DE. The IL-1 family: regulators of immunity. Nat Rev Immunol 2010;10:89–102. [PubMed: 20081871]
10. Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. J Exp Med 2006;203:1685–1691. [PubMed: 16818675]
11. Lee WW, Kang SW, Choi J, Lee SH, Shah K, Eynon EE, Flavell RA, Kang I. Regulating human Th17 cells via differential expression of IL-1 receptor. Blood 2010;115:530–540. [PubMed: 19965648]
12. Hashimoto S, Billings P, Harris JP, Firestein GS, Keithley EM. Innate immunity contributes to cochlear adaptive immune responses. Audiol Neurootol 2005;10:35–43. [PubMed: 15567913]

13. Henderson C, Goldbach-Mansky R. Monogenic IL-1 mediated autoinflammatory and immunodeficiency syndromes: finding the right balance in response to danger signals. Clin Immunol 2010;135:210–222. [PubMed: 20353899]

14. Hawkins PN, Lachmann HJ, Aganna E, McDermott MF. Spectrum of clinical features in Muckle-Wells syndrome and response to anakinra. Arthritis Rheum 2004;50:607–612. [PubMed: 14872505]

15. Mirault T, Launay D, Cuisset L, Hachulla E, Lambert M, Queyrel V, Quemeneur T, Morell-Dubois S, Hatron PY. Recovery from deafness in a patient with Muckle-Wells syndrome treated with anakinra. Arthritis Rheum 2006;54:1697–1700. [PubMed: 16646042]

16. Mantovani A, Bonecchi R, Martinez FO, Galliera E, Perrier P, Allavena P, Locati M. Tuning of innate immunity and polarized responses by decoy receptors. Int Arch Allergy Immunol 2003;132:109–115. [PubMed: 14600422]

17. Mantovani A, Muzio M, Ghezzi P, Colotta C, Introna M. Regulation of inhibitory pathways of the interleukin-1 system. Ann N Y Acad Sci 1998;840:338–351. [PubMed: 9629261]

18. Amano Y, Lee SW, Allison AC. Inhibition by glucocorticoids of the formation of interleukin-1 alpha, interleukin-1 beta, and interleukin-6: mediation by decreased mRNA stability. Mol Pharmacol 1993;43:176–182. [PubMed: 8429822]

19. Lorenz RR, Solares CA, Williams P, Sikora J, Pelfrey CM, Hughes GB, Tuohy VK. Interferon-gamma production to inner ear antigens by T cells from patients with autoimmune sensorineural hearing loss. J Neuroimmunol 2002;130:173–178. [PubMed: 12225899]

20. Dickensheets HL, Donnelly RP. IFN-gamma and IL-10 inhibit induction of IL-1 receptor type I and type II gene expression by IL-4 and IL-13 in human monocytes. J Immunol 1997;159:6226–6233. [PubMed: 9550426]

21. Docagne F, Campbell SJ, Bristow AF, Poole S, Vigues S, Guaza C, Perry VH, Anthony DC. Differential regulation of type I and type II interleukin-1 receptors in focal brain inflammation. Eur J Neurosci 2005;21:1205–1214. [PubMed: 15813930]

22. Vambutas A, DeVoti J, Goldofsky E, Gordon M, Lesser M, Bonagura V. Alternate splicing of interleukin-1 receptor type II (IL1R2) in vitro correlates with clinical glucocorticoid responsiveness in patients with AIED. PLoS ONE 2009;4:e5293. [PubMed: 19401759]

23. Orlando S, Sironi M, Bianchi G, Drummond AH, Boraschi D, Yabes D, Mantovani A. Role of metalloproteases in the release of the IL-1 type II decoy receptor. J Biol Chem 1997;272:31764–31769. [PubMed: 9395521]

24. Neumann D, Kollewe C, Martin MU, Boraschi D. The membrane form of the type II IL-1 receptor accounts for inhibitory function. J Immunol 2000;165:3350–3357. [PubMed: 10975853]

25. Yokoo T, Kitamura M. Dual regulation of IL-1 beta-mediated matrix metalloproteinase-9 expression in mesangial cells by NF-kappa B and AP-1. Am J Physiol 1996;270:F123–F130. [PubMed: 8769830]

26. Cundall M, Sun Y, Miranda C, Trudeau JB, Barnes S, Wenzel SE. Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids. J Allergy Clin Immunol 2003;112:1064–1071. [PubMed: 14657859]

27. Benesová Y, Vasku A, Novotná H, Litzman J, Stourac P, Beránek M, Kadanka Z, Bednarík J. Matrix metalloproteinase-9 and matrix metalloproteinase-2 as biomarkers of various courses in multiple sclerosis. Mult Scler 2009;15:316–322. [PubMed: 19153173]

28. Hu W, Metselaar J, Ben LH, Cravens PD, Singh MP, Frohman EM, Eagar TN, Racke MK, Kieseier BC, Stüve O. PEG minocycline-liposomes ameliorate CNS autoimmune disease. PLoS ONE 2009;4:e4151. [PubMed: 19127301]

29. Yamaguchi M, Jadhav V, Obenaus A, Colohan A, Zhang JH. Matrix metalloproteinase inhibition attenuates brain edema in an in vivo model of surgically-induced brain injury. Neurosurgery 2007;61:1067–1075. discussion 1075–1076. [PubMed: 18091283]

30. Corbacella E, Lanzoni I, Ding D, Previati M, Salvi R. Minocycline attenuates gentamicin induced hair cell loss in neonatal cochlear cultures. Hear Res 2004;197:11–18. [PubMed: 15504599]

31. Brown EA, Dare HA, Marsh CB, Wewers MD. The combination of endotoxin and dexamethasone induces type II interleukin 1 receptor (IL-1r II) in monocytes: a comparison to interleukin 1 beta

(IL-1 beta) and interleukin 1 receptor antagonist (IL-1ra). Cytokine 1996;8:828–836. [PubMed: 9047079]

32. Liu C, Hart RP, Liu XJ, Clevenger W, Maki RA, De Souza EB. Cloning and characterization of an alternatively processed human type II interleukin-1 receptor mRNA. J Biol Chem 1996;271:20965–20972. [PubMed: 8702856]
33. Moscicki RA, San Martin JE, Quintero CH, Rauch SD, Nadol JBJ Jr, Bloch KJ. Serum antibody to inner ear proteins in patients with progressive hearing loss: correlation with disease activity and response to corticosteroid treatment. JAMA 1994;272:611–616. [PubMed: 8057517]
34. Distler JH, Jüngel A, Huber LC, Seemayer CA, Reich CF III, Gay RE, Michel BA, Fontana A, Gay S, Pisetsky DS, Distler O. The induction of matrix metalloproteinase and cytokine expression in synovial fibroblasts stimulated with immune cell microparticles. Proc Natl Acad Sci USA 2005;102:2892–2897. [PubMed: 15701693]
35. Lee SW, Tsou AP, Chan H, Thomas J, Petrie K, Eugui EM, Allison AC. Glucocorticoids selectively inhibit the transcription of the interleukin 1 beta gene and decrease the stability of interleukin 1 beta mRNA. Proc Natl Acad Sci USA 1988;85:1204–1208. [PubMed: 3257575]
36. Granowitz EV, Vannier E, Poutsiaka DD, Dinarello CA. Effect of interleukin-1 (IL-1) blockade on cytokine synthesis. II. IL-1 receptor antagonist inhibits lipopolysaccharide-induced cytokine synthesis by human monocytes. Blood 1992;79:2364–2369. [PubMed: 1533323]
37. Granowitz EV, Clark BD, Vannier E, Callahan MV, Dinarello CA. Effect of interleukin-1 (IL-1) blockade on cytokine synthesis. I. IL-1 receptor antagonist inhibits IL-1-induced cytokine synthesis and blocks the binding of IL-1 to its type II receptor on human monocytes. Blood 1992;79:2356–2363. [PubMed: 1533322]
38. Hirose K, Discolo CM, Keasler JR, Ransohoff R. Mononuclear phagocytes migrate into the murine cochlea after acoustic trauma. J Comp Neurol 2005;489:180–194. [PubMed: 15983998]
39. Fujioka M, Kanzaki S, Okano HJ, Masuda M, Ogawa K, Okano H. Proinflammatory cytokines expression in noise-induced damaged cochlea. J Neurosci Res 2006;83:575–583. [PubMed: 16429448]
40. Tabuchi K, Murashita H, Sakai S, Hoshino T, Uemaetomari I, Hara A. Therapeutic time window of methylprednisolone in acoustic injury. Otol Neurotol 2006;27:1176–1179. [PubMed: 16980917]
41. Solares CA, Edling AE, Johnson JM, Baek MJ, Hirose K, Hughes GB, Tuohy VK. Murine autoimmune hearing loss mediated by CD4+ T cells specific for inner ear peptides. J Clin Invest 2004;113:1210–1217. [PubMed: 15085200]
42. Baek MJ, Park HM, Johnson JM, Altuntas CZ, Jane-Wit D, Jaini R, Solares CA, Thomas DM, Ball EJ, Robertson NG, et al. Increased frequencies of cochlin-specific T cells in patients with autoimmune sensorineural hearing loss. J Immunol 2006;177:4203–4210. [PubMed: 16951386]
43. Kern JA, Lamb RJ, Reed JC, Daniele RP, Nowell PC. Dexamethasone inhibition of interleukin 1 beta production by human monocytes: posttranscriptional mechanisms. J Clin Invest 1988;81:237–244. [PubMed: 3257219]
44. Goleva E, Hauk PJ, Boguniewicz J, Martin RJ, Leung DY. Airway remodeling and lack of bronchodilator response in steroid-resistant asthma. J Allergy Clin Immunol 2007;120:1065–1072. [PubMed: 17900681]
45. Goleva E, Hauk PJ, Hall CF, Liu AH, Riches DW, Martin RJ, Leung DY. Corticosteroid-resistant asthma is associated with classical antimicrobial activation of airway macrophages. J Allergy Clin Immunol 2008;122:550–559. e3. [PubMed: 18774390]
46. Markova S, Nakamura T, Makimoto H, Ichijima T, Yamamori M, Kuwahara A, Iwaki K, Nishiguchi K, Okamura N, Okumura K, Sakaeda T. IL-1beta genotype-related effect of prednisolone on IL-1beta production in human peripheral blood mononuclear cells under acute inflammation. Biol Pharm Bull 2007;30:1481–1487. [PubMed: 17666808]
47. Barnes PJ I, Adcock M. Glucocorticoid resistance in inflammatory diseases. Lancet 2009;373:1905–1917. [PubMed: 19482216]
48. Charron CE, Chou PC, Coutts DJ, Kumar V, To M, Akashi K, Pinhu L, Griffiths M, Adcock IM, Barnes PJ, Ito K. Hypoxia-inducible factor 1alpha induces corticosteroid-insensitive inflammation

via reduction of histone deacetylase-2 transcription. J Biol Chem 2009;284:36047–36054. [PubMed: 19880520]

49. Ito K, Yamamura S, Essilfie-Quaye S, Cosio B, Ito M, Barnes PJ, Adcock IM. Histone deacetylase 2-mediated deacetylation of the glucocorticoid receptor enables NF-kappaB suppression. J Exp Med 2006;203:7–13. [PubMed: 16380507]
50. Webster JC, Oakley RH, Jewell CM, Cidlowski JA. Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative beta isoform: a mechanism for the generation of glucocorticoid resistance. Proc Natl Acad Sci USA 2001;98:6865–6870. [PubMed: 11381138]
51. von Wedel-Parlow M, Wölte P, Galla HJ. Regulation of major efflux transporters under inflammatory conditions at the blood-brain barrier in vitro. J Neurochem 2009;111:111–118. [PubMed: 19656257]
52. Saito T, Zhang ZJ, Tsuzuki H, Ohtsubo T, Yamada T, Yamamoto T, Saito H. Expression of P-glycoprotein in inner ear capillary endothelial cells of the guinea pig with special reference to blood-inner ear barrier. Brain Res 1997;767:388–392. [PubMed: 9367275]
53. Lu MC, Lai NS, Li KJ, Hsieh SC, Wu CH, Yu CL. Increased multidrug resistance-associated protein activity in mononuclear cells of patients with systemic lupus erythematosus. Clin Exp Rheumatol 2008;26:638–645. [PubMed: 18799096]
54. Diaz-Borjon A, Richaud-Patin Y, Alvarado de la Barrera C, Jakez-Ocampo J, Ruiz-Argüelles A, Llorente L. Multidrug resistance-1 (MDR-1) in rheumatic autoimmune disorders. II. Increased P-glycoprotein activity in lymphocytes from systemic lupus erythematosus patients might affect steroid requirements for disease control. Joint Bone Spine 2000;67:40–48. [PubMed: 10773967]

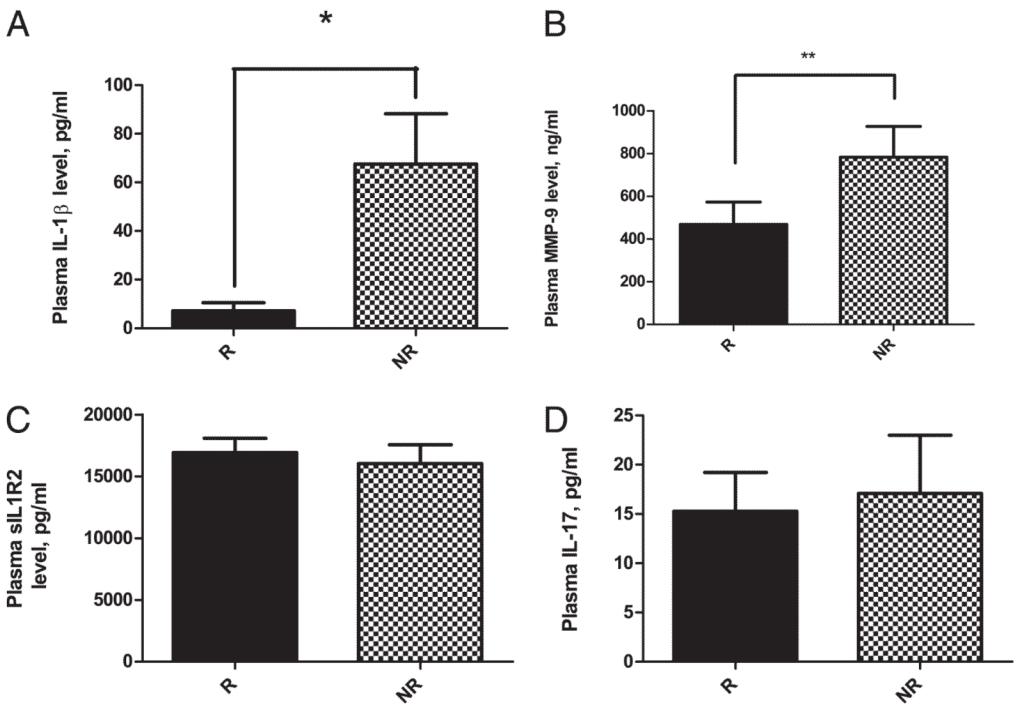

FIGURE 1.

IL-1β and MMP-9 are over-expressed in plasma of patients with corticosteroid-resistant hearing loss. Plasma values for 47 patients who received corticosteroid therapy for an acute decline in hearing. The majority of the patients had autoimmune inner ear disease, whereas a few had a SSNHL of suspected immunologic origin. Plasma values for IL-1β, MMP-9, sIL-1R2, and IL-17 were measured for clinical corticosteroid responders and nonresponders. Corticosteroid nonresponders demonstrated significantly higher levels of IL-1β than nonresponders. *$p = 0.0008$ (*A*). Similarly, a significantly higher level of MMP-9 was observed in corticosteroid nonresponders when compared with responders. **$p = 0.037$ (*B*). Neither sIL-1R2 (*C*) nor IL-17 (*D*) plasma values correlated with clinical corticosteroid responsiveness.

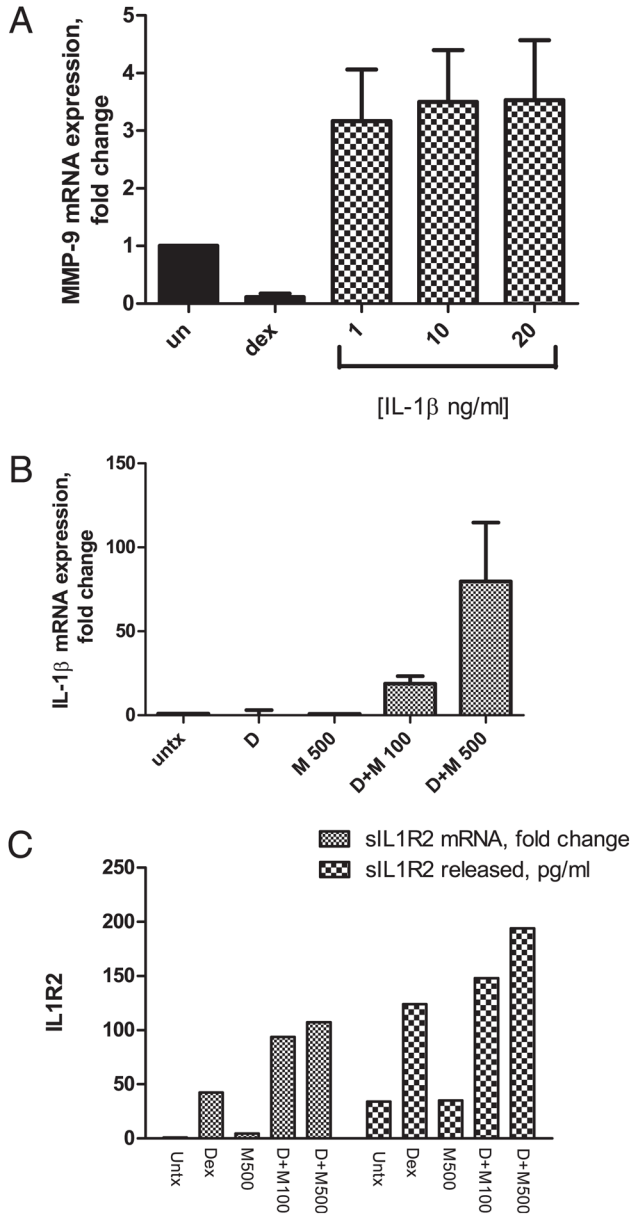

FIGURE 2.

IL-1β induces MMP-9 expression; however, dexamethasone is required for MMP-9 to reciprocally induce IL-1β PBMC from control patients were obtained to determine whether IL-1β induces expression of MMP-9. RNA expression was measured by Q-RT-PCR. PBMC from three control subjects were treated with either dexamethasone or increasing amounts of IL-1β (*A*). Fold change is shown relative to the unstimulated condition. IL-1β clearly induces MMP-9 mRNA expression, $p = 0.004$, by a repeated measures ANOVA. Conversely, control PBMC were treated with either the catalytic domain of MMP-9 alone, or in combination with dexamethasone (D+M). The catalytic domain was used at either 100 or 500 ng/ml (*B*). Although MMP-9 alone had no effect on IL-1β transcription (data not shown), increasing the MMP-9 concentration in combination with dexamethasone resulted in a strong induction of IL-1β transcription. Similarly, MMP-9 alone had no effect on

sIL-1R2 transcription or release; however, in combination with dexamethasone, increased MMP-9 concentration augmented both sIL-1R2 transcription and release (*C*).

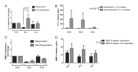

FIGURE 3.

Dexamethasone fails to reduce IL-1β transcription and release in patients unresponsive to corticosteroid therapy. PBMC from seven corticosteroid responders and seven nonresponders were evaluated prior to clinical treatment. In this figure, IL-1β and MMP-9 RNA expression (measured by Q-RT-PCR) and IL-1β and MMP-9 release into the culture supernatant (measured by ELISA) were evaluated in response to anakinra, minocycline, and dexamethasone as compared with unstimulated PBMC. Results for RNA expression are shown as fold change relative to unstimulated for IL-1β (*A*) and MMP-9 (*C*). Release of IL-1β (*B*) and MMP-9 (*D*) was measured for each of these culture conditions. Strikingly, in clinical corticosteroid nonresponders, coculture with dexamethasone resulted in increased IL-1β mRNA production, $*p = 0.05$, Mann–Whitney *U* test (*A*). And it failed to prevent IL-1β release, $**p = 0.05$, Mann–Whitney *U* test (*B*). In these nonresponders, anakinra effectively reduced IL-1β release, suggesting a role for anakinra therapy in corticosteroid nonresponders. No significant difference was observed for MMP-9 mRNA expression (*C*) or release (*D*), n.s. (not significant).

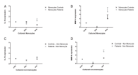

FIGURE 4.

IL-1β mRNA expression is not reduced by dexamethasone in monocytes. Monocyte and nonmonocyte fractions were isolated from a group of AIED patients (n = 3) and a group of controls (n = 2). mRNA expression was determined by Q-RT-PCR, and expression was measured as fold change relative to the unstimulated condition. mRNA expression for IL-1β (*A*) and MMP-9 (*B*) in monocytes was performed. The nonmonocyte fraction was also evaluated for IL-1β (*C*) and MMP-9 (*D*) expression. Unstimulated monocytes from patients demonstrated substantially greater IL-1β mRNA than in either the nonmonocyte fraction of patients, or from either fraction of controls (detection threshold by Q-RT-PCR 19.4 as compared with 24.0 for nonmonocytes and 25.9 and 26.2 for controls). Dexamethasone had no substantial ability to repress IL-1β mRNA expression in monocytes from AIED patients (*A*). Given that transcriptional repression of IL-1β was observed in the PBMC of corticosteroid responders, it is possible that monocyte–T cell interactions may be necessary for the observed reduction in IL-1β transcription. MMP-9 transcription was repressed by dexamethasone in both fractions.

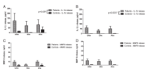

FIGURE 5.

IL-1β is predominantly released from monocytes. Release of IL-1β and MMP-9 into conditioned media was determined by ELISA from the monocyte and nonmonocyte fractions in AIED patients and controls from Fig. 4. Monocytes clearly produce the majority of IL-1β (*A*, mean 122.7 pg/ml in patients versus 15.1 pg/ml in controls) as compared with the nonmonocyte fraction (*B*, mean 60.0 pg/ml in patients versus 1.2 pg/ml in controls), although both monocyte and nonmonocyte fractions were statistically significantly different in AIED patients than controls (monocyte fractions AIED versus controls, $*p = 0.05$; nonmonocyte fractions, AIED versus control, $**p = 0.003$). Anakinra was comparable to dexamethasone in its ability to reduce IL-1β release from monocytes (*A*). MMP-9 release from monocytes (*C*) and nonmonocyte fractions (*D*) was similar. Presence of minocycline, anakinra, and dexamethasone in the culture has no significant effect on MMP-9 release from either fraction.

Table I

Clinical features of patients with AIED

Age	Sex	Systemic Autoimmune Disease	Hear Δ Posttreatment (PTA in dB)	Concurrent Immunosuppressive Therapy
Responders				
63	F	No	13	No
54	M	Psoriasis	15	No
76	M	No	33	No
37	F	No	35	No
47	M	No	*a*	Corticosteroids
61	M	No	56	No
70	F	No	11	No
31	F	Multiple sclerosis	13	No
58	F	No	5 dB, 32% SDS	No
70	F	No	7	No
61	M	No	30	No
36	M	No	48	No
60	F	Sarcoidosis	6 dB	Methotrexate
14	F	No	18	Methotrexate
55	M	No	32	No
59	F	No	20	No
40	M	No	13	No
66	F	No	24	Corticosteroids, remicade
32	F	Hashimoto’s	19	No
57	F	Hashimoto’s	34	Methotrexate
73	M	No	21	No
50	F	No	8	No
43	F	No	30	No
14	M	No	*a*	Corticosteroids, methotrexate
71	F	No	45	No
58	F	No	0 dB, 28% SDS	No
66	M	No	59	No
74	M	No	10	No
51	F	No	22	No
Nonresponders				
49	F	No	−6	No
50	M	No	−4	No
38	F	No	1	No
62	F	No	1	No
52	M	No	−2	No
31	M	No	−2/+5	No
55	F	No	1	No
42	M	No	−1	No

Age	Sex	Systemic Autoimmune Disease	Hear Δ Posttreatment (PTA in dB)	Concurrent Immunosuppressive Therapy
53	F	No	−4	No
47	F	Multiple sclerosis	−4/−11	No
66	F	No	−1	No
40	M	No	−4	No
64	M	No	−6/+3	No
47	F	Premature ovarian failure	2	No
73	M	No	−5	No
56	F	Psoriatic arthritis	2	No
61	M	No	24 dB, 0% SDS	No
54	F	Hashimoto's	−3	No

Clinical data on 47 patients treated with corticosteroids, separated by clinical response measured by hearing improvement. The change in (Δ) hearing is the difference between the posttest PTA and the pretest PTA. A positive clinical response (responder) was measured as ≥5 dB improvement in the PTA (average of 250, 500, 1000, 2000, and 4000 Hertz), or >24% improvement in the SDS. Conversely, if the patient had a pretreatment SDS of 0%, clinical response required improvement of the SDS, regardless of PTA improvement.

[a]Denotes two patients that were steroid dependent, in which weaning resulted in hearing deterioration: thus, Δ hearing could not be accurately measured. Presence of concurrent systemic autoimmune disease is described, as is concurrent use of other immunosuppressive agents, including corticosteroids at the time of PBMC acquisition.

Table II

Primer sequences for Q-RT-PCR

Gene	Position	Primer Sequence	GenBank No.	Universal Probe: Human
Actin	425–442	5′-CCAACCGCGAGAAGATGA-3′	NM_001101.3	64
	502–521	5′-CCAGAGGCGTACAGGGATAG-3′		
IL-1β	640–659	5′-CTGTCCTGCGTGTTGAAAGA-3′	NM_000576.2	78
	687–709	5′-TTGGGTAATTTTTGGGATCTACA-3′		
MMP-9	129–145	59-GAACCAATCTCACCGACAGG-39	NM_004994.2	06
	173–192	5′-GCCACCCGAGTGTAACCATA-3′		