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

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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 nonresponders with anakinra resulted in repression of IL-1β release, suggesting that IL-1β blockade may be a viable therapy for these patients. The Journal of Immunology, 2011, 186: 1870–1879.

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 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-1R2 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-autoimmune 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 without affected 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 cells 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 2°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

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**Table I. Clinical features of patients with AIED**

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Systemic Autoimmune Disease</th>
<th>Hear Δ Posttreatment (PTA in dB)</th>
<th>Concurrent Immunosuppressive Therapy</th>
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<tr>
<td>Responders</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63 F</td>
<td>No</td>
<td>13</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>54 M</td>
<td>No</td>
<td>15</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>76 M</td>
<td>No</td>
<td>33</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>37 F</td>
<td>No</td>
<td>35</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>47 M</td>
<td>No</td>
<td>11</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>61 M</td>
<td>No</td>
<td>11</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>31 F</td>
<td>Multiple sclerosis</td>
<td>13</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>58 F</td>
<td>No</td>
<td>5 dB, 32% SDS</td>
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<td>No</td>
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<tr>
<td>70 F</td>
<td>No</td>
<td>7</td>
<td>No</td>
<td>No</td>
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<td>No</td>
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<td>No</td>
<td>No</td>
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<td>36 M</td>
<td>No</td>
<td>48</td>
<td>No</td>
<td>No</td>
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<tr>
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<td>6 dB</td>
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</tr>
<tr>
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<td>No</td>
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<td>Methotrexate</td>
<td>No</td>
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<td>No</td>
<td>No</td>
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<tr>
<td>59 F</td>
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<td>20</td>
<td>No</td>
<td>No</td>
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<tr>
<td>40 M</td>
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<td>No</td>
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</tr>
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<td>24</td>
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<td>No</td>
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<tr>
<td>57 F</td>
<td>Hashimoto’s</td>
<td>34</td>
<td>Methotrexate</td>
<td>No</td>
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<tr>
<td>73 M</td>
<td>No</td>
<td>21</td>
<td>No</td>
<td>No</td>
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<tr>
<td>50 F</td>
<td>No</td>
<td>11</td>
<td>No</td>
<td>No</td>
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<tr>
<td>43 F</td>
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<td>30</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>14 M</td>
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<td>24</td>
<td>Corticosteroids, methotrexate</td>
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<td>No</td>
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<td>0 dB, 28% SDS</td>
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<td>59</td>
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</tr>
<tr>
<td>51 F</td>
<td>No</td>
<td>22</td>
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<td>−6</td>
<td>−6</td>
</tr>
<tr>
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<td>No</td>
<td>−4</td>
<td>−4</td>
<td>−4</td>
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<tr>
<td>38 F</td>
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<td>1</td>
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<td>1</td>
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<td>1</td>
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<tr>
<td>52 M</td>
<td>No</td>
<td>−2</td>
<td>−2</td>
<td>−2</td>
</tr>
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<td>−2−4.5</td>
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<td>−2−4.5</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>42 M</td>
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<td>−1</td>
<td>−1</td>
</tr>
<tr>
<td>53 F</td>
<td>No</td>
<td>−4</td>
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</tr>
<tr>
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<td>Multiple sclerosis</td>
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<td>−4−−11</td>
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<tr>
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<td>−1</td>
<td>−1</td>
<td>−1</td>
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<td>No</td>
<td>−4</td>
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</tr>
<tr>
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<td>No</td>
<td>−6−4.3</td>
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<td>−6−4.3</td>
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<tr>
<td>47 F</td>
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<td>No</td>
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<td>No</td>
<td>−5</td>
<td>−5</td>
<td>−5</td>
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<tr>
<td>56 F</td>
<td>Psoriatic arthritis</td>
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<td>2</td>
</tr>
<tr>
<td>61 M</td>
<td>No</td>
<td>24 dB, 0% SDS</td>
<td>No</td>
<td>24 dB, 0% SDS</td>
</tr>
<tr>
<td>54 F</td>
<td>Hashimoto’s</td>
<td>−3</td>
<td>No</td>
<td>−3</td>
</tr>
</tbody>
</table>

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.

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.
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/ulp/adc.jsp) from
mRNA sequences submitted to GenBank. Table II lists the primer se-
quences, nucleotide position number, and GenBank accession numbers
for IL-1β, MMP-9, and actin (Table I). Primers for the sIL-1R2 and mL-
1R2 were previously described (22). Q-RT-PCR was conducted using an
ABI PRISM 7900 HT machine (Applied Biosystems) under the following
amplification 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 du-
pendent triplicate or triplicate for each sample), cycle threshold
(Ct) value was determined from the linear region of the amplification plot and normalized
by subtraction of the Ct value for actin (generating a ΔCt value). The re-
sponse to the experimental gene of interest was determined by subtraction of
the ΔCt 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 (where Ct was converted to an absolute value), and
downregulated genes were represented as a value <1.

ELISA

Plasma and conditioned supernatants 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 repro-
ducibility.

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 BIORAD 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 [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

sIL-1R2

Plasma levels of sIL-1R2 were quantified by ELISA (Quantikine colori-
metric sandwich ELISA; sensitivity <10 pg/ml; R&D Systems, Minne-
apolis, 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 Immunochemistry Systems, San Jose, CA). Purity for these experiments exceeded 80%.

Statistical analysis

Data analysis was performed using GraphPad Prism version 5.00 for Win-
dows, GraphPad Software (San Diego, CA; http://www.graphpad.com). In
each figure, the mean and SEM are shown. Statistically significant com-
parisons 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 oc-
curred at the time of active hearing decline, prior to corticoсте-
roid therapy. For some patients, recruitment occurred shortly af-
after corticosteroid therapy commenced, and rarely during disease
quiescence. Patients on immunosuppressants, including cortico-
steroids, 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.
1A–D). Strikingly, steroid nonresponders demonstrated a signifi-
cantly higher level of IL-1β than corticosteroid responders (Fig.
1A, 67.6 versus 7.2 pg/ml [p = 0.0008, Mann–Whitney U test]).
Similarly, for MMP-9, corticosteroid nonresponders also demon-
strated statistically greater MMP-9 levels when compared with
responders (Fig. 1B, 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. 1C), consistent with previously reported results (22).

Table II. Primer sequences for Q-RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position</th>
<th>Primer Sequence</th>
<th>GenBank No.</th>
<th>Universal Probe: Human</th>
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<td>Actin</td>
<td>425–442</td>
<td>5’-CCACCGCAGAGATGA-3’</td>
<td>NM_001101.3</td>
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<td>502–521</td>
<td>5’-CCACGGCTACCGGAT-3’</td>
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<tr>
<td>IL-1β</td>
<td>640–659</td>
<td>5’-CTGCTTGGTGTGGAAA-3’</td>
<td>NM_000576.2</td>
<td>78</td>
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<td>687–709</td>
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<td>173–192</td>
<td>5’-GCCCACCGGAGTGAACCATA-3’</td>
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</table>
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. 1D), 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. 2A). 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. 2B). 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.

**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. 3A, 3C). 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. 3A). 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. 3A, 3C), and dexamethasone also failed to prevent IL-1β release (Fig. 3B), 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. 3C). It did not repress release of MMP-9 in nonresponders (Fig. 3D); however, differences between corticosteroid responders and nonresponders were not statistically significant.
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. 3C). We hypothesize that a reduction of circulating IL-1β indirectly mediated this response.

Monocytes are the primary producers of IL-1β

Given the notable 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 nonmonocytes 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. 4A–D), and culture supernatants were analyzed by ELISA (Fig. 5A–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 cycles (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. 4A, 4C). 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. 5A, 5B), 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. 5A), 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. 4B, 4D), but MMP-9 release into the supernatant was not altered in either fraction (Fig. 5C, 5D). Although patients’ monocytes treated with

Anakinra represses IL-1β release in clinical nonresponders

We also examined the effect of anakinra on transcription of IL-1β (Fig. 3A) and in the prevention of IL-1β release in responders and nonresponders as compared with dexamethasone (Fig. 3B). In clinical responders, anakinra reduced IL-1β transcription (Fig. 3A). In clinical responders and nonresponders, anakinra effectively prevented IL-1β release (Fig. 3B). 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

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).
anakinra increased MMP-9 RNA expression (Fig. 4B), this did not result in increased release of MMP-9 into the culture supernatant (Fig. 5C).

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 methylprednisolone 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 nonresponders 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 nonresponders. 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 nonresponders 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 nonresponders 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 that 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

![FIGURE 3](http://www.jimmunol.org/)
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

![Figure 5](http://www.jimmunol.org/)

**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.
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 methasone. Anakinra proved effective in repressing both IL-1β with either AIED or SSNHL who do not respond to steroids. Our resistant SNHL exists is under investigation. The role for p-glycoprotein in those patients with chronic inflammatory remission as compared with active disease, suggesting a critical activity was reduced in lymphocytes in those patients with clinical responsiveness in lymphocytes in those patients with clinical progression (47). Hypoxia in macrophages appears to result in reduced transcription and release in these nonresponders. Taken together, 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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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.

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