

## BRIEF REPORT

# Role of Interleukin-1 $\beta$ in *NLRP12*-Associated Autoinflammatory Disorders and Resistance to Anti-Interleukin-1 Therapy

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**Objective.** A new class of autoinflammatory syndromes called *NLRP12*-associated disorders (*NLRP12AD*) has been associated with mutations in *NLRP12*. Conflicting data on the putative role of *NLRP12* in interleukin-1 $\beta$  (IL-1 $\beta$ ) signaling have been found in *in vitro* analyses. This prospective study was undertaken to assess the secretion of IL-1 $\beta$  and 3 IL-1 $\beta$ -induced cytokines (IL-1 receptor antagonist [IL-1Ra], IL-6, and tumor necrosis factor  $\alpha$  [TNF $\alpha$ ]) in patients' peripheral blood mononuclear cells (PBMCs) cultured *ex vivo* and to evaluate the patients' response to IL-1Ra (anakinra), a major drug used in the treatment of autoinflammatory disorders.

**Methods.** Patients' disease manifestations and cytokine measurements were recorded before anakinra treatment was started, during 14 months of therapy, and after discontinuation of anakinra treatment.

**Results.** Spontaneous secretion of IL-1 $\beta$  by patients' PBMCs was found to be dramatically increased

(80–175 fold) compared to healthy controls. Consistent with these findings, anakinra initially led to a marked clinical improvement and to a rapid near-normalization of IL-1 $\beta$  secretion. However, a progressive clinical relapse occurred secondarily, associated with an increase in TNF $\alpha$  secretion, persistent elevated levels of IL-1Ra and IL-6, and a reactivation of IL-1 $\beta$  secretion. Anakinra was discontinued after 14 months of therapy.

**Conclusion.** Our findings provide *in vivo* evidence of the crucial role of IL-1 $\beta$  in the pathophysiology of *NLRP12AD*. This is the first time anakinra has been used to treat this disorder. This study provides new insights into the mechanisms underlying resistance to anti-IL-1 therapy observed in a few patients with autoinflammatory syndromes. Our data also point to the potential of *ex vivo* cytokine measurements as predictors of response to treatment.

Recurrent fever syndromes are autoinflammatory disorders characterized by recurrent episodes of fever, systemic inflammation, sterile peritonitis, arthritis, and/or cutaneous manifestations. Among them, cryopyrin-associated periodic syndromes have been associated with mutations in *NLRP3*, a gene of myelomonocytic expression. Peripheral blood mononuclear cells (PBMCs) from patients carrying *NLRP3* mutations secrete excessive amounts of interleukin-1 $\beta$  (IL-1 $\beta$ ) (e.g., refs. 1 and 2), a crucial cytokine in systemic inflammation. IL-1 $\beta$  acts on multiple organs and amplifies inflammation by inducing the expression of proinflammatory cytokines (including IL-1 $\beta$  itself) and numerous genes involved in inflammatory processes. In patients with cryopyrin-associated periodic syndromes, there is constitutive activation of a multiprotein complex, called the *NLRP3* inflammasome, which triggers the cleavage of procaspase 1 into caspase 1 and leads to the conversion of proIL-1 $\beta$  to mature IL-1 $\beta$ . Once

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**Table 1.** Clinical characteristics of the patients with *NLRP12*-associated disorder before, during, and after anakinra treatment

	Patient 1						Patient 2					
	Before treatment*			Treatment period			Before treatment*			Treatment period		
	Months 0–2	Months 3–8	Months 9–14	After discontinuation†	Months 0–2	Months 3–8	Months 9–14	After discontinuation†	Months 0–2	Months 3–8	Months 9–14	After discontinuation†
Frequency of crises (per 2 months)	0	<1	>3	1	0	<1	>3	1	0	<1	>3	1
Duration of crises, days	–	<7	4–5	2	–	5–7	2–3	2	–	5–7	2–3	3–10
Fever	39–40°C	38–38.5°C	38–38.5°C	38–39.5°C	38–38.5°C	38–38.5°C	38–38.5°C	38–39.5°C	38–38.5°C	38–38.5°C	38–38.5°C	38–39.5°C
Arthralgia	Severe	No	Yes	Yes	No	No	Yes	Yes	No	Yes	Yes	Yes
Myalgia	Yes	No	Severe continuous	Yes	No	Severe continuous	Severe continuous	Yes	Severe continuous	Severe continuous	Severe continuous	Yes
Cutaneous manifestations	A few episodes of urticaria	Generalized pruritus after injections	Local injection site reactions	Moderate pustular rash, maculo-urticarial rash	Generalized pruritus after injections	Local injection site reactions	Local injection site reactions	Moderate pustular rash, maculo-urticarial rash	Generalized pruritus after injection, lower limb pustulosis	Local injection site reactions	Severe pustular rash, maculo-urticarial rash	Moderate pustular rash, maculo-urticarial rash
Headaches	No	No	No	No	No	No	No	No	Yes	No	No	Yes
Hearing impairment, right/left ACPTA‡	5/27 dB	5/27 dB	22/27 dB	5/27 dB	5/27 dB	5/27 dB	22/27 dB	5/27 dB	70/67 dB	74/67 dB	70/68 dB	61/73 dB
Ocular manifestations	No	No	No	No	No	No	No	No	No	No	No	No

\* Phenotypic features reported in ref. 4 were reevaluated before anakinra treatment was started; in particular, C-reactive protein levels were found to be moderately elevated.

† Characteristics measured during the first 6 months after discontinuation of anakinra.

‡ Air-conduction pure-tone average (ACPTA) thresholds in the conversational frequencies (0.5, 1, 2, and 4 kHz) were calculated for each ear and were used to define the severity of the deafness as mild (20 < ACPTA  $\leq$  39 dB), moderate (40 < ACPTA  $\leq$  69 dB), severe (70 < ACPTA  $\leq$  89 dB), or profound (90 < ACPTA  $\leq$  119 dB). Hearing impairment can be considered stable if the loss between 2 measurements is <10 dB.

secreted, IL-1 $\beta$  competes with IL-1 receptor antagonist (IL-1Ra) for binding to IL-1 receptor type I. Consistent with this pathophysiologic mechanism, daily injections of recombinant IL-1Ra (anakinra) improve manifestations in many patients (3).

*NLRP12*, another *NLRP* family member expressed in PBMCs, has recently been shown to be involved in autoinflammatory syndromes evocative of cryopyrin-associated periodic syndromes (4,5). Several NLRP proteins have been shown to be intracellular sensors of the innate immune system regulating inflammatory processes. With regard to *NLRP12*, somewhat conflicting data have been reported on its role in IL-1 $\beta$  signaling. *NLRP12* expressed in COS-7L cells was found to activate proIL-1 $\beta$  secretion (6); activated THP-1 cells transduced with *NLRP12* small interfering RNA were shown to secrete increased levels of IL-1 $\beta$  (7), whereas activated dendritic cells from *Nlrp12*-deficient mice displayed normal IL-1 $\beta$  secretion (8).

To gain insight into the mechanisms responsible for *NLRP12*-associated disorders (*NLRP12AD*), we examined the release of IL-1 $\beta$  and of several cytokines whose expression is induced by IL-1 $\beta$  in PBMCs from patients in whom an *NLRP12* mutation had been identified (4). The data obtained prompted us to treat patients with anakinra and to evaluate the effects of this biologic therapy.

## PATIENTS AND METHODS

**Patients.** Monozygotic twin brothers carrying an *NLRP12* mutation (p.Arg284X) (4) were included in this prospective study. The family was informed that anakinra had been approved only for adults with rheumatoid arthritis but was effective in other autoinflammatory syndromes. Written informed consent for the children's participation in the study was obtained from their parents. The study was approved by the local ethics committee (CCPPRB Henri Mondor, France; PHRC AOM97201). Disease manifestations were evaluated before treatment, at regular intervals over a period of 14 months during anakinra therapy (1 mg/kg/day), and 6 months after discontinuation of anakinra. PBMCs from 4 healthy donors were used as controls.

**Culture of PBMCs.** Fresh heparinized blood was fractionated by density gradient. PBMCs were cultured in 96-well plates for 24 hours in 200  $\mu$ l of RPMI 1640 ( $10^6$  cells/ml), supplemented with Glutamax-I, fetal calf serum (10%), penicillin, and streptomycin. Cells were stimulated with lipopolysaccharide (LPS; 10 ng/ml) or were left untreated. Each condition was performed in duplicate. Supernatants were collected and cleared by centrifugation.

**Cytokine measurements.** Cytokines were measured in cell supernatants by enzyme-linked immunosorbent assay (ELISA). Cytokines measured were IL-1 $\beta$  and IL-1Ra

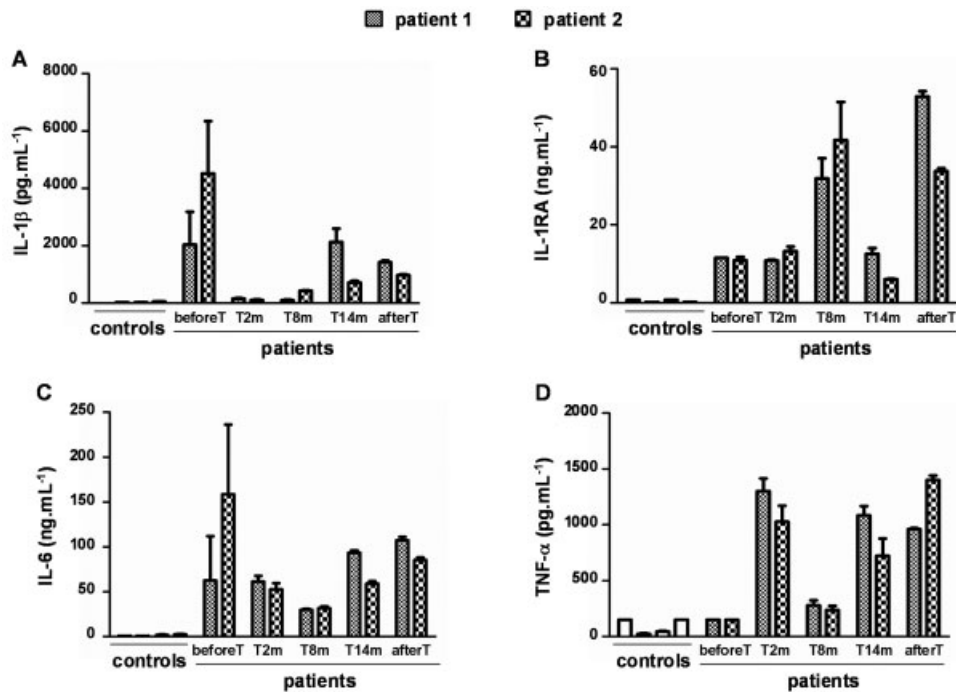
(matched antibody pairs for ELISA; R&D Systems) and IL-6 and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (Eli-pair; Diaclone).

**Statistical analysis.** Student's *t*-tests were used to compare the means of cytokine secretion levels. Statistical analyses (2-tailed) were conducted using GraphPad software. *P* values  $\leq$  0.05 were considered significant.

## RESULTS

**Manifestations before anakinra treatment.** The twin brothers (patient 1 and patient 2) had been presenting with *NLRP12AD* symptoms since birth, with several attacks per month, lasting 3–15 days and triggered by generalized exposure to cold. Episodes were associated with high fever (39–40°C), arthralgia, myalgia, and urticaria in both patients, and headaches in patient 2 (Table 1). Both children had sensorineural hearing loss. Elevation of C-reactive protein levels (32 mg/liter) and neutrophilia ( $23.5 \times 10^9$ /liter) were observed in patient 2 soon after the onset of an attack; no autoantibodies were identified. In addition to their usual followup, the patients had regular consultations in a reference center for autoinflammatory syndromes, where the disease manifestations were reevaluated and cytokine studies were performed; notably, patients were not experiencing attacks at these times, and biologic parameters were within the normal range.

Since, as shown in cryopyrin-associated periodic syndromes, IL-1 $\beta$  was undetectable in the sera of the patients (data not shown), the spontaneous secretion of cytokines was measured in supernatants from PBMCs cultured *ex vivo*. The effect of LPS was also assessed, since PBMCs from patients with cryopyrin-associated periodic syndromes show increased IL-1 $\beta$  secretion in either the absence (e.g., ref. 1) or the presence (e.g., ref. 2) of LPS stimulation. Spontaneous IL-1 $\beta$  secretion was found to be much higher in patients than in controls ( $P < 0.01$ ), with an 80-fold increase in patient 1 and a 175-fold increase in patient 2 (Figure 1A). IL-1Ra levels were also much higher in patients than in controls (20–25-fold increase;  $P < 1 \times 10^{-4}$ ) (Figure 1B). This result was expected, since IL-1Ra release represents a homeostatic response to limit the deleterious effects of IL-1 $\beta$ , as observed in patients with cryopyrin-associated periodic syndromes. The levels of IL-6 and TNF $\alpha$ , 2 proinflammatory cytokines that are also induced by IL-1 $\beta$ , were measured concomitantly. IL-6 levels were much higher in patients than in controls (43-fold higher in patient 1 and 110-fold higher in patient 2) ( $P < 0.02$ ) (Figure 1C); however, TNF $\alpha$  levels were close to basal values in the 2 patients (Figure 1D).



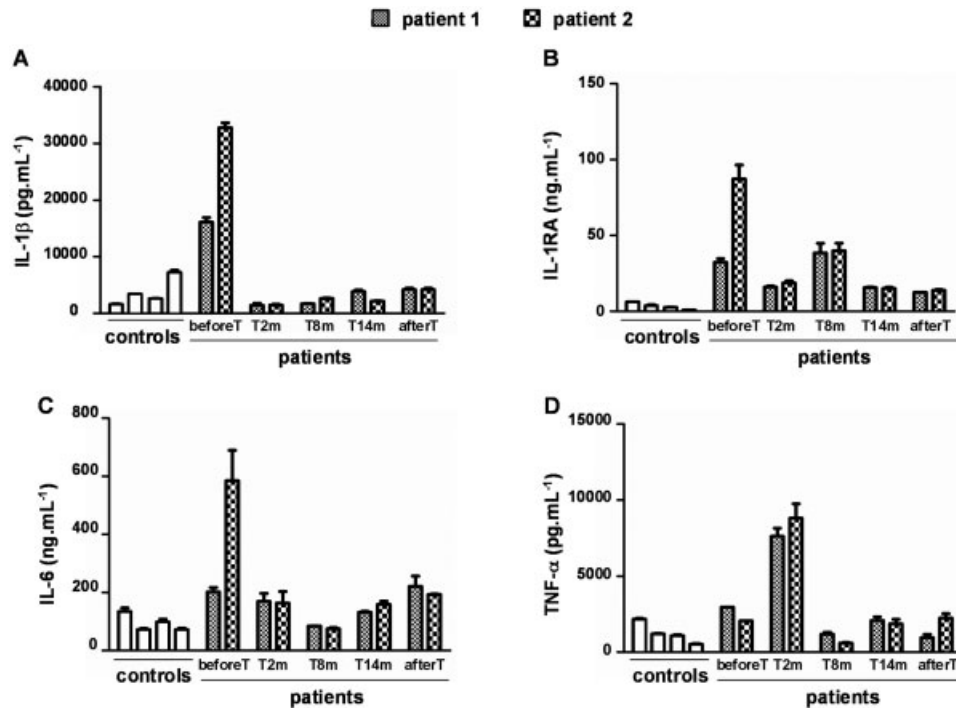
**Figure 1.** Spontaneous secretion of inflammatory cytokines interleukin-1 $\beta$  (IL-1 $\beta$ ) (A), IL-1 receptor antagonist (IL-1Ra) (B), IL-6 (C), and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (D) by peripheral blood mononuclear cells (PBMCs) from healthy controls and from 2 patients with *NLRP12*-associated disorder before treatment (beforeT), after 2 months of treatment (T2m), after 8 months of treatment, after 14 months of treatment, and 6 months after discontinuing treatment (afterT) with anakinra. Supernatants of PBMCs cultured for 24 hours were collected, and cytokine concentrations were determined using enzyme-linked immunosorbent assays. Bars show the mean  $\pm$  SEM.

Stimulation of PBMCs with LPS further stimulated IL-1 $\beta$  secretion, although to a different extent in patients (7–8-fold) than in controls (144-fold) (Figure 2); nevertheless, absolute production of IL-1 $\beta$  remained 5–10-fold higher in PBMCs from patients despite their limited response. Similar results were obtained for IL-1Ra, IL-6, and TNF $\alpha$ , whose release was also elicited by LPS (Figure 2).

**Initiation of anakinra therapy.** The major increase in the patients' levels of IL-1 $\beta$  secretion, and the known efficacy of anti-IL-1 in autoinflammatory syndromes, prompted us to treat the patients with anakinra. After the first injection, they experienced generalized pruritus, which was readily explained by the fact that the syringe needle covers of anakinra contain natural rubber and the patients were allergic to latex. After anakinra reconditioning in latex-free syringes, the patients experienced only a local erythematous skin reaction, an adverse event frequently observed with anakinra treatment (e.g., ref. 3). Most importantly, the children and their parents reported a marked clinical improvement within 2 weeks of treatment.

**Followup at 2 months.** A followup visit at 2 months revealed that the patients had not experienced any new flares, with only a few days of isolated and moderate fever (38–38.5°C); skin and musculoskeletal manifestations had disappeared. Consistent with the clinical findings, the level of spontaneous IL-1 $\beta$  secretion in PBMCs was nearly normalized (Figure 1A). However, although we expected a major drop in IL-6 and IL-1Ra secretion following the decrease in IL-1 $\beta$  secretion, the levels of these 2 cytokines remained elevated (Figures 1B and C). In addition, levels of spontaneous secretion of TNF $\alpha$  increased 7–9 fold compared to levels measured before anakinra treatment and were significantly higher in patients than in controls ( $P < 5 \times 10^{-4}$ ) (Figure 1D).

**Results of treatment from month 3 to month 14.** During months 3–8 of treatment, each of the patients experienced 2 new bouts of moderate fever (38–38.5°C) and systemic inflammation. Most importantly, patients developed chronic severe disabling myalgia, which was not present before initiation of the biologic therapy (Table 1). Over the subsequent 6 months (treatment



**Figure 2.** Lipopolysaccharide (LPS)-induced secretion of inflammatory cytokines IL-1 $\beta$  (A), IL-1Ra (B), IL-6 (C), and TNF $\alpha$  (D) by PBMCs from healthy controls and from 2 patients with *NLRP12*-associated disorder before treatment, after 2 months of treatment, after 8 months of treatment, after 14 months of treatment, and 6 months after discontinuing treatment with anakinra. Supernatants of PBMCs cultured for 24 hours in the presence of LPS (10 ng/ml) were collected, and cytokine concentrations were determined using enzyme-linked immunosorbent assays. Bars show the mean  $\pm$  SEM. See Figure 1 for other definitions.

months 9–14), the efficacy of anakinra was further reduced. Each of the 2 children experienced  $\sim$ 10 episodes of fever, arthralgia, and myalgia, and skin manifestations reappeared. However, the duration of the episodes remained shorter and the temperature remained lower than before anakinra treatment was started (Table 1).

The decline in response to treatment coincided with a progressive reactivation of the spontaneous secretion of IL-1 $\beta$  (Figure 1A); in patient 1, the levels of IL-1 $\beta$  secreted after 14 months of anakinra reached the levels measured before treatment (83-fold higher than in controls) (Figure 1A). During months 3–14 of treatment, a persistence of elevated levels of other cytokines was also observed. IL-1Ra, IL-6, and TNF $\alpha$  were 10–85-, 20–65-, and 2.5–15-fold higher in patients than in controls, respectively (Figures 1B–D). As described above (Figure 2), treatment of PBMCs with LPS elicited further secretion but minimized the differences observed between patients and controls.

**Discontinuation of anakinra.** Considering the final moderate clinical improvement and the biologically proven resistance to anti-IL-1 therapy, anakinra treatment was discontinued. Within the 6 months following anakinra discontinuation, the 2 patients each experienced 3 attacks. Notably, clinical manifestations returned to those observed before anti-IL-1 therapy (Table 1). The duration of the episodes increased, reaching 10 days in patient 2. Fever was also higher ( $>39^{\circ}\text{C}$ ), while severe myalgia disappeared. The spontaneous cytokine secretion from the patients' PBMCs followed the tendency observed in the previous months, with increased levels compared to controls. IL-1 $\beta$  was 40–55-fold higher, IL-1Ra was 65–110-fold higher, IL-6 was 60–75-fold higher, and TNF $\alpha$  was 10–15-fold higher (Figure 1).

## DISCUSSION

The identification of several autoinflammatory syndrome genes has greatly helped in understanding the



pathophysiology of these disorders and in the development of targeted therapies, although in vitro functional assays of autoinflammatory syndrome proteins have produced some conflicting results. This study, which investigated the secretion of IL-1 $\beta$  in patients with *NLRP12AD* and evaluated their response to IL-1Ra, provides in vivo evidence of the crucial role of *NLRP12* in IL-1 $\beta$  signaling.

Patient PBMCs spontaneously released huge amounts of IL-1 $\beta$ , whereas specific stimuli were required to activate IL-1 $\beta$  secretion in normal PBMCs. This strongly suggests that *NLRP12*, when mutated, leads to constitutive inflammasome activation. Although LPS further induced IL-1 $\beta$  release in the patients' PBMCs, the relative response was less important than that in controls, probably due to the already high level of spontaneous secretion. Similar results have been reported in patients with cryopyrin-associated periodic syndromes (e.g., ref. 1).

The inflammatory phenotype of our 2 patients revealed that natural IL-1Ra may slightly temper the effects of IL-1 $\beta$ , but it is not sufficient to abrogate them, since a significant molar excess of IL-1Ra is required to completely inhibit IL-1 $\beta$ -induced responses. In addition, the patients' good clinical response soon after the introduction of anakinra was consistent with the down-modulation of IL-1 $\beta$  secretion by their PBMCs. Taken together, these data confirm that poor control of IL-1 $\beta$  release is a hallmark of many autoinflammatory syndromes, including *NLRP12AD*. During the revision of this manuscript, Borghini et al reported an accelerated secretion of IL-1 $\beta$  in autoinflammatory syndrome patients carrying an *NLRP12* missense mutation (9). All of these data underline the similarities between *NLRP12AD* and cryopyrin-associated periodic syndromes. Both of these autoinflammatory syndromes are associated with mutations in *NLRP* genes, and they share phenotypic features (4,5). In both syndromes, *NLRP12* and *NLRP3* interact with ASC and activate caspase 1 processing (e.g., refs. 5 and 6), and the patients' PBMCs secrete abnormally elevated levels of IL-1 $\beta$ .

*NLRP12AD* represents a newly identified class of disorders, with only a few cases reported worldwide. Prior to this study, the efficacy of anakinra in *NLRP12AD* had not been assessed. The 2 brothers examined in this study developed resistance to anakinra within a few months of treatment. Since the patients also experienced severe disabling myalgia as a side effect, and since cytokine measurements revealed resistance to anti-IL-1 blockade, the treatment was discontinued after 14 months without any dosage adjustment, which has

been shown to be effective in certain patients with cryopyrin-associated periodic syndromes (e.g., refs. 1 and 3). Although the present study reveals failure of anakinra, anti-IL-1 treatment may remain a therapeutic choice for other patients with this disorder; indeed, although a few patients with cryopyrin-associated periodic syndromes failed to respond to anakinra treatment (10), this therapy has proven benefits in many of them (3).

When trying to identify the mechanism that accounts for anakinra resistance, we should keep in mind that cytokine networks are homeostatic systems and that the level of any cytokine can be interpreted only by taking into account the levels of other synergistic and inhibitory cytokines. The marked increase in TNF $\alpha$  levels observed in the patients in the present study after 2 months of treatment may play a role in this resistance process, since both TNF $\alpha$  and IL-1 $\beta$  stimulate inflammatory cytokines, including IL-6 and IL-1Ra, as well as IL-1 $\beta$  and TNF $\alpha$ . The initial anakinra-induced IL-1 $\beta$  down-modulation observed in patients may therefore be partially counterbalanced by the increase in TNF $\alpha$  levels, which may in turn circumvent the action of anakinra and eventually lead to the reactivation of IL-1 $\beta$  hypersecretion. Consistent with this idea, the levels of IL-1Ra and IL-6 remained elevated throughout the course of the study.

Because the 2 children were treated by splenectomy for a pyropoikilocytosis, anti-TNF therapy, which is more immunosuppressive than anakinra and is associated with an increased risk of adverse events, was not proposed. Although we cannot categorically exclude the possible involvement of anti-anakinra antibodies in the resistance process, it is worth noting that neutralizing antibodies are rarely found in patients with autoinflammatory syndrome treated with anakinra; in addition, when observed, these antibodies are transient and of uncertain clinical relevance (e.g., ref. 11). Taken together, the data obtained reveal that the 2 patients were not intrinsically resistant to treatment but acquired resistance at some point during the course of anakinra therapy.

This prospective study shows the pivotal role of IL-1 $\beta$  in the pathogenesis of *NLRP12AD*. It represents one of the few examples of resistance to anti-IL-1 therapy in autoinflammatory syndromes and reveals a striking correlation between the patients' clinical and biologic course. The study of key inflammatory cytokines provides the first clues to understanding the mechanisms leading to such resistance. In addition, our findings point to the potential use of ex vivo cytokine measurements as predictors of the response to anti-IL-1 treatment.

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### AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Amselem had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Jéru, Hentgen, Amselem, Lecron.

**Acquisition of data.** Jéru, Hentgen, Normand, Duquesnoy, Cochet, Delwail, Marlin, Lecron.

**Analysis and interpretation of data.** Jéru, Hentgen, Normand, Duquesnoy, Grateau, Amselem, Lecron.

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### Erratum

In the article by Lee et al in the August 2009 issue of *Arthritis & Rheumatism* (pages 2325–2332), the type II collagen primer sequences used were reported incorrectly (at the top of page 2327). The actual primer sequences for type II collagen were 5'-CTCCTGGAGCATCTGGAGAC-3' (sense) and 5'-ACC-ACGATCACCCCTTGACTC-3' (antisense). None of the results reported in the article are affected by this correction.

We regret the error.