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BRIEF REPORT

Etanercept Inhibits the Tumor Necrosis Factor α–Driven Shift of Th17 Lymphocytes Toward a Nonclassic Th1 Phenotype in Juvenile Idiopathic Arthritis

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Objective. **To evaluate the effects of etanercept on the phenotype of CD4+ T helper lymphocytes from patients with juvenile idiopathic arthritis (JIA).**

Methods. **We compared the proportions of various Th cell subsets in peripheral blood (PB) from etanercept-treated and untreated JIA patients. An in vitro study was performed on PB mononuclear cells (PBMCs) from 15 children with untreated JIA, in which we evaluated the proliferative response of these cells, as well as their cytokine production profile, in the presence of various stimuli with or without etanercept.**

Results. **We found lower proportions of CD4+ CD161+ (nonclassic) Th1 lymphocytes in the PB of patients treated with etanercept than in untreated patients. In vitro, etanercept inhibited the proliferative response induced by either polyclonal or recall antigen stimulation of PBMCs. Moreover, etanercept increased the proportion of CD4+CD161+ Th17/Th1 and Th17 cells in vitro while decreasing the proportions of nonclassic Th1 cell subsets, leaving CD4+CD161− (classic) Th1 cells unaffected. We also found that tumor necrosis factor α (TNFα) was able to induce transition of Th17 lymphocytes toward the nonclassic Th1 phenotype in vitro, probably due to the high expression of TNF receptor type II observed in Th17 cells.**

Conclusion. **We have previously demonstrated the occurrence of a shifting of CD4+CD161+ Th17 cells to the nonclassic Th1 phenotype in children with JIA. The present findings suggest that etanercept can exert its disease-modifying action by interfering with this shifting.**

Juvenile idiopathic arthritis (JIA) is the most common form of persistent arthritis in children. The cause of the disease is still poorly understood (1), but T cells are certainly involved in its pathogenesis, as indicated by the synovial infiltrates predominantly consisting of CD4+ T cells belonging to the Th1 subset (2,3). Following the discovery of Th17 cells, some investigators focused their attention on these as being possibly involved in the joint inflammation (4). Recently, we reported an accumulation of CD4+CD161+ cells, belonging to either the Th1 or the Th17/Th1 subset, in the inflamed joints of JIA patients, and we showed that their proportions in synovial fluid (SF) positively correlated with parameters of disease activity (5). More importantly, a shifting of CD4+CD161+ cells from the Th17 to the nonclassic Th1 phenotype has also been shown to occur in the SF of children with JIA (6,7).

Current treatment of JIA includes nonsteroidal antiinflammatory drugs, corticosteroids, and disease-modifying antirheumatic drugs such as tumor necrosis factor α (TNFα) antagonists. One of these antagonists is etanercept, a soluble dimeric fusion protein that binds soluble TNFα. The efficacy of etanercept in the treatment of JIA has been demonstrated in randomized clinical trials, as well as in long-term observational registries (8).

The aim of the present work was to evaluate the effects of etanercept on the phenotype of CD4+ T helper cell subsets in JIA patients. Our findings indicate that, beyond its antiinflammatory activity, etanercept

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also possesses some immunomodulatory properties that can contribute to its strong impact on the natural course of JIA.

PATIENTS AND METHODS

Subjects. Peripheral blood (PB) samples for in vivo experiments were obtained from 18 patients with oligoarticular JIA, 8 of whom had been treated for at least 6 months with etanercept only (mean ± SEM 22.1 ± 3.1 months [range 6–32]) and 10 of whom were untreated. Etanercept-treated patients were administered the drug at 0.4 mg/kg twice weekly as a subcutaneous injection, with an interval of 3–4 days between doses. PB mononuclear cells (PBMCs) were evaluated no more than 3 days after the last administration of the drug. JIA in all of the etanercept-treated patients was responsive to treatment, according to the American College of Rheumatology response criteria (9). For in vitro experiments, PB samples were obtained from 15 untreated children with oligoarticular-onset JIA, as well as from 5 healthy adult volunteers.

All procedures followed in the study were in accordance with the ethics standards of the Regional Committee on Human Experimentation. Informed consent was obtained by parents or guardians.

Proliferation assay. PBMCs (10^5) from JIA patients were stimulated with anti-CD3 plus anti-CD28 monoclonal antibody (mAb) (each at 5 μg/ml), tetanus toxoid (10 μg/ml), or streptokinase (5,000 units/ml), in the presence or absence of etanercept (5 μg/ml). On day 5, interleukin-2 (IL-2), IL-4, IL-6, IL-10, IL-17, and interferon-γ (IFNγ) levels in the supernatants were measured by cytometric bead assay (BD Biosciences). Cells were pulsed for 8 hours with 0.5 μCi of tritiated thymidine (PerkinElmer) and harvested, and radionuclide uptake was measured by scintillation counting.

The concentration of etanercept used for the in vitro experiments was selected based on data reported from the European Medicines Agency in the "Summary of Product Characteristics" for Enbrel (Pfizer). Serum levels of etanercept were measured (ProMonitor-ETN ELISA Kit; Menarini) in 4 of the 8 etanercept-treated patients included in the in vivo study; the mean ± SEM level was 4.4 ± 0.78 μg/ml.

Short-term T cell lines. PBMCs (5×10^5) from JIA patients were stimulated with anti-CD3 plus anti-CD28 in the presence or absence of etanercept (5 μg/ml). On day 7, cells were stimulated with phorbol myristate acetate plus ionomycin (Sigma-Aldrich) for 6 hours (the last 4 hours in the presence of brefeldin A) and evaluated for intracellular cytokine production using a BD LSRII flow cytometer (BD Biosciences).

Sorting for pure IL-17–producing cells in PB from healthy donors was performed by cytokine capture assay and fluorescence-activated cell sorting, as previously described (7). The recovered subsets were cultured in the presence or absence of TNFα (1 ng/ml) or IL-12 (2 ng/ml) (both from R&D Systems), with or without etanercept (5 μg/ml). All fluorochrome-conjugated or unconjugated mAb were from BD Biosciences, with the exception of anti–IL-17 (eBioscience).

RNA isolation, complementary DNA synthesis, and real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR). T cell clones were obtained as previously described (10). Total RNA was extracted using an RNeasy Micro kit (Qiagen), and TaqMan RT-PCR was performed as previously described (11). Primers and probes were purchased from Applied Biosystems. Quantification was performed on 10^4 cells, and results were reported as $-\Delta C_t$, i.e., $-[C_t$ of the target gene (TNF receptor type II [TNFRII]) minus C_t of the endogenous control (GAPDH)].

Statistical analysis. For analysis of the significance of differences between conditions or groups, the Mann-Whitney U test or the Wilcoxon test for paired samples was used. P values less than 0.05 were considered significant.

RESULTS

Reduced proportion of nonclassic Th1 lymphocytes in the PB of etanercept-treated JIA patients versus untreated patients. Based on our previous results showing that CD4+CD161+ T cells are involved in maintain-

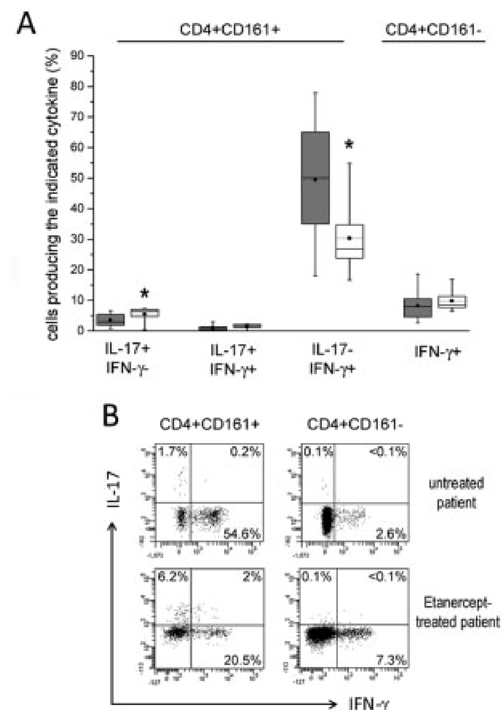

Figure 1. Decrease in the proportion of nonclassic Th1 lymphocytes in the peripheral blood (PB) of juvenile idiopathic arthritis (JIA) patients after etanercept treatment. **A,** Proportions of various T cell subsets in the PB of 10 untreated patients (gray boxes) and 8 etanercept-treated patients (white boxes). Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Solid squares (on lines) inside the boxes represent the mean. Lines outside the boxes represent the minimum and maximum values. $* = P < 0.05$ versus untreated patients. **B,** Cytometric plots depicting the intracellular detection of interferon-γ (IFNγ) and interleukin-17 (IL-17) in CD4+CD161+ and CD4+CD161− lymphocytes from the PB of 1 representative untreated JIA patient and 1 representative etanercept-treated JIA patient, after in vitro polyclonal stimulation.

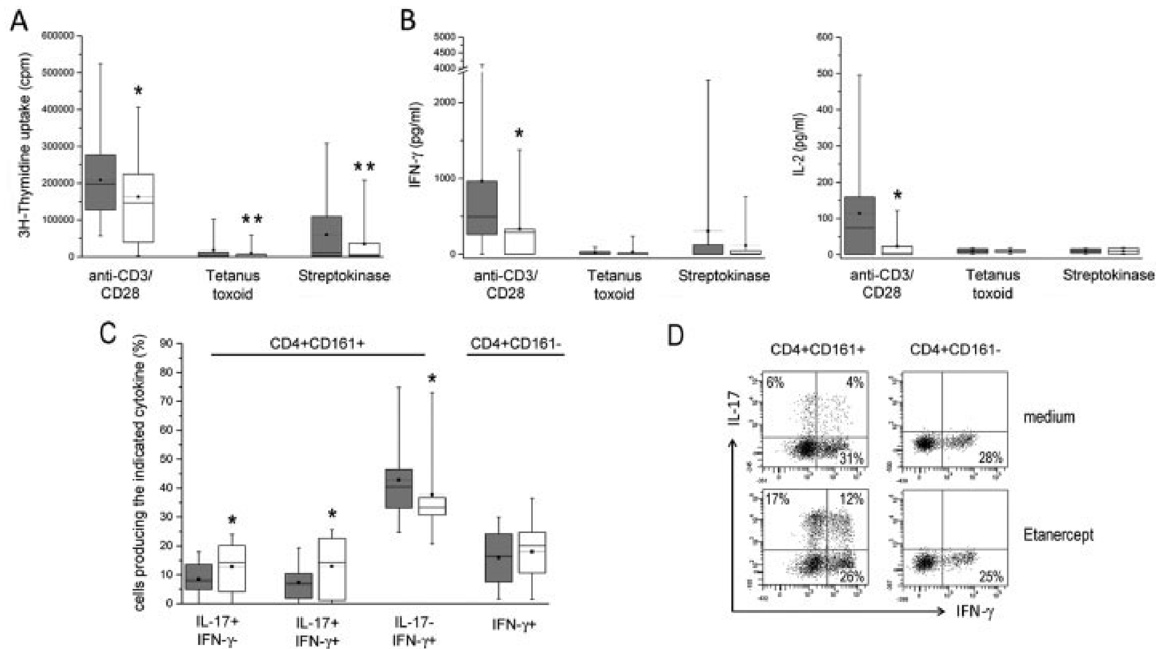

Figure 2. Etanercept reduces both T cell proliferation and cytokine production in vitro, and reduces the proportions of Th1 cells. **A,** Proliferative response to anti-CD3/anti-CD28 and recall antigen (tetanus toxoid and streptokinase) stimulation in PB mononuclear cells (PBMCs) from 15 JIA patients, in the absence (gray boxes) or presence (white boxes) of etanercept. **B,** Cytokine levels in culture supernatants of anti-CD3/anti-CD28– and recall antigen–stimulated PBMCs from 10 JIA patients, in the absence (gray boxes) or presence (white boxes) of etanercept. **C,** Proportions of various T cell subsets, based on their IL-17 and IFNγ expression, in polyclonally activated short-term T cell lines obtained from the PB of JIA patients, in the absence (gray boxes) or presence (white boxes) of etanercept. In **A–C,** each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Solid squares (on lines) inside the boxes represent the mean. Lines outside the boxes represent the minimum and maximum values. $* = P < 0.05$; $** = P < 0.01$, versus absence of etanercept. **D,** Flow cytometric plots depicting IL-17 and IFNγ expression in CD161+ and CD161− Th cells from polyclonally activated short-term T cell lines (1 representative patient). See Figure 1 for other definitions.

ing the inflammatory process in JIA and that during the course of the disease a shift from Th17 to nonclassic Th1 cells usually occurs (7), we investigated the proportions of various Th cell subsets in untreated and etanercept-treated JIA patients. We found that, compared with PB from untreated patients, PB from patients treated with etanercept had significantly lower proportions of nonclassic Th1 cells and higher proportions of Th17 cells (Figure 1).

Etanercept inhibits the in vitro proliferative response and IFNγ production by CD4+ T cells from JIA patients. From ex vivo observations, we hypothesized that etanercept is able to interfere with the expression of different functional phenotypes among CD4+ Th lymphocytes. In experiments to test this, etanercept reduced the proliferative response to both polyclonal and recall antigen stimulation in PBMCs from patients with JIA (Figure 2A). In polyclonally stimulated cultures, etanercept reduced IFNγ and IL-2 levels (Figure 2B), whereas the reduction of IL-6 was not statistically significant and levels of IL-4, IL-10, and IL-17 were very low or undetectable in all cultures (data not shown).

To ascertain whether the inhibitory effect of etanercept on IFNγ production was due to a modification of the Th cell phenotype, polyclonally activated T cell lines were generated from the PB of JIA patients, in the presence or absence of the drug. Addition of etanercept to the cultures increased the proportions of CD161+ Th17 and CD161+ Th17/Th1 cells and reduced those of CD161+ nonclassic Th1 cells. Of note, etanercept did not exhibit any effect on the CD161− subset in terms of proportions of Th1 cells (Figures 2C and D).

Etanercept inhibits the TNFα-driven shift of Th17 lymphocytes toward the nonclassic Th1 phenotype. To assess the possibility that TNFα could act differentially on CD161+ and CD161− Th lymphocyte subsets, the expression of TNFα receptors on a panel of

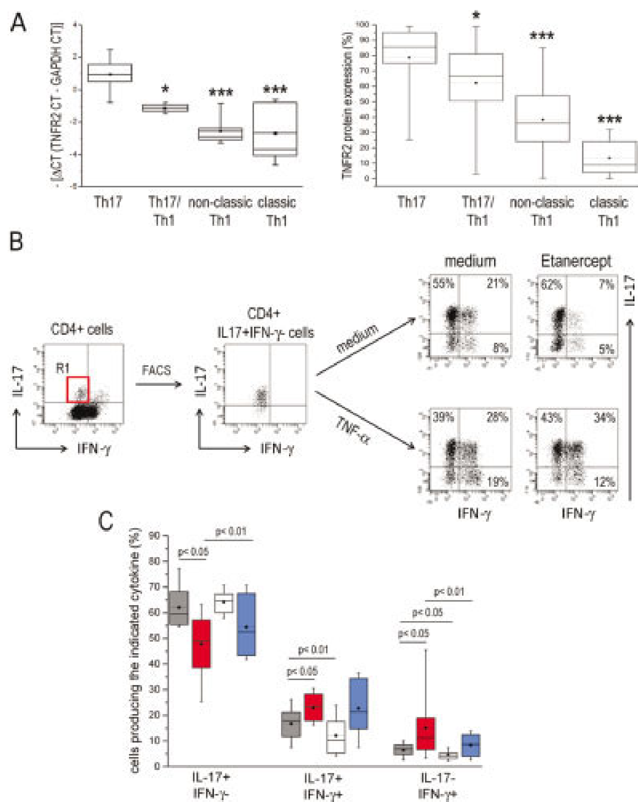

Figure 3. Inhibitory effect of etanercept on the tumor necrosis factor α (TNFα)–driven shift of Th17 cells toward the Th1 phenotype. **A,** TNF receptor type II (TNFRII) expression was evaluated at both the mRNA level (with quantification of cell numbers and results normalized to GAPDH expression) and the protein level (by flow cytometry), in Th17, Th17/Th1, Th1 CD161+, and Th1 CD161− established T cell clones from healthy donors (10 clones from each phenotype). $* = P < 0.05$; $*** = P < 0.001$, versus Th17 clones. **B,** Th17 cells from the PB of healthy donors were sorted by fluorescence-activated cell sorting (FACS) and cultured in vitro for 7 days under the indicated conditions. Cultures were then assessed by flow cytometry for their ability to produce IL-17 and IFNγ. Results of 1 representative experiment are depicted. **C,** Proportions of T cells producing the indicated cytokines in the same cultures as described in **B** (5 separate experiments) in the presence of medium alone (gray boxes), TNFα (red boxes), etanercept (white boxes), or both TNFα and etanercept (blue boxes) were determined. In **A** and **C,** each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Solid squares (on lines) inside the boxes represent the mean. Lines outside the boxes represent the minimum and maximum values. See Figure 1 for other definitions.

Th17, Th17/Th1, and Th1 (CD161+ and CD161−) clones from healthy donors was evaluated. Expression of TNFRII was found to be higher in Th17 than in other types of T cell clones at both the messenger RNA and the protein levels (Figure 3A), whereas TNFRI expression was low or undetectable in all clones examined (data not shown). To test whether TNFα could play a role in the shift toward the nonclassic Th1 phenotype, we isolated pure Th17 lymphocytes from the PB of healthy donors by cytokine capture assay and cultured them in the presence of TNFα, etanercept, or both. As seen in Figures 3B and C, TNFα induced a polarization of Th17 cells toward the nonclassic Th1 phenotype, and etanercept inhibited this shift. Moreover, etanercept also inhibited the spontaneous shift of Th17 cells toward a Th1 phenotype, probably due to its action on TNFα produced by Th17 cells themselves (12). Of note, etanercept was unable to affect the well-known ability of IL-12 to induce IFNγ production in Th17 cells (data not shown).

DISCUSSION

CD4+CD161+ T cells play a crucial role in JIA pathogenesis, as indicated by the abundance of these cells in SF from inflamed joints (7). Most of these cells are Th17/Th1 or nonclassic Th1 cells, which have been shown to be generated from a shifting of Th17 cells (7). Based on these findings, we undertook the present study to assess the phenotype of CD4+CD161+ T lymphocytes in the PB of untreated and etanercept-treated JIA patients, and observed lower proportions of nonclassic Th1 cells and higher proportions of Th17 cells in the latter. This observation prompted us to study the effect of etanercept in vitro on the phenotype and function of CD4+ T cells.

First, we found that etanercept reduced the proliferative response to either polyclonal antigen– or recall antigen–induced stimulation of PBMCs, thus supporting the concept that the drug exerts immunosuppressive activity. Moreover, etanercept was able to reduce IFNγ and IL-2 production in the same cultures, suggesting that this agent might be able to interfere mainly with the activity of Th1 lymphocytes, which usually exhibit a strong proliferative response. We therefore assessed the effect of etanercept on the functional phenotype of CD4+ Th lymphocyte subsets obtained from the PB of patients with JIA. Etanercept was found to exert a modulatory effect only on CD4+CD161+ T lymphocytes, which have been reported to be one of the hallmarks of joint inflammation in JIA (7). Etanercept inhibited the expansion of Th1 cells and increased the proportions of both the Th17 and the Th17/Th1 cell subsets, as has been reported in rheumatoid arthritis (13).

There are at least 2 possible explanations for these phenomena. The first is that the suppressive

activity is more marked on cell populations with high rates of proliferation, such as the Th1 subset. This possibility is consistent with our recent observation that Th17 and Th17/Th1 cells, despite their well-known pathogenetic role in some inflammatory disorders, are very rare at sites of inflammation, due to their inability to expand because of abnormalities in the molecular pathway that allows IL-2 production (11). A second possibility (which would not preclude the first one) is that etanercept can interfere with the transition of CD161+ Th17 lymphocytes toward the nonclassic Th1 phenotype, which occurs in the course of JIA (7). Since etanercept acts by blocking TNFα activity, we wondered whether this cytokine could be involved in the shift of the Th17 phenotype toward the nonclassic Th1 cell phenotype. In a study of a wide panel of established T cell clones generated from healthy subjects, we found higher expression of TNFRII in Th17 than in Th17/1 and Th1 cells (both the nonclassic and the classic subsets) (14). To assess whether this differential receptor expression could have any biologic significance, we derived CD161+IL-17+IFNγ− (Th17) cells from the PB of healthy donors and then stimulated these cells in the presence of TNFα or etanercept. Etanercept inhibited the shifting of Th17 lymphocytes toward the nonclassic Th1 phenotype both in the presence and in the absence of exogenous TNFα.

The ability of etanercept to block the effects of exogenous TNFα indicates an activity of the drug on soluble TNFα. Additionally, we found that etanercept was able to bind to a fraction of CD4+ T cells, belonging prevalently to the CD161+ subset, in vitro (data not shown). This could represent a further potential mechanism by which the drug may affect the cell response. Moreover, the inhibition of the shift toward nonclassic Th1 cells in the absence of exogenous TNFα indicates that Th17 lymphocytes themselves produce TNFα, which can thus drive their transition to the Th1 phenotype by either an autocrine or a paracrine mechanism (7). The finding that TNFα can drive the shift from Th17 to Th1 cells has not been previously reported, and it may represent an additional indication for the use of TNFα inhibitors in diseases in which this shift can play a pathogenic role. We did not investigate whether TNFα inhibitors other than etanercept show a similar behavior in JIA, but we recently reported that adalimumab inhibits the shifting of CD161+ T cells to the Th1 phenotype in Crohn's disease (15).

Taken together, the results of this study support previous observations on the possible role of CD4+ CD161+ T lymphocytes in the pathogenesis of JIA (16). More importantly, they provide the first evidence of a previously unreported mechanism of action of etanercept, in which the drug appears to be able to inhibit the TNFα-induced transition of CD161+ Th17 lymphocytes to the Th17/Th1 and the Th1 phenotype, thus possibly contributing to the dampening of inflammation in JIA and arresting disease progression.

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. Annunziato 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. L. Maggi, Cimaz, Santarlasci, Liotta, Romagnani, E. Maggi, Annunziato, Cosmi.

Acquisition of data. L. Maggi, Capone, Santarlasci, Querci, Simonini, Nencini.

Analysis and interpretation of data. L. Maggi, Liotta, Romagnani, E. Maggi, Annunziato, Cosmi.

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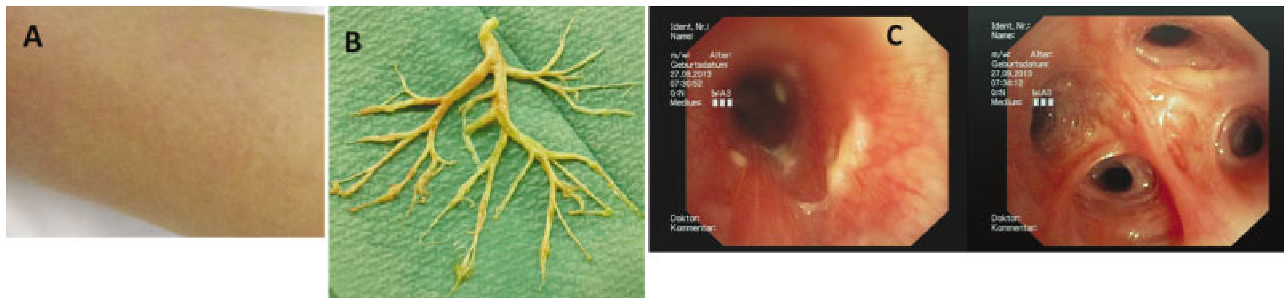
Clinical Images: Urticaria, fever, and hypofibrinogenemia



The patient, a 12-year-old girl, presented with a history of recalcitrant urticarial rash, arthralgias, and febrile episodes for >2 years. She repeatedly developed high-spiking fever and systemic inflammation unresponsive to antibiotics and antipyretic medication. Positron emission tomography revealed symmetric cervical and mediastinal lymphadenopathy. The chronic urticaria in combination with the recurrent fever and elevated serum amyloid A levels prompted us to search for a mutation in the *NLRP3* gene. Heterozygosity for the V198M substitution encoded by exon 3 confirmed the suspected diagnosis of a cryopyrin-associated periodic syndrome (CAPS) inherited from her asymptomatic father. This mutation was recently described as having reduced penetrance, causing variable CAPS phenotypes (1,2). No mutations in 2 other genes known to cause autoinflammation (*TNFRSF1A* and *MEFV*) were found. In the context of chronic inflammation, the patient developed a macrophage activation syndrome (MAS) with serositis, hyperferritinemia, hypofibrogenemia, and bicytopenia as well as hemophagocytosis, which was controlled with anakinra and dexamethasone treatment. The urticarial rash (**A**) vanished within 1 week. The patient's disease remained stable for >1 year while she received canakinumab, but she experienced a relapse when the treatment intervals were prolonged. Surprisingly, she expectorated bronchial casts (**B**) for >6 months. Bronchoscopy revealed thick mucus in the left main bronchus (left) and left upper bronchus (right), partially with obstructing mucus impaction (**C**). The bronchial cast specimens consisting of fibrin vividly document the degree of hyperinflammation caused by CAPS and likely precipitated by MAS.

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