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High Levels of DEK Autoantibodies in Sera of Patients With Polyarticular Juvenile Idiopathic Arthritis and With Early Disease Flares Following Cessation of Anti–Tumor Necrosis Factor Therapy

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Objective. The nuclear oncoprotein DEK is an autoantigen associated with juvenile idiopathic arthritis (JIA), especially the oligoarticular subtype. DEK is a secreted chemotactic factor. Abundant levels of DEK and DEK autoantibodies are found in inflamed synovium in JIA. We undertook this study to further characterize the nature of DEK autoantibodies in screening serum samples from 2 different cohorts that consisted mostly of patients with JIA.

Methods. DEK autoantibody levels were analyzed in sera from 33 JIA patients, 13 patients with other inflammatory conditions, and 11 healthy controls, as well as in 89 serum samples from JIA patients receiving anti–tumor necrosis factor (anti-TNF) therapy. Recombinant His-tagged full-length DEK protein (1–375 amino acids [aa]) and the 187–375-aa and 1–350-aa His-tagged DEK fragments made in a baculovirus system were used for enzyme-linked immunosorbent assay (ELISA) and immunoblotting. The C-terminal 25-aa fragment of DEK was expressed in a glutathione S-transferase–tagged vector. ELISA results were calculated as area under the curve by the trapezoidal rule.

Results. DEK autoantibody levels were significantly higher in patients with polyarticular JIA than in those with oligoarticular JIA, and were higher in patients with polyarticular JIA who had more active disease after cessation of anti-TNF therapy. Immunoblotting against the C-terminal 25-aa fragment of DEK confirmed that this section of the DEK molecule is the most immunogenic domain.

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Conclusion. **DEK autoantibody levels are higher in patients with polyarticular JIA than in those with oligoarticular JIA, and higher in patients who have disease flares after cessation of anti-TNF therapy. The C-terminal 25-aa fragment is the most immunogenic portion of DEK. These findings are significant with respect to the nature of DEK autoantibodies, their contribution to JIA pathogenesis, and their implications for JIA management.**

Juvenile idiopathic arthritis (JIA) is a chronic inflammatory condition that includes a group of heterogeneous autoimmune diseases affecting children under the age of 16 years. It is the most common rheumatic condition in children and may lead to short- or long-term disability. Subtypes of JIA include oligoarticular arthritis (involving ≤4 joints), polyarticular arthritis (involving ≥5 joints, rheumatoid factor [RF] positivity or negativity), systemic-onset arthritis, enthesitis-related arthritis, psoriatic arthritis, and undifferentiated arthritis (1,2). While the pathogenesis of JIA is unknown, discovery of DEK autoantibodies in serum of JIA patients as well as DEK protein and DEK autoantibodies in synovial fluid of JIA patients has sparked investigation of DEK's potential contribution to the pathogenesis of JIA (3–6).

DEK is a nuclear phosphoprotein that was initially characterized as part of the *dek-can* fusion oncogene resulting from a (6;9) translocation in a subset of patients with acute myelogenous leukemia (7,8). DEK is involved in various pathways, including transcriptional regulation, modulation of chromatin architecture, DNA replication, and messenger RNA processing (9–11). DEK can also be secreted and may play a role as an extracellular inflammatory cytokine (12,13).

Autoantibodies to DEK are detectable not only in the serum of patients with JIA, but also in serum of patients with granulomatous diseases (e.g., sarcoidosis, tuberculosis) and several autoimmune diseases, including systemic lupus erythematosus (SLE), scleroderma, and idiopathic uveitis. Thus, DEK autoantibodies are associated with clinical conditions characterized by abnormal immune activation (4,6,14). In view of the limited understanding of JIA, the mechanism by which DEK autoantibodies develop, their specificity, and their contribution to the pathogenesis of JIA are of great interest.

Because nonspecific autoantibodies are not generally characteristic of JIA, the presence of DEK autoantibodies in JIA is particularly intriguing. DEK and DEK autoantibodies contribute directly to joint inflammation via the generation of immune complexes, and acetylation of the DEK protein enhances its immunogenicity (3). In addition, DEK protein can be secreted by monocytes and released by apoptotic T cells acting as an extracellular chemoattractant, which suggests that DEK is a proinflammatory factor recruiting inflammatory cells to the synovium (12,13). We recently found that DEK also contributes directly to the formation of neutrophil extracellular traps (NETs) (15). NETs are chromatin structures that are released by activated neutrophils in response to inflammation in order to clear bacteria or fungal infection (16,17). An excess of NETs can contribute to chronic inflammatory conditions such as rheumatoid arthritis or SLE (18). Indeed, synovial neutrophils from JIA patients spontaneously generate NETs containing DEK that is recognized by DEK autoantibodies purified from the synovial fluid of JIA patients (15). DEK autoantibodies from the synovial fluid of JIA patients have been found to predominantly recognize the C-terminus of DEK (3).

Thus, we hypothesized that anti-DEK antibodies and DEK protein form immune complexes by recognition of the C-terminal portion of the DEK protein, further augmenting the inflammatory process in the joint. In this study we also show that anti-DEK antibodies are found at a particularly high level in patients with polyarticular JIA. These levels are higher than those in patients with oligoarticular JIA, in whom anti-DEK antibody levels were previously thought to be highest.

The treatment of JIA has recently been improved by biologic response modifiers such as anti–tumor necrosis factor (anti-TNF) therapy (19–21). However, anti-TNF therapy is associated with significant adverse events, including infections (22–24) and a possible increased risk of cancer (25). Its long-term effects on children are uncertain, and biologic agents are very expensive. Thus, it is important to ascertain when one can safely discontinue anti-TNF therapy in children with disease in clinical remission without significant risk of relapse. Having ascertained that patients with polyarticular JIA have high titers of anti-DEK antibodies, we measured anti-DEK autoantibody levels in serum samples from patients with polyarticular JIA who participated in a multicenter trial designed to enable better understanding of when anti-TNF therapy could be safely stopped. We found significantly higher levels of DEK autoantibodies upon flare after cessation of anti-TNF therapy. Further, we determined that the C-terminal 25–amino acid (25-aa) sequence is the most immunogenic portion of DEK in a significant percentage of JIA patient sera.

PATIENTS AND METHODS

Patients. Forty-six children (mean age 11.7 years, mean disease duration 6.2 years) were enrolled by the Pediatric Rheumatology clinic at the University of Michigan.

These patients had oligoarticular, polyarticular (RF-positive and RF-negative), systemic-onset, spondyloarthritis, and psoriatic subtypes. Control subjects without JIA included children with chronic pain, fatigue, low-titer antinuclear antibody (ANA) positivity, scleroderma, SLE, and juvenile dermatomyositis, but without joint involvement (no arthritis). Patient recruitment was performed under a protocol approved by the University of Michigan Institutional Review Board (IRB) (HUM00014692).

In a second patient cohort for a multicenter study aiming to identify biomarkers to indicate when it is safe to stop anti-TNF therapy, 137 patients with polyarticular JIA were enrolled (mean age 11.3 years, mean disease duration 5.0 years). Parent's/patient's consent (assent, if appropriate) was obtained and screening for eligibility was performed at the participating site at which the patient was recruited. Each patient's eligibility was validated via eligibility case report forms sent immediately to the Pediatric Rheumatology Collaborative Study Group Coordinating Center at Cincinnati Children's Hospital Medical Center (see Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40404/abstract). The Coordinating Center served as the clinical research organization for this study and oversaw all matters related to regulatory, financial, and clinical affairs, as well as data management, quality assurance, and analysis. The multicenter study was approved by the IRB at each participating site and was conducted in compliance with the Declaration of Helsinki Principles for Human Experimentation.

Study design. The first patient cohort included randomly enrolled patients treated at the Pediatric Rheumatology clinic at the University of Michigan. Most JIA patients were treated with methotrexate and nonsteroidal antiinflammatory drugs (NSAIDs), and several were also treated with TNF inhibitors and other biologic agents. Sera were collected at the time of enrollment.

In the second group, patients were enrolled at 16 pediatric rheumatology centers, and serum samples were collected from 137 children with polyarticular JIA who were receiving anti-TNF therapy, as approved by IRBs (HUM00033009). Therapy was stopped after a minimum of 6 months for patients with persistent clinically inactive disease. Clinically inactive disease was defined using the American College of Rheumatology (ACR) provisional criteria (26). Disease activity was then monitored prospectively for an additional 8 months or until disease flare. The primary outcome for this study was disease flare using a variation of the validated criteria, defined as 30% worsening in ≥3 of any of the 6 juvenile rheumatoid arthritis ACR core set variables (27), with no more than 1 improving by >30% (28). The 6 core set variables include the physician's global assessment of disease activity on a 10-cm visual analog scale (VAS), the parent's/patient's assessment of overall well-being on a 10-cm VAS, functional ability measured by the Childhood Health Assessment Questionnaire (C-HAQ) (29), the number of joints with active arthritis, the number of joints with limited range of motion, and an acute-phase reactant (the erythrocyte sedimentation rate [ESR]).

Because enrolled subjects began the second phase with clinically inactive disease, a 30% worsening could represent a less than clinically important change. Thus, for this study the patient was considered to have had a disease flare if their disease worsened by 30% and by at least the following amounts: increases of at least 2 units on a 21-numbered circle VAS (30)

for the physician's assessment and parent's/patient's assessment, increases of at least 2 joints with active arthritis and at least 2 joints with limited range of motion, a minimum increase of 0.125 on the C-HAQ score, and an increase in the ESR from normal to abnormal.

Laboratory assessment. Whole blood samples were obtained from patients seen in the Pediatric Rheumatology clinic at the University of Michigan under a protocol approved by the IRB. Serum in a standard vacutainer blood collection tube was prepared by allowing the blood sample to clot at room temperature for 15–30 minutes followed by centrifugation at 1,000–2,000g for 10 minutes. For patients in the anti-TNF study, an additional 8.5-cc P100 tube to collect plasma was used for anti-DEK antibody analysis of all subjects weighing >20 kg at visits 1, 2, and 3 and at disease flare/end of the study. Samples were processed as described above, stored, and shipped to the University of Michigan.

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with full-length (1–375-aa) or 1–350-aa recombinant DEK (50 μl of 125 ng/well) and incubated overnight at 21°C. Plates were blocked with phosphate buffered saline plus 0.25% bovine serum albumin and 0.05% Tween 20 for 30 minutes at room temperature, followed by 3 washes with double-distilled water, 10 minutes of blocking, and 3 additional washes. Serum samples from JIA patients and controls (dilutions of 1:200, 1:400, 1:800, 1:1,600, and 1:3,200 in blocking buffer) were added to the plates for 2 hours at room temperature, followed by 3 washes, 10 minutes of blocking, and 3 additional washes. Biotinylated goat anti-human secondary antibody (Jackson ImmunoResearch) at a concentration of 1:200,000 in dilution buffer (50 μl/well) was added for 2 hours of incubation at room temperature, followed by 3 washes and 10 minutes of blocking and washing. Streptavidin (1:300 in dilution buffer; 50 μl/well) was added for 1 hour of incubation at room temperature, followed by 5 washes. We added 3,3′,5,5′-tetramethylbenzidine substrate (50 μl/well) to develop the plate for 5–15 minutes prior to stopping the reaction with 1N H_2SO_4 (50 μl/well). The optical density (OD) at 450 nm was read within 20 minutes.

Statistical analysis of ELISA results. Anti-DEK antibody levels in sera from JIA patients, measured at 5 different dilutions (1:200, 1:400, 1:800, 1:1,600, and 1:3,200), were compared to those in sera from non-JIA patients and healthy controls and expressed as fold change over those in healthy controls in each individual experiment. Fold changes were calculated by OD. Results were also plotted as the area under the dilution curve (AUDC) of each sample, calculated using the trapezoidal rule. Analysis of variance models were used to compare AUDC between groups of patients. Statistical significance was defined as a 2-sided P value of less than 0.05. Receiver operating characteristic (ROC) curve analysis was used to assess the ability of the AUDC to discriminate among groups. Area under the ROC curve (AUC) was calculated, as was its 95% confidence interval (95% CI). The marker showed good discrimination ability if the interval's lower limit was >0.5.

To analyze samples from patients participating in the TNF inhibition study at different time points, we took additional steps to normalize across the different assays using anti-DEK monoclonal antibody (BD Biosciences) as a reference. The anti-DEK AUC values in JIA patients were standardized against those in healthy controls. We analyzed the difference in the AUDC values. Student's t-test was used to compare patients

who had disease flares and those who did not by the 8-month follow-up visit after discontinuation of anti-TNF therapy.

Cloning and expression of the C-terminal 25-aa domain of DEK. Expression and purification of His-tagged full-length DEK and of the 187–375-aa and 1–350-aa His-tagged DEK fragments was performed as described previously (31,32). The C-terminal 25-aa portion of the human DEK protein was amplified from the His-tagged full-length DEK vector via polymerase chain reaction (PCR) using primers containing 5′ *Eco* RI and 3′ *Xho* I restriction sites. The PCR product was identified and eluted from an agarose gel prior to digestion with *Eco* RI and *Xho* I. The pGEX-4T1 vector containing glutathione S-transferase (GST) was also digested with *Eco* RI and *Xho* I, and the 25-aa DEK fragment was ligated into the pGEX vector. DNA from clones containing the correct sequence was used to express the protein in the GST-tagged vector.

Immunoblotting. DEK-specific polyclonal antibodies were purified as described (32). Protein aliquots (3.5 μg) were separated by 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was probed with a 1:400 dilution of patient sera or a 1:1,000 dilution of rabbit anti-DEK antibody followed by a horseradish peroxidase–conjugated secondary goat anti-human antibody or goat anti-rabbit antibody and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

RESULTS

Association of high levels of DEK autoantibodies with JIA. Previous studies had shown elevated titers of anti-DEK antibodies in JIA patients, especially those with oligoarticular disease (4,14). We collected 57 serum samples from 11 healthy control individuals (the basis

**Table 1.** Demographic information on the pediatric patients recruited into both studies*

	University of Michigan cohort		TNF study†	
	JIA patients (n = 33)	Non-JIA patients (n = 13)	Patients with flares (n = 39)	Patients without flares (n = 67)
Age, mean ± SD years	11.7 ± 3.7	14.0 ± 4.0	11.13 ± 5.05	11.18 ± 4.22
Male:female ratio	9:24	5:8	10:29	21:46
Duration of disease, mean ± SD years	6.2 ± 4.2	2.6 ± 1.7	6.37 ± 4.55	4.48 ± 3.14
Diagnosis				
Oligoarticular JIA	8	–	–	–
Extended oligoarticular JIA	1	–	8	9
RF-positive polyarticular JIA (RA)	2	–	2	7
RF-negative polyarticular JIA	12	–	36	59
Systemic-onset JIA	3	–	–	–
Psoriatic arthritis	3	–	–	–
Spondyloarthritis	1	–	–	–
Undifferentiated arthritis	3	–	–	–
UCTD	–	1	–	–
SLE	–	2	–	–
Juvenile DM	–	2	–	–
MCTD	–	2	–	–
Localized scleroderma	–	2	–	–
Other (Kawasaki disease and pericarditis, all ANA positive)	–	3	–	–
Uveitis	4 (12)	1 (8)	–	–
ANA positive	13 (39)	8 (62)	22 (20)	31 (29)
Active arthritis	16 (48)	1 (8)	–	–
Current medications				
Methotrexate	17 (52)	4 (31)	13 (12)	28 (26)
Glucocorticoids	2 (6)	4 (31)	–	–
Hydroxychloroquine	9 (27)	5 (36)	–	–
NSAIDs	24 (73)	2 (15)	–	–
Mycophenolate mofetil	1 (3)	4 (31)	–	–
Leflunomide	3 (9)	0 (0)	–	–
Sulfasalazine	6 (18)	0 (0)	–	–
TNF inhibitors	5 (15)	1 (8)	39 (37)	67 (63)
Infliximab	3	1	3	2
Adalimumab	0	0	4	10
Etanercept	2	0	32	55
Other biologic agents (anakinra)	2 (6)	0 (0)	–	–
Cyclosporine	2 (6)	1 (8)	–	–
IVIG	1 (3)	1 (8)	–	–

* Except where indicated otherwise, values are the number or number (%). JIA = juvenile idiopathic arthritis; TNF = tumor necrosis factor; RF = rheumatoid factor; RA = rheumatoid arthritis; UCTD = undifferentiated connective tissue disease; SLE = systemic lupus erythematosus; DM = dermatomyositis; MCTD = mixed connective tissue disease; ANA = antinuclear antibody; NSAIDs = nonsteroidal antiinflammatory drugs; IVIG = intravenous immunoglobulin.
† Percentages were calculated based on the total number of patients (those with and those without disease flares).

for statistical calculations), 13 non-JIA control patients, and 33 JIA patients who were enrolled in our study at the Pediatric Rheumatology Clinic at the University of Michigan. The mean ± SD age of the JIA patients was 11.7 ± 3.7 years, and their mean ± SD disease duration was 6.2 ± 4.2 years. The majority of JIA patients were female (24 female, 9 male) (Table 1), and among all 12 healthy controls recruited for the study, 5 were female

and 7 were male (see Supplementary Table 2, http://onlinelibrary.wiley.com/doi/10.1002/art.40404/abstract). The distribution of JIA subtypes included 24% with oligoarticular arthritis, 36% with RF-negative polyarticular arthritis, 9% with psoriatic arthritis, 9% with systemic-onset JIA, 6% with RF-positive polyarticular arthritis, and 3% with spondyloarthritis. ANA positivity was noted in 39% of patients. Twelve percent of patients had uveitis.
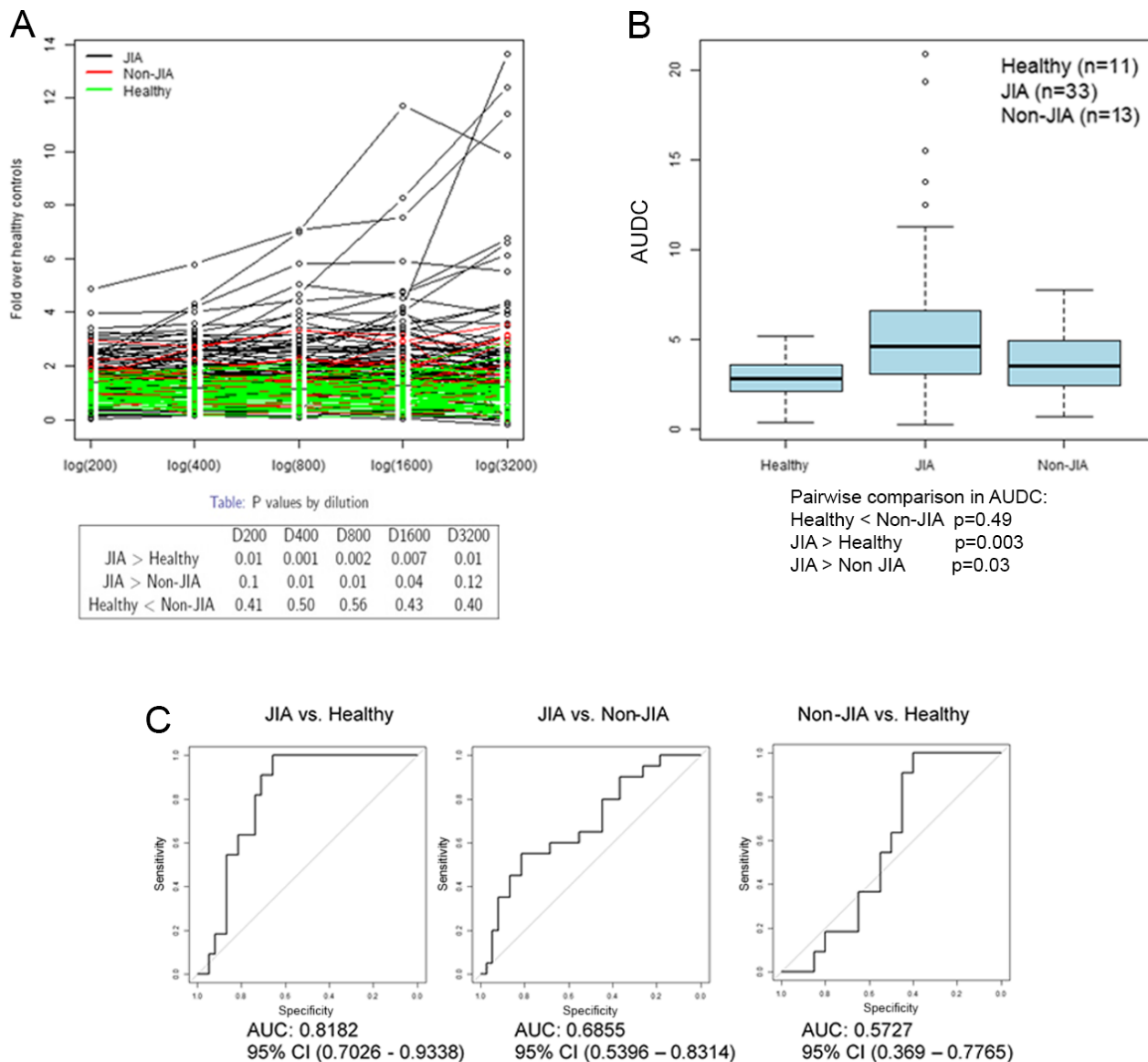

Figure 1. High levels of DEK autoantibodies in patients with juvenile idiopathic arthritis (JIA). A, Serum samples from patients with JIA (n = 33), patients with other rheumatic diseases (n = 13), or healthy controls (n = 11) were serially diluted as indicated and tested for anti-DEK antibody levels by enzyme-linked immunosorbent assay (ELISA). Lines represent individual samples. Samples were compared to those from 5 normal healthy controls. Results shown are the average of 2–8 independent ELISAs. B, Shown are pairwise comparisons, based on area under the dilution curve (AUDC), among healthy subjects, JIA patients, and non-JIA patients. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Lines outside the boxes represent the 10th and 90th percentiles. Circles indicate outliers. C, The area under the receiver operating characteristic curve (AUC) was calculated along with its 95% confidence interval (95% CI) to assess the performance of the AUDC measurements in distinguishing between different groups of patients.

With regard to medications used by patients at the time of study enrollment and blood sampling, 52% of patients were being treated with methotrexate, 27% with hydroxychloroquine, 73% with NSAIDs, 6% with glucocorticoids, 9% with leflunomide, 18% with sulfasalazine, 15% with TNF inhibitors, and 6% with anakinra (primarily for systemic-onset JIA). Of these JIA patients, 53% had active disease at the time of blood collection.

The mean ± SD age of the non-JIA patients with rheumatic disease at the time of serum collection was 14.0 ± 4.0 years, and their mean ± SD disease duration was 2.6 ± 1.7 years. There was a fairly equal distribution of other autoimmune diseases in this group, including relatively equal numbers of patients with SLE, juvenile dermatomyositis, mixed connective tissue disease, and localized scleroderma. One patient with idiopathic uveitis who was receiving steroid-sparing agents was also in this group. The healthy control group was made up of healthy college student volunteers with a mean ± SD age of 20 ± 5 years.

Sera were analyzed by ELISA for anti-DEK antibody levels using recombinant DEK protein. Serum samples were serially diluted and were tested for anti-DEK antibody levels as described in Patients and Methods. Figure 1A depicts the average results of 2–8 independent ELISAs. Samples were compared to those from 5 normal healthy controls. As shown in Figure 1A and Supplementary Figure 1 (http://onlinelibrary.wiley.com/doi/10.1002/art.40404/abstract), JIA patients had significantly higher levels of anti-DEK antibodies than did non-JIA patients and healthy controls. Although there appeared to be slightly higher levels of anti-DEK antibodies detected by ELISA among non-JIA patients than among healthy controls, the difference was not statistically significant. Figure 1B demonstrates ELISA antibody titers as pairwise comparisons based on the AUDC values among healthy
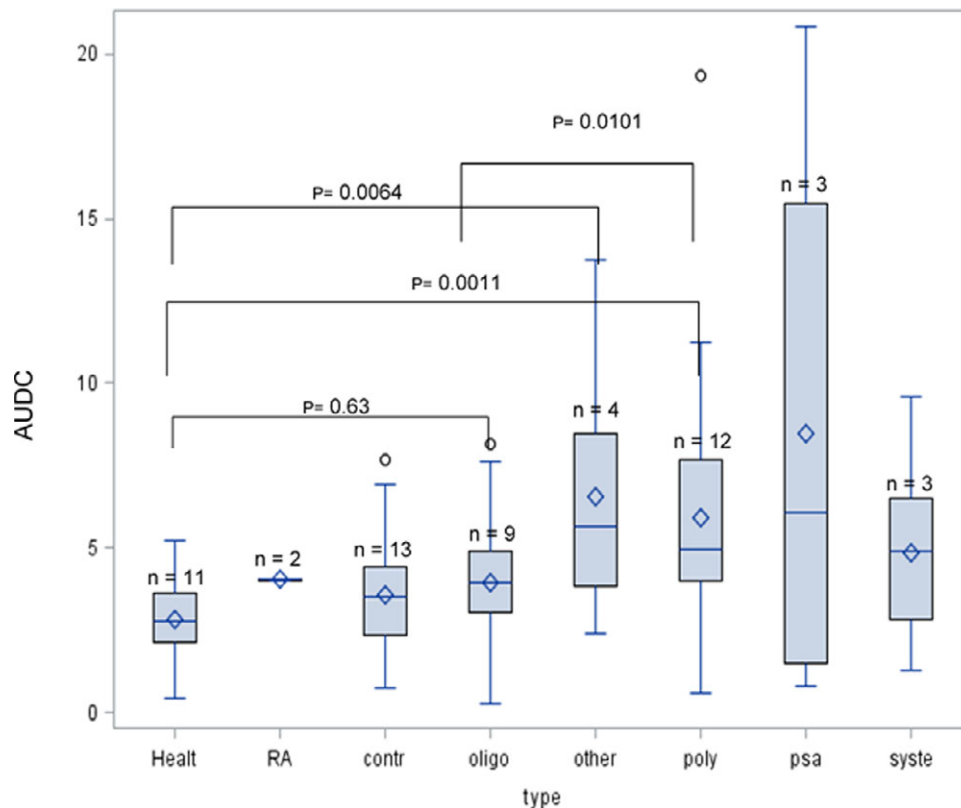
Figure 2. DEK autoantibody levels are significantly higher in patients with polyarticular arthritis (poly) than in those with oligoarticular arthritis (oligo) or in healthy controls (Healt). DEK autoantibody levels in the sera of patients with different juvenile idiopathic arthritis (JIA) subtypes, as analyzed by anti-DEK antibody enzyme-linked immunosorbent assay, were compared to those in healthy controls. All levels of antibodies to DEK were compared to those in sera from healthy individuals based on area under the dilution curve (AUDC). Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Diamonds represent the mean. Lines outside the boxes represent the 10th and 90th percentiles. Circles indicate outliers. RA = patients with rheumatoid arthritis; contr = patients seen in the rheumatology clinic who did not in fact have arthritis; other = patients with no definitive JIA subtype at the time of diagnosis; PsA = patients with psoriatic arthritis; syste = patients with systemic-onset arthritis. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40404/abstract.

subjects, JIA patients, and non-JIA patients. JIA patients had a significantly higher AUDC than did either non-JIA patients ($P = 0.03$) or healthy controls ($P = 0.003$). To test the specificity of our assay, we used the ROC test as shown in Figure 1C. DEK autoantibody levels showed good discrimination among JIA patients, non-JIA patients, and healthy subjects, confirming the presence of increased levels of anti-DEK antibodies in patients with JIA.

Significantly higher DEK autoantibody levels in patients with polyarticular arthritis than in patients with oligoarticular arthritis or in healthy individuals. Previous reports have suggested that anti-DEK antibodies are especially found in JIA patients with the oligoarticular form of the disease. Therefore, DEK autoantibody levels in sera from patients with different JIA subtypes were compared to levels in sera from healthy controls and analyzed by anti-DEK antibody ELISA (Figure 2). All anti-DEK antibody levels were compared to levels in sera from healthy individuals and were assessed as AUDC. As shown in Figure 2, there was a statistically significant difference in

AUDC between healthy controls and patients with polyarticular JIA ($P = 0.0011$) and between healthy controls and patients with other forms of JIA ($P = 0.0064$). No significant differences were found between patients with oligoarticular JIA and healthy individuals ($P = 0.63$), but a significant difference was noted between patients with oligoarticular JIA and those with polyarticular JIA ($P = 0.0101$), with the latter group being statistically more likely to have higher levels of DEK autoantibodies. High levels of DEK autoantibodies were also detected in 2 patients with spondyloarthritis and 2 patients with arthritis as a manifestation of their undifferentiated connective tissue disease (categorized as "other").

DEK autoantibody levels in patients receiving anti-TNF therapy. Anti-TNF therapy has proven to be a very valuable modality in the treatment of JIA (19–21). However, it is expensive, and its immunosuppressive effects can lead to opportunistic infections, skin disorders, colitis, and malignancies (33,34). Further, the long-term effects of anti-TNF therapy on children remain unclear (35). Therefore,
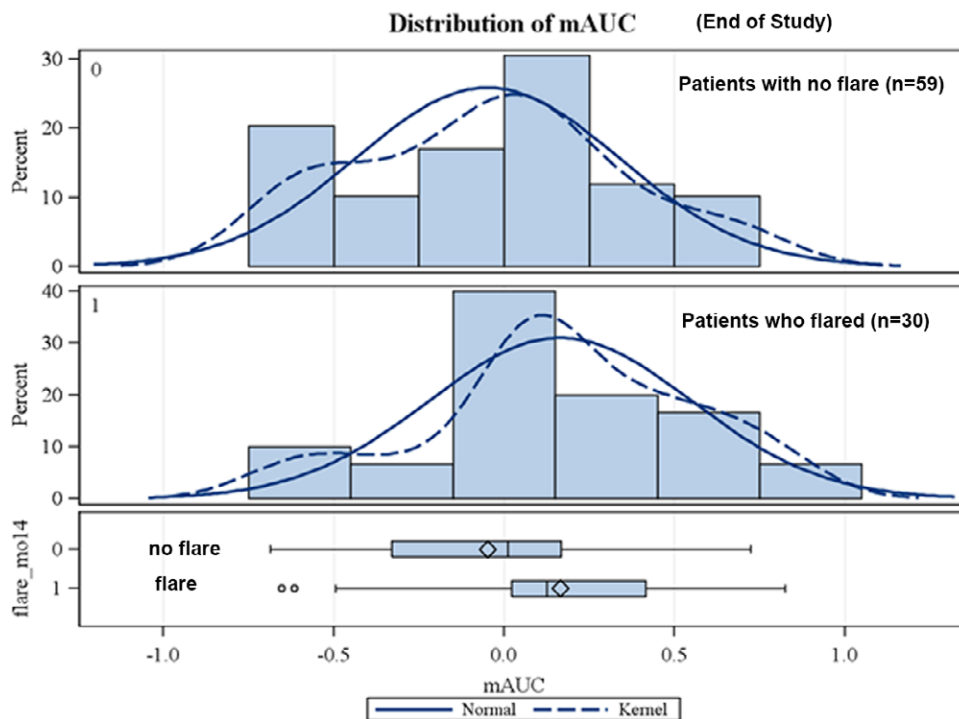

Figure 3. Increase in anti-DEK antibodies is evident in patients with juvenile idiopathic arthritis who have disease flares within 8 months of stopping anti–tumor necrosis factor therapy. Top and middle, Shown are data on sera from 89 of 106 patients who were analyzed for anti-DEK antibody levels. High levels of anti-DEK antibodies (mean ± SD difference in area under the dilution curve [AUDC] values [mAUC] 0.164 ± 0.39 [95% confidence interval {95% CI} 0.02, 0.31]) were detected in the 30 patients who had disease flares within 8 months (middle), while lower levels of anti-DEK antibodies (mean ± SD difference in AUDC values –0.05 ± 0.39 [95% CI –0.15, 0.05]) were detected in the 59 patients who had no disease flares for at least 8 months and up to 14 months (flare_mo14) (top) ($P = 0.016$ by Student's t-test). The difference between the 2 populations is demonstrated by a normal distribution, and the same trend is shown by a kernel density distribution to demonstrate the distribution of the data based on the actual spread/density of the results. Bottom, Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the median. Diamonds represent the mean. Lines outside the boxes represent the 10th and 90th percentiles. Circles indicate outliers. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40404/abstract.

strategies are much needed for deciding when to stop treatment after it is initiated. Accordingly, we measured DEK autoantibodies in a larger cohort of JIA patients who received anti-TNF therapy (Table 1; also see Supplementary Table 1, http://onlinelibrary.wiley.com/doi/10.1002/art.40404/abstract). We enrolled 103 female patients and 34 male patients with polyarticular JIA (mean age 11.3 years, mean disease duration 5.0 years). Seventy-seven percent were receiving etanercept, 18% were receiving adalimumab, 5% were receiving infliximab, and 40% were receiving concomitant methotrexate. Thirty-one patients discontinued the study for various reasons, including reactivation of disease during therapy.

Within 8 months of stopping therapy, 39 patients had disease flares but 67 patients did not. Anti-DEK antibody levels at the time of stopping anti-TNF therapy did not differ significantly between these 2 groups of patients (see Supplementary Figure 2, http://onlinelibrary.wiley.com/doi/10.1002/art.40404/abstract). However, at the end of the study, either after 8 months of no disease flare without therapy or at the time of disease flare without therapy, high levels of anti-DEK antibodies (mean $\pm$ SD difference in AUDC values 0.164 ± 0.39 [95% CI 0.02, 0.31]) were detected in 30 of the patients who had disease flares, while lower levels of anti-DEK antibodies (mean $\pm$ SD difference in AUDC values -0.05 ± 0.39 [95% CI -0.15, 0.05]) were measured in 59 of the patients with no disease flares ($P = 0.016$ by Student's t-test) (Figure 3). Thus, retrospectively, patients who experienced disease flares within 8 months of stopping anti-TNF therapy had significantly higher levels of anti-DEK antibodies than did patients with clinically inactive disease until the end of the study.

Requirement of the C-terminal portion of the DEK protein for autoantibody recognition. Previous studies with a very limited number of sera and synovial fluid samples suggested that anti-DEK antibodies target the last 25
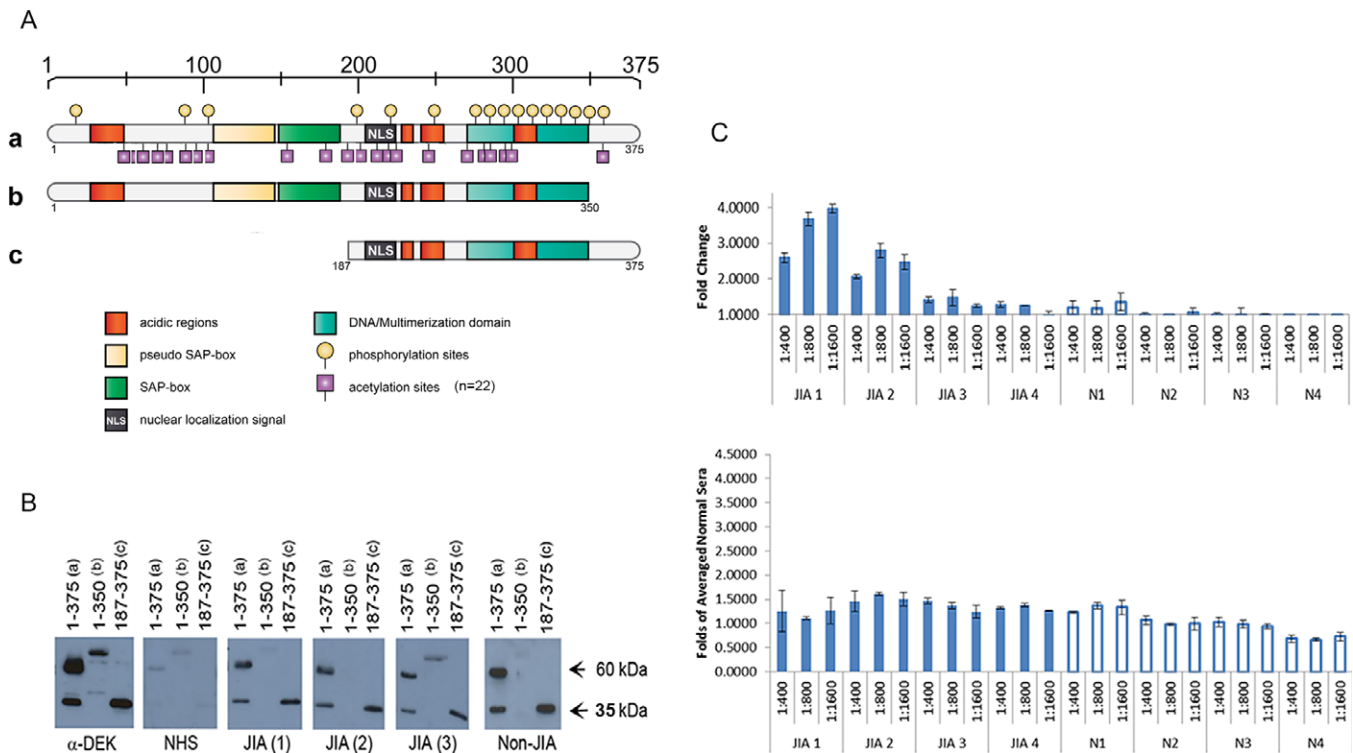

Figure 4. Antibodies in sera from patients with juvenile idiopathic arthritis (JIA) recognize the C-terminal domain of the DEK protein. **A,** Illustration of the different fragments and domains of recombinant histidine-tagged DEK with posttranslational modifications previously identified in primary human macrophages and HeLa cells (3). **B,** Results of Western blot analysis of DEK fragments (1 μg protein per lane) expressed in Sf9 cells. DEK protein was probed with serum samples from a normal healthy control subject (NHS), from 3 JIA patients, or from a rheumatology patient without JIA, or with DEK-specific antibody as a positive control. **C,** Levels of anti-DEK antibodies. Sera from 4 JIA patients and either sera or plasma from 4 healthy controls (N1–N4) were serially diluted and tested for antibodies to full-length (1–375–amino-acid) recombinant DEK (top) or the 1–350–amino-acid DEK fragment (bottom) by enzyme-linked immunosorbent assay (ELISA). Values are the mean $\pm$ SD fold change compared to another set of sera from 4 different healthy controls. $P = 0.0337$ by Student's t-test for recognition of full-length DEK by sera from JIA patients versus sera from healthy controls (top). $P = 0.009$ by Student's t-test for recognition of full-length DEK by sera from JIA patients (top) versus recognition of the 1–350–amino-acid DEK fragment by sera from JIA patients (bottom). Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40404/abstract.

amino acids of the molecule (3). To address this question further, full-length, 1–350-aa, and 187–375-aa fragments of recombinant His-tagged DEK were first expressed in Sf9 insect cells. The overlapping fragments were designed to investigate the immunogenic importance of the C-terminal portion of DEK, specifically the C-terminal 25 amino acids, as shown in Figure 4A. DEK protein fragments were then purified and analyzed by immunoblotting (Figure 4B). DEK protein was probed with serum samples from a normal healthy control subject, from 3 JIA patients, or from a rheumatology patient without JIA, or with DEK-specific antibody as a positive control.

As shown in Figure 4B, the DEK-specific antibody strongly detected all DEK fragments. The full-length DEK protein was detected as expected primarily at 55 kd; a previously identified breakdown form was also detected at 35 kd (12). (The 1–350-aa DEK runs slightly higher than the full-length DEK, perhaps due to changes in its 3-dimensional structure.) Serum from the normal healthy

control did not notably detect DEK or its fragments. Sera from all 3 JIA patients readily detected full-length DEK protein as well as the 187–375-aa fragment, which contains the C-terminal 25 amino acids, consistent with our previous findings using autoantibodies found in synovial fluid samples from patients (3). The non-JIA patient sera detected the full-length and 187–375-aa DEK fragments, consistent with the observation that patients with other autoimmune diseases can also have antibodies to DEK. In contrast, the 1–350-aa DEK fragment lacking only the last 25 amino acids could not be recognized by most patient sera (3 of 4 serum samples did not recognize the truncated fragment). These results are consistent with our previous findings that 5 of 8 JIA patients' synovial fluid antibodies that recognized DEK (>50%) failed to recognize DEK when the C-terminal 25 amino acids were deleted as in the 1–350-aa DEK mutant (3).

ELISAs were performed to determine further if autoantibodies in patient sera indeed primarily recognize
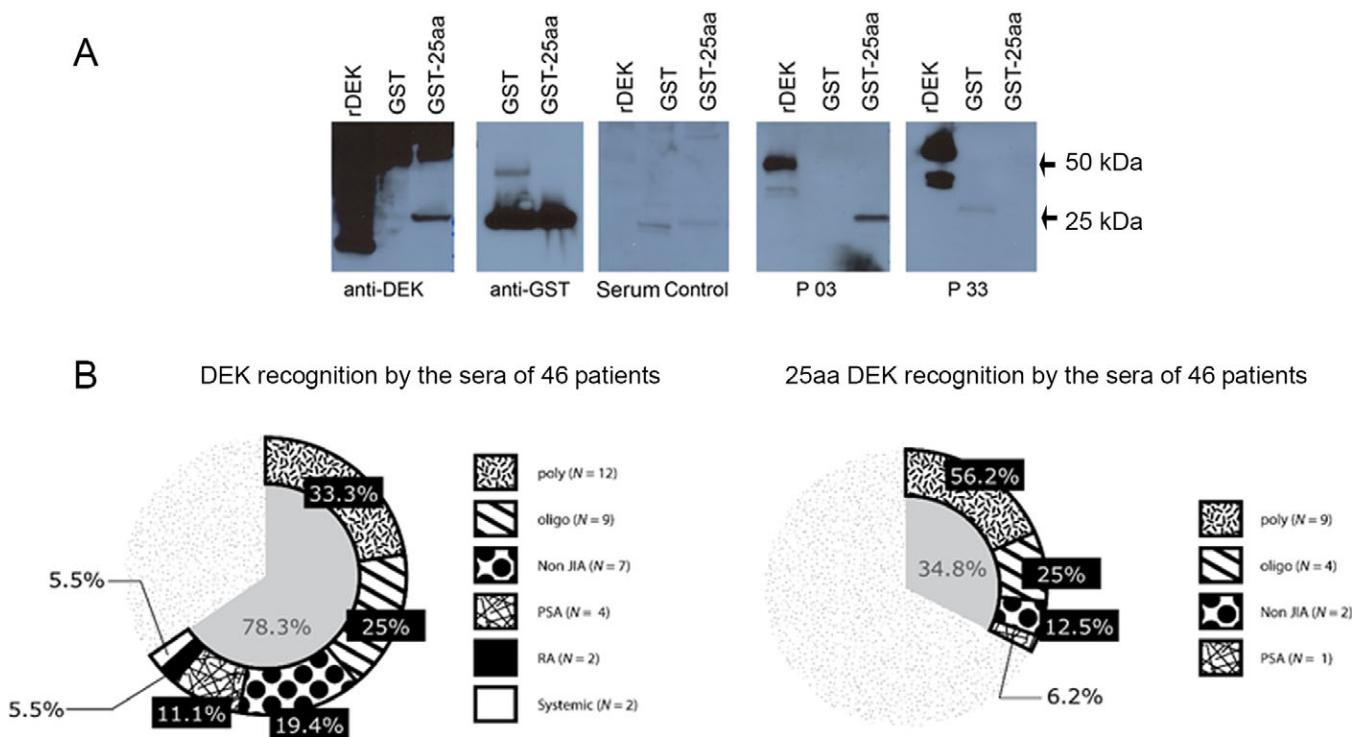
Figure 5. The last 25 amino acids (aa) of DEK are sufficient for recognition by the autoantibodies of a substantial percentage of patients with juvenile idiopathic arthritis (JIA) who have autoantibodies to full-length DEK. **A,** Recombinant full-length DEK (rDEK), glutathione S-transferase (GST) control protein, and the C-terminal GST-tagged 25 amino acids of DEK (GST-25aa) were purified and analyzed by Western blotting and densitometry. Results from 2 different representative JIA patient sera (P03 and P33) are shown in addition to a representative control serum from a healthy individual. **B,** Pie chart demonstrates the percentage of patients who had autoantibodies to full-length DEK as detected in sera from patients with polyarticular JIA (poly), oligoarticular JIA (oligo), psoriatic arthritis (PsA), rheumatoid arthritis (RA), systemic-onset JIA, and non-JIA rheumatic diseases (control) (left). From the 46 patients screened, we also calculated the percentage of patients whose sera recognized the last 25 amino acids of DEK alone, also divided into the different JIA subtypes and non-JIA rheumatic diseases (right). Note that overall, approximately half of the patients' sera that recognized full-length DEK also recognized the isolated C-terminal 25 amino acids of the protein. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40404/abstract.

the last 25 amino acids of DEK. Sera from 4 patients with JIA and either sera or plasma from 4 healthy controls were serially diluted and tested for antibodies to full-length (1–375-aa) recombinant DEK or to DEK containing amino acids 1–350 (the entire DEK protein except for the C-terminal 25 amino acids). DEK was not recognized by sera from healthy controls, while sera from JIA patients were able to recognize wild-type DEK (Figure 4C). Although levels of DEK recognition varied among JIA patients, all levels of DEK recognition among JIA patients were significantly greater than among healthy controls. This difference disappeared, however, when ELISA was performed using the 1–350-aa DEK fragment, which was not recognized by sera from JIA patients or controls (Figure 4C).

Antibodies in sera from JIA patients recognize the last 25 amino acids of the DEK protein. To determine if DEK's autoantigenicity truly resides in its terminal 25 amino acids, we produced the last 25 amino acids of DEK with a GST tag. Recombinant full-length DEK, GST control protein, and the C-terminal GST-tagged 25 amino acids of DEK were purified and analyzed using a DEK-specific polyclonal antibody, an antibody to GST, serum from a representative healthy control, and sera from 3 different JIA patients (Figure 5A).

The serum from the healthy control did not detect DEK protein, while sera from all of the JIA patients detected full-length DEK. Sera from 2 representative JIA patients also detected the GST-tagged 25-aa fragment, indicating recognition of the C-terminal portion of the DEK protein but not of the GST control protein. We next screened all 46 patient samples for recognition of full-length DEK and the GST-tagged 25-aa DEK fragment (Figure 5B). Approximately four-fifths (78.3%) of the sera from JIA patients recognized full-length DEK, but only half of those sera (one-third of the sera from the total patient cohort) recognized the C-terminal portion of DEK. Stated another way, of the patients with antibodies to full-length DEK, 50% had antibodies that recognized the C-terminal 25-aa fragment alone. Most of the patients with autoantibodies recognizing the C-terminus of DEK had the polyarticular subtype. Therefore, in some but not all cases, the C-terminal 25-aa DEK fragment can be sufficient to generate DEK autoantibodies. Taken together, these findings demonstrate that the C-terminal 25 amino acids are usually necessary, but only sometimes sufficient, to generate autoantibodies to DEK.

DISCUSSION

DEK, originally identified as a nuclear protein, is a key factor in the modulation of global chromatin structure (10). In addition to that significant function, the DEK protein plays a role in immunity and is also recognized as an autoantigen in JIA and other autoimmune diseases. Our research group found that DEK binds to a specific sequence in the Y box of the HLA–DQA1 promoter (36), an allele that predisposes children in northern European populations to the development of oligoarticular-onset JIA (37,38). We also previously demonstrated that DEK is actively secreted by human macrophages and passively released by apoptotic T cells, attracting leukocytes into the inflamed area (12,13). DEK and DEK autoantibodies are abundant in synovial fluid of patients with JIA, leading to the development of immune complexes in the affected joints (3). Autoantibodies to DEK also show increased affinity for acetylated and poly(ADP-ribosyl)ated DEK (13). We have also recently shown that DEK is not only secreted by activated macrophages but is also released by activated neutrophils, and it was found to be an important component of NETs. Indeed, DEK-knockout mice develop much less joint inflammation after zymosan injection due to decreased formation of NETs, and inflammation in the joints can be reduced by neutralizing DEK with specific anti-DEK aptamers (15). Thus, DEK and DEK autoantibodies appear to contribute to joint inflammation by attracting inflammatory cells, generating immune complexes, and supporting NET formation.

The DEK protein was initially described as an autoantigen in 1991 by Szer et al (4,5). With the exception of ANAs, autoantibodies are commonly absent in children with JIA (14). The discovery of antibodies to the DEK nuclear antigen in JIA therefore holds promise for improving our understanding of the pathogenesis and management of JIA. Reactivity to anti-DEK antibodies was found to be most strongly associated with onset of any JIA subtype before the sixth birthday, particularly early-onset oligoarticular JIA and iridocyclitis. However, DEK autoantibody levels were previously not found to have a correlation with disease severity (14). Using recombinant full-length DEK, we have screened multiple sera from JIA patients from 2 different cohorts, 46 patients from the Pediatric Rheumatology clinic at the University of Michigan and 89 patients with polyarticular JIA receiving anti-TNF therapy from a study coordinated by the Cincinnati Children's Hospital Medical Center. In both patient cohorts, high levels of DEK autoantibodies were significantly correlated with polyarticular arthritis (surprisingly, greater than in oligoarticular arthritis) (Figure 2), and higher levels of DEK autoantibodies were found to be correlated with disease flare within the 8 months after cessation of anti-TNF therapy ($P = 0.016$) (Figure 3). These findings show that DEK autoantibody levels correlate with disease activity and might contribute

to disease pathogenesis. However, while at this point we have shown that anti-DEK antibody levels correlate with active disease, they cannot yet be used to predict whether it is safe to discontinue anti-TNF therapy.

We have now also demonstrated in a large group of patients that the C-terminal 25-aa sequence of DEK is a major autoantigenic region and is sometimes sufficient to generate an autoimmune response. These findings suggest that more refined ELISAs using the C-terminal 25-aa sequence of DEK might prove to be of future use. Taken together, it appears that by understanding the action of DEK and anti-DEK antibodies, improvements can be made in the management of JIA.

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. Drs. Mor-Vaknin and Markovitz had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
Study conception and design. Mor-Vaknin, Rivas, Johnson, Huang, Zhao, Spalding, Jung, Mehta, Giannini, Adams, Lovell, Markovitz.
Acquisition of data. Mor-Vaknin, Rivas, Legendre, Yuanfan, Mau, Johnson, Zhao, Kimura, Spalding, Morris, Gottlieb, Onel, Olson, Edelheit, Shishov, Jung, Cassidy, Prahalad, Passo, Beukelman, Mehta, Giannini, Adams, Lovell, Markovitz.
Analysis and interpretation of data. Mor-Vaknin, Rivas, Legendre, Mohan, Mau, Huang, Zhao, Mehta, Adams, Lovell, Markovitz.

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