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Article type : Full Length

High levels of DEK autoantibodies in sera of polyarticular JIA patients and in early flare following cessation of anti-TNF therapy

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This work was supported by grants to D.M.M. from the National Institutes of Health (NIH), R01 AI087128, R01 AI062248, and R01 DK109188; the Burroughs Wellcome Fund Clinical Scientist Award in Translational Research; and a grant from the Rheumatology Research Foundation. R03 AR056748-01 and K01 AR055620 from the NIH supported N.M.V. L.D.J and H.B. were supported by R01 AI087128.

ABSTRACT

Objective. The nuclear oncoprotein DEK is an autoantigen associated with juvenile idiopathic arthritis (JIA), especially the oligoarticular subtype. DEK is a secreted chemoatactic factor. Abundant DEK and DEK autoantibodies are found in the inflamed JIA synovium. In screening sera samples from two different JIA patient cohorts for DEK autoantibodies, we now further characterize the nature of DEK autoantibodies.

Methods. DEK autoantibody levels were analyzed in sera from 33 JIA patients, 13 patients with other inflammatory conditions, and 11 healthy controls and 89 sera samples from JIA patients undergoing anti-TNF therapy. Recombinant His-tagged DEK proteins (1-375 amino acid (aa), 187-375 aa, and 1-350 aa) made in a baculovirus system were used for ELISA and immunoblotting. The last 25 aa of DEK was expressed in a GST-tagged vector. ELISA results were calculated as area under the curve (AUC) by the Trapezoidal Rule.

Results. DEK autoantibody levels were significantly higher in polyarticular JIA patients compared to oligoarticular JIA patients, and were higher in polyarticular JIA patients with more active disease after cessation of anti-TNF therapy. Immunoblot against DEK's C-terminal 25 aa confirmed that the last 25 amino acids of the DEK molecule is the most immunogenic domain.

Conclusions. DEK autoantibody levels are higher in patients with polyarticular, rather than oligoarticular, JIA and in patients who flare after cessation of anti-TNF therapy. The C-terminal 25 aa is the most immunogenic portion of DEK. These findings are of significance with respect to the nature of DEK autoantibodies and their contribution to the pathogenesis and management of JIA.

Juvenile idiopathic arthritis (JIA) is a chronic inflammatory condition that includes a group of heterogeneous autoimmune diseases affecting children under the age of 16. It is the most common rheumatologic condition in children, and may lead to short-or long-term disability. Subtypes of JIA include oligoarticular arthritis (4 or fewer joints), polyarticular arthritis (5 or more joints, positive or negative rheumatoid factor), 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 the 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 mRNA 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 in the serum of patients not only with JIA, but also in 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 directly contribute 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, suggesting 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 (RA) 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 synovial fluids of JIA patients have been found to predominantly recognize the C-terminus of DEK (3). Thus, we hypothesize 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. Here we also show that anti-DEK antibodies are found at a particularly high level in patients with polyarticular JIA. These levels are higher than in 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-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 biological agents are very expensive. Thus, it is important to ascertain when one can safely discontinue anti-TNF therapy in children who are in clinical remission without significant risk of relapse. Having ascertained that pateints with polyarticular JIA have high titers of anti-DEK antibody, we measured DEK autoantibody levels in serum samples from polyarticular JIA patients who participated in a multi-center trial designed to better understand when anti-TNF therapy treatment 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 aa sequence is the most immunogenic portion of DEK in a significant percentage of JIA patients' sera.

PATIENTS AND METHODS

Patients

Forty six children with JIA, mean age of 11.7 and disease duration of 6.2 years, were enrolled by the Pediatric Rheumatology clinic at the University of Michigan, including patients with oligoarticular, polyarticular (RF positive and negative), systemic-onset, spondyloarthropathy, and psoriatic subtypes. Non-JIA control subjects include children with chronic pain, fatigue, low titer positive anti-nuclear antibody test or 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 (HUM00014692).

In a second patient cohort for a multi-center study aiming to identify biomarkers to indicate when it is safe to stop anti-TNF therapy, 137 patients with polyarticular JIA were enrolled, with a mean age of 11.3 years and disease duration of 5.0 years. Parent/patient consent (assent, if appropriate) and screening for eligibility were done at the participating site at which the patient was recruited. Each patient's eligibility was validated via eligibility case report forms immediately sent to the Pediatric Rheumatology Collaborative Study Group (PRCSG) Coordinating Center (CC) at Cincinnati Children's Hospital Medical Center (Supplementary Material Table 2). The CC 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 multi-center study was approved by the IRB at each participating site and was conducted to be in compliance with the Helsinki Declaration: http://www.wma.net/en/20activities/10ethics/10helsinki/index.html).

Study design

The first patient cohort includes randomly enrolled patients treated at the Pediatric Rheumatology clinic at the University of Michigan. Most JIA patients were treated with

Methotraexate and NSAIDs, and several were also treated with TNF inhibitors and other biologics. Sera were collected at the time of enrollment.

In the second group, patients were enrolled at 16 pediatric rheumatology centers, and sera samples were collected from 137 children with polyarticular JIA on anti-TNF therapy as approved by Institutional Review Boards (HUM00033009). Therapy was stopped after a minimum of 6 months for patients with persistent clinically inactive disease (CID). CID was defined using the 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 or more of any of the 6 JIA 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 (PhGA), parent/patient assessment of overall well-being (PaGA), functional ability measured by the Childhood Health Assessment Questionnaire (CHAQ), the number of joints with active arthritis, the number of joints with limitation of motion, and an acute phase reactant (ESR). Because enrolled subjects began the second phase with CID, a 30% worsening could represent a less than clinically important change. Thus, for this study the patient was considered to have flared if they worsened by 30%, and by at least the following amounts: PhGA and PaGA increase by at least 2 units on a 21 circle VAS (29), increase in the active joint count and joints with limited motion by at least 2, an increase by a minimum of +0.125 on the CHAQ, 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 Institutional Review Board. Serum in a standard vacutainer blood collection tube was prepared by allowing the blood sample to clot at room temperature for 15-30 min followed by centrifugation at 1,000-2,000 x g for 10 minutes. For patients in the anti-TNF study, an additional 8.5 cc P100 tube to collect plasma was used for DEK antibody analysis on all subjects >20 kg

at visits 1, 2, 3, and at flare/end of study. Samples were processed as described above, stored, and shipped to the University of Michigan.

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 PBS + 0.25% bovine serum albumin and 0.05% Tween-20 for 30 min at room temperature, followed by three washes with ddH_2O, 10 minutes of blocking, and three additional washes. Serum samples from JIA patients and controls (dilutions of 1:200, 1:400, 1:800, 1:1600, and 1:3200 in blocking buffer) were added to the plates for two hours at room temperature, followed by three washes, 10 minutes of blocking, and three additional washes. Goat anti-human biotinylated secondary antibody (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) at a concentration of 1:2 x10^5 in dilution buffer (50 µl/well) was added for two hours of incubation at room temperature, followed by three washes, and 10 min of blocking and washing. Streptavidin (1:300 in dilution buffer; 50 µl/well) was added for one hour incubation at room temperature, followed by five washes. 3,3',5,5'- Tetramethylbenzidine (TMB) substrate (50 µl/well) was added to develop the plate for 5 to 15 minutes prior to stopping the reaction by 1 N H_2SO_4 (50 µl/well). Optical density was read at 450 nm within 20 minutes.

ELISA statistics

DEK antibody levels in sera from JIA patients, measured at five different dilutions (1:200, 1:400, 1:800, 1:1600, 1:3200), were compared to non-JIA patients and healthy controls and expressed as fold change over healthy controls in each individual experiment. The fold changes were calculated by optical density (O.D). Results were also plotted as area under the dilution curve (AUDC) of each sample, calculated using the trapezoidal rule. ANOVA models were used to compare AUDCs between groups of

patients. Statistical significance was defined as a two-sided *P*-value <0.05. Receiver operating curve (ROC) analysis was used to assess the ability of the AUDCs to discriminate among groups. Area under the ROC curve (AUC) was calculated, as was its 95% confidence interval. The marker showed good discrimination ability if the interval's lower bound was higher than 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 DEK monoclonal antibody (BD Bioscience) as a reference. The DEK AUC values in JIA patients were standardized against the healthy controls. We analyzed the difference in the AUDC values (mAUCs). Student T-test was used for the comparisons between the patients who flared versus those who did not by the eight month follow-up after the discontinuation of the 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 the 187-375 and 1-350 His-tagged DEK-fragments, was performed as described previously (30, 31). 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' EcoR1 and 3' Xho I restriction sites. The PCR product was identified and eluted from an agarose gel prior to digestion with EcoR1 and Xho1. The pGEX-4T1 vector containing GST was also digested with EcoR1 and Xho1, and the 25 aa DEK fragment 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 (31). 3.5 µg protein aliquots were separated by 4-20% SDS-PAGE and transfered to a nitrocellulose membrane. The membrane was probed with a 1:400 dilution of patient sera or 1:1000 dilution of rabbit anti-DEK antibody followed by a secondary goat anti-human antibody or goat anti-rabbit antibody, conjugated with HRP and detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

RESULTS

High levels of DEK autoantibodies are associated 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 including eleven healthy control individuals, 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. Mean age of the JIA patients was 11.7 ± 3.7 years, and mean disease duration was 6.1 ± 4.2 years. The majority of the JIA patients were female (24 females and 9 males; see Table 1 and supplemental Table 1). The distribution of JIA subtypes included 27% with oligoarticular arthritis, 36% with rheumatoid factor (RF) negative polyarticular arthritis, 9% with psoriatic JIA, 9% with systemic JIA, 6% with RF positive polyarticular arthritis, and 1% with spondyloarthropathy. ANA positivity was noted in 39% of patients. Four percent of the patients had uveitis.

With regard to patient medications at the time of study enrollment and blood sampling, 53% of patients were being treated with methotrexate, 29% with hydroxychloroquine, 73% with NSAIDs, 8% with glucocorticoids, 10% with leflunomide, 20% with sulfasalazine, 16% 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.

Among the non-JIA patients with rheumatologic disease, mean age at the time of serum collection was 14.5 ± 3.6 years, with disease duration of 2.5 ± 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 on steroid-sparing agents was also in this group. The healthy control group was made up of healthy college student volunteers aged 20 ± 5 years.

Sera were analyzed by ELISA for DEK antibody levels using recombinant DEK protein. Serum samples were serially diluted and were tested for DEK antibody levels as described in Methods. Figure 1A depicts the average of 2-8 independent ELISAs. Samples were compared to 5 normal healthy controls. As seen in Figure 1A, and Supplemental Figure 1, JIA patients had significantly higher levels of DEK antibody as compared to non-JIA patients and healthy controls. Although there appear to be slightly higher levels of DEK antibody detected by ELISA among non-JIA patients as compared to healthy controls, this was not statistically significant. Figure 1B demonstrates ELISA antibody titers as pairwise comparisons based on area under the curve (AUC) among healthy subjects, JIA patients, and non-JIA patients. JIA patients showed significantly higher AUC than either non-JIA patients (p=0.03) or healthy controls (p=0.003). To test the specificity of our assay, we used the Receiver Operating Curve (ROC) test as shown in Figure 1C. DEK autoantibody levels show good discrimination between JIA patients, non-JIA patients, and healthy subjects, confirming the presence of increased levels of anti-DEK antibodies in patients with JIA.

DEK autoantibody levels are significantly higher in patients with polyarticular arthritis as compared to patients with oligoarticular arthritis or healthy individuals

Previous literature has suggested that anti-DEK antibodies are especially found in JIA patients with the oligoarticular form of the disease. DEK autoantibody levels in sera from different JIA subtypes were thus compared to healthy controls and were analyzed by DEK antibody ELISA (Figure 2). All DEK antibody levels were compared to sera from healthy individuals and were assessed as area under the dilution curve (log AUDC). As seen in Figure 2, there is a statistically significant difference in AUDC

between healthy controls and patients with polyarticular JIA (p=0.0011) or patients with other forms of JIA (p=0.0064) (Figure 2). No significant differences were found between oligoarticular JIA and healthy individuals (p=0.64), but a significant difference was noted between patients with oligoarticular JIA versus polyarticular JIA (p=0.0101), with the latter being statistically more likely to have higher levels of DEK autoantibodies than patients with the oligoarticular form. High levels of DEK autoantibodies were also detected in two patients with spondyloarthropathy and two patients with arthritis as a manifestation of their undifferentiated connective tissue disease (UCTD) (categorized as "others").

DEK autoantibody levels in patients treated with 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 (32, 33). Further, long-term effects of anti-TNF therapy on children remain unclear (34). Thus, strategies for deciding when to stop treatment after it is initiated are much needed. Therefore, we measured DEK autoantibodies in a larger cohort of JIA patients treated with anti-TNF therapy (Table 1 and Supplemental Table 2). Female (103) and male (34) patients with polyarticular JIA were enrolled, with a mean age of 11.3 years and disease duration of 5.0 years. (77% of the patients were on etanercept, 18% adalimumab, 5% infliximab, and 40% were on concurrent methotrexate). Thirty one patients discontinued the study for various reasons, including loss of clinically inactive disease (CID) during therapy. Within eight months of stopping therapy, 39 patients flared, but 67 subjects had no flare within those eight months. DEK antibody levels at the time of stopping anti-TNF therapy were not significantly different between patients that flared or did not flare within eight months after stopping the therapy (Supplemental Figure 2). However, at the end of the study, either after eight months of no flare or at time of disease flare off therapy, high levels of DEK antibodies (mAUCs mean and SD of 0.164 ± 0.39, with a 95% confidence interval of (0.02, 0.31)), were detected in 30 of the patients that flared as compared to lower levels of DEK antibodies (mAUCs -0.05 ± 0.39, 95% confidence interval of (-0.15, ± 0.05)) measured in 59 of the patients with no disease flare (Student-T, P=0.016)

(Figure 3). Thus, retrospectively, patients who experienced flare within 8 months of stopping anti-TNF therapy had significantly higher levels of DEK antibodies as compared to patients who maintained their CID until the end of the study.

The C-terminal portion of the DEK protein is necessary for autoantibody recognition.

Previous studies with a very limited number of sera and synovial fluids suggested that anti-DEK antibodies target the last 25 amino acids (aa) of the molecule (3). To address this question further, full length, 1-350 aa, and 187-375 aa fragments of the recombinant His-tagged DEK were first expressed in 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 immunoblot (Figure 4B). DEK protein was probed with sera samples from a normal healthy control (NHS) subject, JIA patients [JIA(1), JIA(2), JIA(3)], serum from a rheumatology patient without JIA' or with DEK specific antibody (α-DEK) as a positive control. As seen in Figure 4B, the anti-DEK specific antibody strongly detected all DEK fragments. The full-length DEK protein is detected as expected primarily at 55 kDa; a previously identified breakdown form is also detected at 35 kDa (12). (The 1-350 aa DEK runs slightly higher than the full-length DEK, pherhaps 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 three 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 employing autoantibodies found in the synovial fluids of patients (3). The non-JIA patient sera detected full-length and the 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 that is lacking only the last 25 aa cannot be recognized by most patient sera (3 out of 4 do not recognize the truncated fragment). These results are in agreement with our previous findings that the synovial fluid antibodies of 5 out of 8 JIA patients (more than 50%) that recognized DEK failed to

recognized DEK when the C-terminal 25 aa were deleted as in the 1-350 aa DEK mutant (3).

ELISA assays were performed to further determine if patient autoantibodies in sera indeed primarily recognize the last 25 amino acids of DEK. Sera from four patients with JIA (JIA1 to JIA4) and either sera or plasma from four healthy controls (N1 to N4) 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 the sera from healthy control patients, whereas sera from patients with JIA were able to recognize wild-type DEK (Figure 4C, upper panel). Although levels of DEK recognition varied among the patients with JIA, all levels were significantly greater than in healthy controls. This difference disappeared, however, when ELISA was performed using the 1-350 DEK fragment, which was not recognized by sera from JIA patients or controls (Figure 4C, lower panel).

Antibodies from sera of JIA patients recognize the last 25 amino acids (aa) of the DEK protein.

To determine if DEK's autoantigenicity truly resides in its terminal 25 amino acids, we produced the last 25 aa of DEK with a GST tag. Recombinant full-length DEK, GST control protein, and GST-tagged 25 aa DEK were purified and analyzed using a DEK-specific polyclonal antibody, a GST antibody, sera from a representative healthy control, and three different JIA patient sera (P16, P03, and P33) (Figure 5A). The sera from healthy controls did not detect DEK protein, whereas all of the patients with JIA detected full-length DEK. Two representative JIA patients also detected the GST-25 aa fragment, indicating recognition of the C-terminal portion of the DEK protein but not the GST control protein. We next screened all 46 patient samples for recognition of full-length DEK (Figure 5B, left panel), and the GST-25 aa DEK, (Figure 5B, right panel). We noted that approximately four-fifths (78.3%) of JIA patients recognize full-length DEK, but only half of those, or one-third of the total cohort of the patients, recognize the C-terminal portion of DEK. Stated another way, of the pateints with antibodies to full-

length DEK, 50% had antibodies that recognize the C-terminal 25 aa alone. Most of those patients with autoantibodies recognizing the C-terminus of DEK belong to the polyarticular subtype (right panel). Therefore, in some cases, but not all, the C-terminal 25 aa of DEK can be sufficient to generate DEK autoantibodies. Taken together, these findings demonstrate that the C-terminal 25 aa 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 (35), an allele that predisposes children in northern European populations to the development of oligoarticular onset JIA (36, 37). 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 fluids 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 intitally described as an autoantigen in 1991 by Szer et al. (4, 5). With the exception of antinuclear antibodies (ANA), 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 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 two different cohorts, 46 from the Pediatric Rheumatology clinic at the University of Michigan and 89 polyarticular JIA patients treated with anti-TNF therapy from a study coordinated by the Cincinnati Children's Hospital. In both JIA 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 autoantibody 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 DEK antibody levels correlate with active disease, they cannot yet be used as predictor of 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 of DEK is a major autoantigenic region and is sometimes sufficient to generate an autoimmune response. These findings suggest that more refined ELISA assays using the C-terminal 25 aa of DEK might prove to be of use in the future. Taken together, it appears that by understanding the action of DEK and anti-DEK antibodies, improvements in the management of JIA can be made.

FIGURE LEGENDS

Figure 1. JIA patients have high levels of DEK autoantibodies. (A). Sera from patients with JIA, other rheumatic diseases, or healthy controls (57 samples: 33 JIA + 11 healthy + 13 non-JIA with other rheumatic diseases) were serially diluted as indicated and tested for DEK antibody levels by ELISA. Results shown are the average of 2-8 independent ELISAs. Samples were compared to five normal healthy controls. (B). Pairwise comparison based on area under the dilution curve (AUDC) between healthy subjects and JIA patients and non-JIA patients. (C). Receiver Operating Curve (ROC) was presented and the area under the ROC (AUC) was calculated to assess the performance of the AUDC measurements in distinguishing between different groups of patients.

Figure 2. DEK autoantibody levels are significantly higher in polyarticular arthritis patients as compared to those with oligoarticular arthritis or healthy individuals. DEK autoantibody levels in the sera of patients with different JIA subtypes were compared to healthy controls as analyzed by DEK antibody ELISA. Subsets included healthy control individuals (Healt); control group (contr) (patients seen in the rheumatology clinic who did not in fact have arthritis); patients with no definitive JIA subtype at time of diagnosis (others), psoriatic arthritis (psa), and systemic arthritis (syste). All levels of antibodies to DEK were compared to sera from healthy individuals based on area under the dilution curve (AUDC).

Figure 3. Increase in DEK antibodies is evident in JIA patients who flare within eight months of stopping anti-TNF therapy. Data above are from the sera of 89 out of 106 patients that were analyzed for DEK antibody levels. High levels of DEK antibodies, mean and SD of 0.164±0.39, with 95% confidence interval of (0.02,0.31), were detected in the 30 patients who flared within eight months as compared to lower levels of DEK antibodies (-0.05 ± 0.39, 95% confidence interval of (-0.15,0.05)) in 59 patients with no disease flare for at least eight months (Student-T, P=0.016). The difference between the two populations is demonstrated by normal distribution (solid line) and with the same trend by Kernel (dashed line) to demonstrate the distribution of the data based on the actual spread/density of the results.

Figure 4. Antibodies from sera of JIA patients recognize the C-terminal domain of the DEK protein. (A). Illustration of the different fragments and domains of recombinant histidine-tagged DEK with post-translational modifications previously identified in primary human macrophages and HeLa cells (3). (B). DEK fragments were expressed in SF-9 cells and analyzed by Western blot (1 µg protein per lane). Detection of DEK by a 1:100 dilution of sera from a healthy control subject (NHS), juvenile idiopathic arthritis patients [JIA(1), JIA(2), JIA(3)], serum from Pediatric Rheumatology clinic patients without JIA, and specific monoclonal antibody to DEK (α-DEK). (C). Levels of DEK antibodies. Sera from patients with JIA and control sera or plasma (4 JIA + 4 healthy controls (N1 to N4)) were serially diluted and tested for antibodies to full length (1-375) recombinant DEK or 1-350 DEK by ELISA. All data are presented as fold change as compared to another set of 4 different healthy controls. (P = 0.0337 when comparing recognition of full-length DEK by JIA patients to healthy control. P = 0.009 when comparing the recognition of full- length DEK (upper panel) to that of DEK that contains only amino acids 1-350 (lower panel) by the sera of JIA patients (1-4)). The p value is calculated using the student t-test.

Figure 5. The last 25 aa of DEK are sufficient for recognition by the autoantibodies of a substantial percentage of JIA patients who have autoantibodies to full-length DEK. (A). Recombinant full-length DEK (rDEK), GST

control protein (GST), and the C-terminal GST-tagged 25 aa of DEK (GST-25aa) were purified and analyzed by Western blot and densitometry. Results from two different representative JIA patient sera (P03 and P33) are shown in addition to a representative serum control from a healthy individual. (B). Pie chart demonstrating the percentage of patients who have autoantibodies to full- length DEK as detected in sera from pateints with polyarticular JIA (poly), oligoarticular JIA (oligo), psoriatic JIA (PSA), rheumatiod arthritis (RA), systemic JIA, and non-JIA rheumatic diseases (control) (left). From the 46 patients screened, we also calculated the percentage of patients whose sera recognize the last 25 amino acids of DEK alone, also divided into the different JIA subtypes (right). Note that overall approximately half of the pateints' sera that recognize full-length DEK also recognize the isolated C-terminal 25 aa of the protein.

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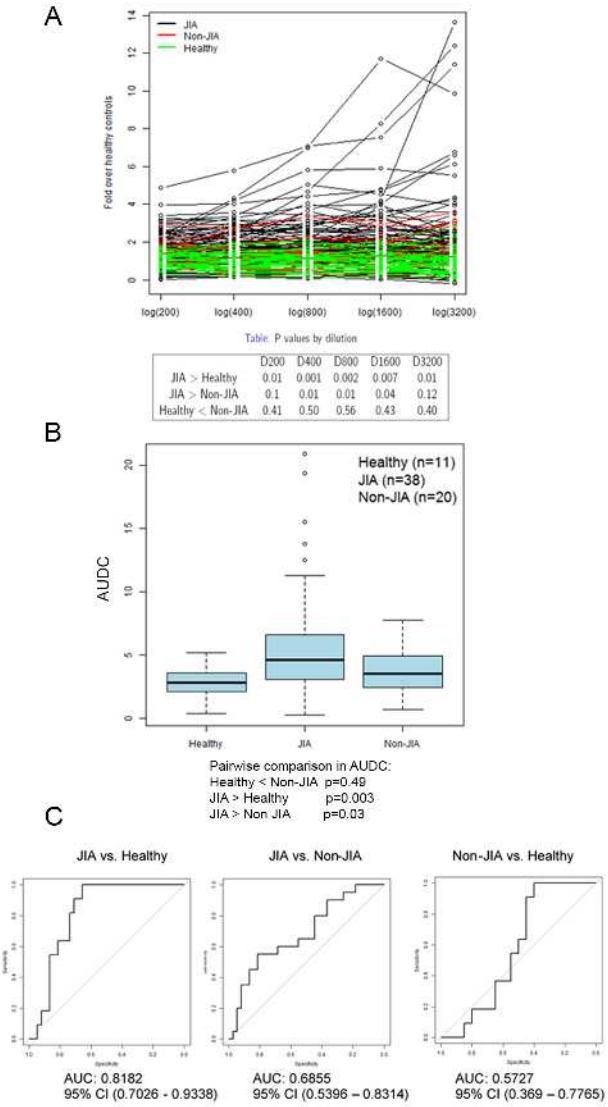
Table 1. Demographic information for pediatric patients recruited into both studies. Data are shown as mean ± standard deviation or N (%).

University of Michigan Cohort			TNF study	
Table 1. Demographics	JIA	Non JIA Diseases	Patients who flared	Patients who did not flare
Total	33	13	39 (37%)	67(63%)
Mean Age, years	11.7 ±3.7	14.0±4.0	11.13±5.05	11.18±4.22
Gender (M:F)	9:24	5:8	10:29	21:46
Mean duration of disease, years	6.2 ±4.2	2.6±1.7	6.37±4.55	4.48±3.14
Diagnosis				
Oligoarticular JIA	8			
Extended Oligo	1		8	9
Rheumatoid Factor-positive polyarticular JIA (RA)	2		2	7
Rheumatoid Factor-negative polyarticular JIA (RA)	12		36	59
Systemic-onset JIA	3			
Psoriatic arthritis	3			
Spondyloarthropathy	1			
undifferentiated arthritis	3			
Undifferentiated Connective Tissue Disease (UCTD)		1		
Systemic Lupus Erythematosus (SLE) (enthesitis related arthritis)		2		
Juvenile dermatomyositis (JDM)		2		
Mixed Connective Tissue Disease (MCTD)		2		
Localized scleroderma		2		
Other (Kawasaki disease, pericarditis, ANA+)		3		
Uveitis	4 (12%)	1 (8%)		
ANA positivity	13 (39%)	8 (62%)	22(42%)	31(58%)
Active arthritis	16 (48%)	1 (8%)		
Current medications				
Methotrexate	17 (52%)	4 (31%)	13(32%)	28(68%)
Glucocorticoids	2 (6%)	4 (31%)		
Hydroxychloroquine (Plaquenil)	9 (27%)	5 (36%)		
NSAIDs	24 (73%)	2 (15%)		
Mycophenolate	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 biologics (anakinra)	2 (6%)	0 (0%)		
Cyclosporine	2 (4%)	1 (8%)		
IVIG	1 (3%)	1(0%)		

Percentages for the TNF study were calculated based on total number of patients (flare and non-flare). All patients were on TNF inhibitors.



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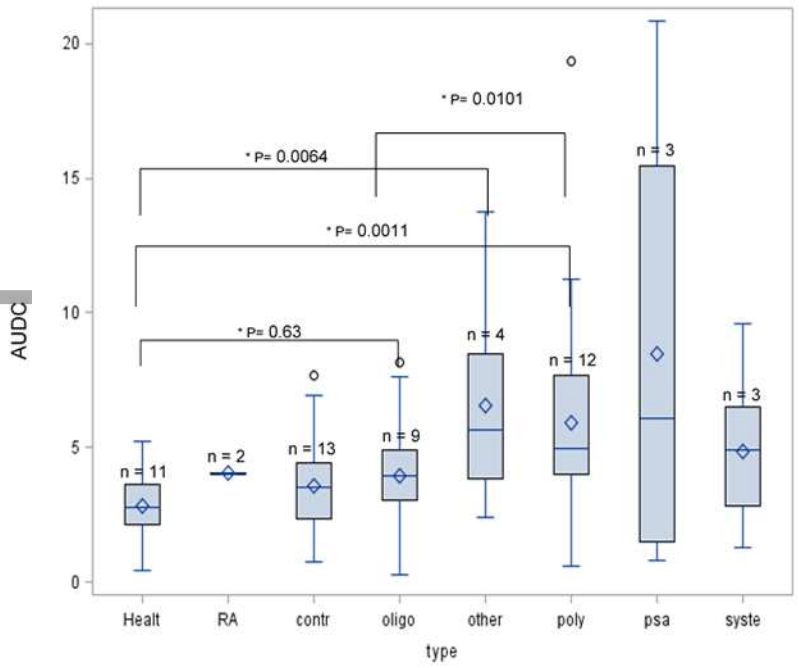

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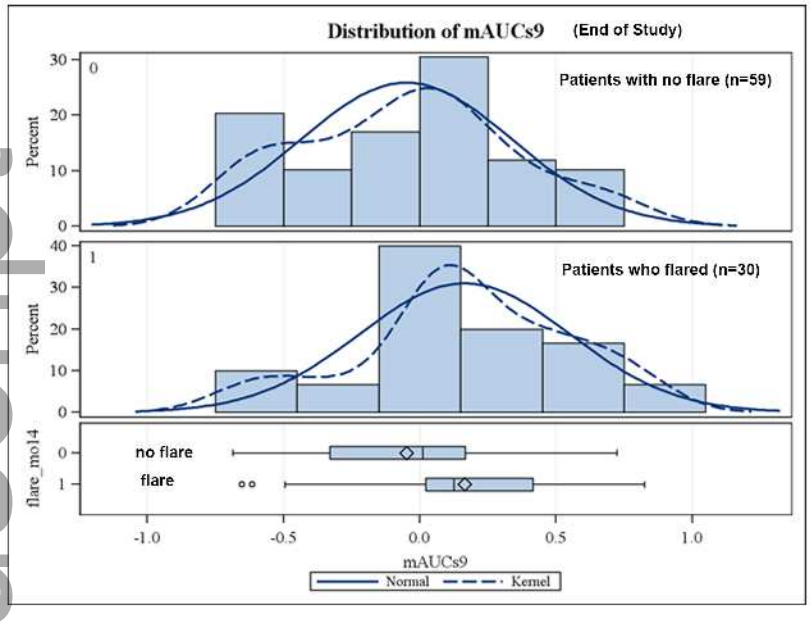

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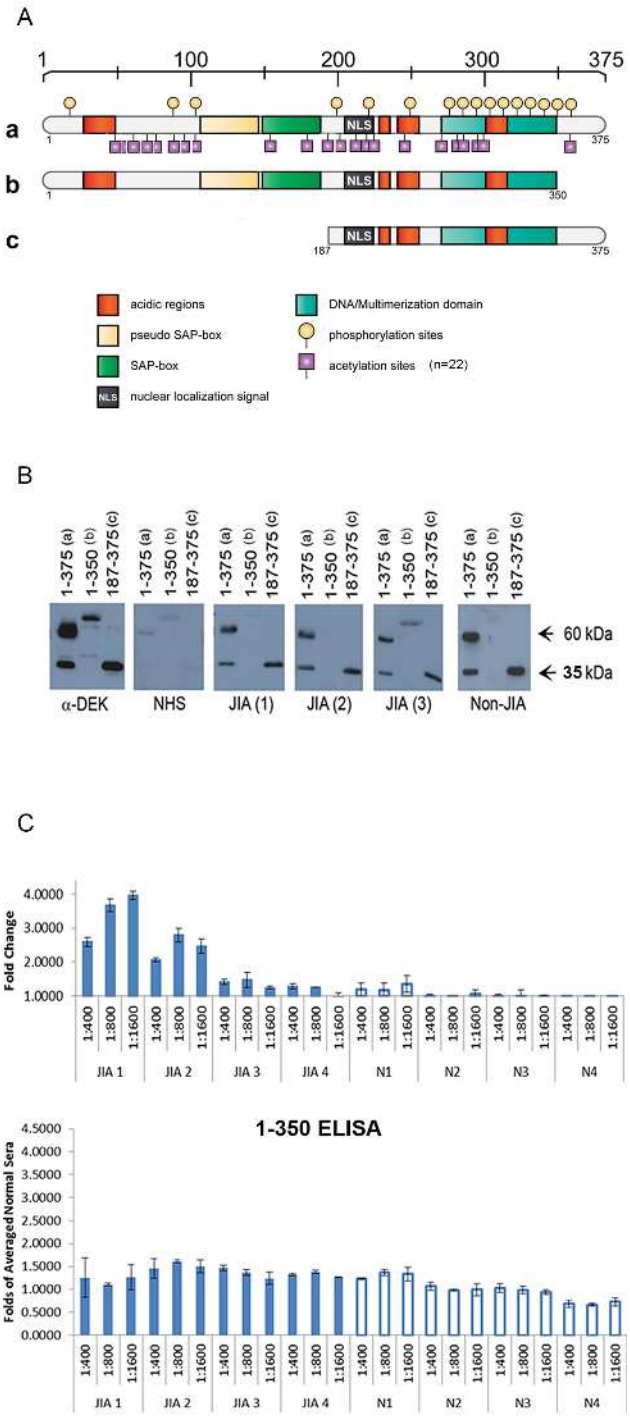

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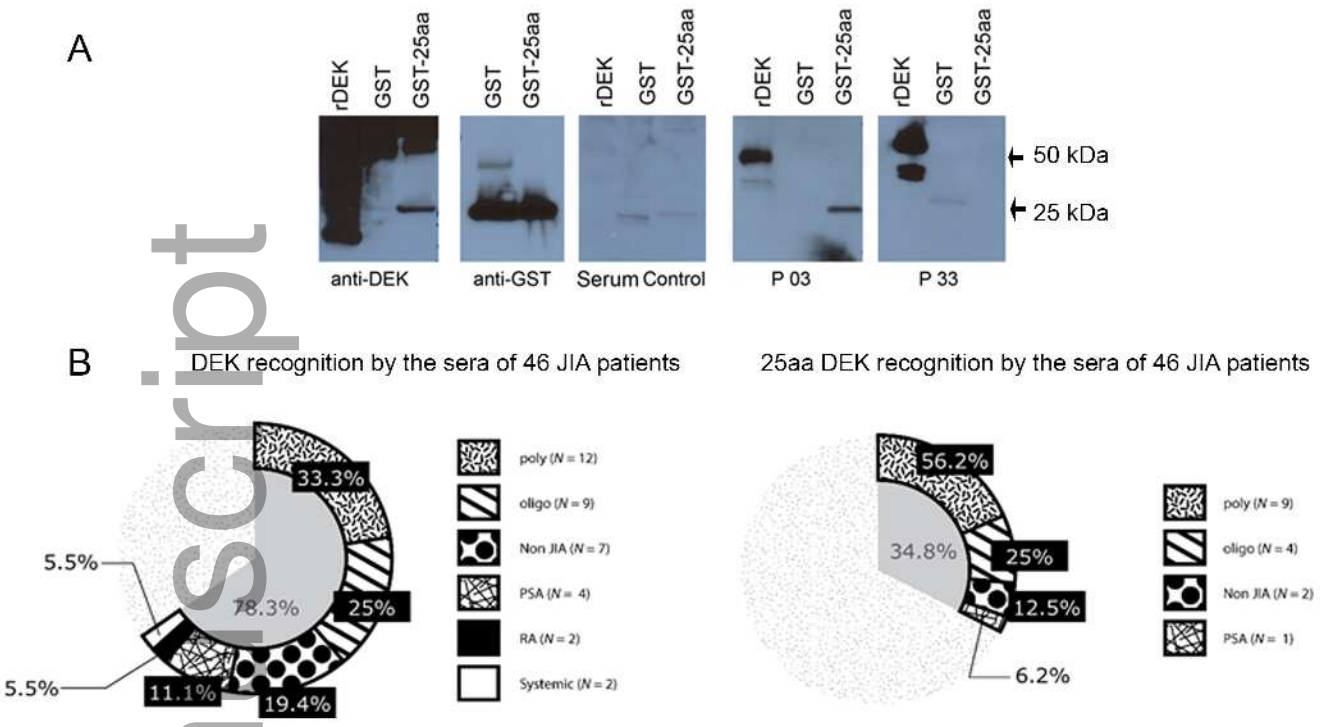

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