DEK in the Synovium of Patients With Juvenile Idiopathic Arthritis

Characterization of DEK Antibodies and Posttranslational Modification of the DEK Autoantigen

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Objective. **DEK is a nuclear phosphoprotein and autoantigen in a subset of children with juvenile idiopathic arthritis (JIA). Autoantibodies to DEK are also found in a broad spectrum of disorders associated with abnormal immune activation. We previously demonstrated that DEK is secreted by macrophages, is released by apoptotic T cells, and attracts leukocytes.**

Since DEK has been identified in the synovial fluid (SF) of patients with JIA, this study was undertaken to investigate how DEK protein and/or autoantibodies may contribute to the pathogenesis of JIA.

Methods. **DEK autoantibodies, immune complexes (ICs), and synovial macrophages were purified from the SF of patients with JIA. DEK autoantibodies and ICs were purified by affinity-column chromatography and analyzed by 2-dimensional gel electrophoresis, immunoblotting, and enzyme-linked immunosorbent assay. DEK in supernatants and exosomes was purified by serial centrifugation and immunoprecipitation with magnetic beads, and posttranslational modifications of DEK were identified by nano–liquid chromatography tandem mass spectrometry (nano–LC-MS/MS).**

Results. **DEK autoantibodies and protein were found in the SF of patients with JIA. Secretion of DEK by synovial macrophages was observed both in a free form and via exosomes. DEK autoantibodies (IgG2) may activate the complement cascade, primarily recognize the C-terminal portion of DEK protein, and exhibit higher affinity for acetylated DEK. Consistent with these observations, DEK underwent acetylation on an unprecedented number of lysine residues, as demonstrated by nano–LC-MS/MS.**

Conclusion. **These results indicate that DEK can contribute directly to joint inflammation in JIA by generating ICs through high-affinity interaction between DEK and DEK autoantibodies, a process enhanced by acetylation of DEK in the inflamed joint.**

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Juvenile idiopathic arthritis (JIA), a polymorphic chronic inflammatory disease of unknown etiology, is

the most common cause of disability in children (1). Although DEK autoantibodies are associated with JIA (2), they are also present in patients with other rheumatic diseases, including systemic lupus erythematosus and linear scleroderma (3). The contribution of DEK protein and DEK antibodies to the pathogenesis of JIA and other autoimmune diseases is not yet known.

DEK is a mammalian nuclear phosphoprotein that was initially identified as an oncoprotein resulting from a t(6;9) translocation in a rare subtype of acute myelogenous leukemia (4). DEK is overexpressed in many malignancies, including hepatocellular carcinoma, glioblastoma, melanoma, bladder cancer, T cell large granular lymphocytic leukemia, and cervical carcinoma; it is also overexpressed in acute myelogenous leukemia independent of the t(6;9) translocation (4–9). Inhibition of apoptosis and senescence by DEK has been shown in recent studies, and DEK has been demonstrated to be a bona fide oncogene (10,11).

DEK bears little resemblance to other known proteins, but it is well conserved among higher eukaryotes. All DEK proteins share a unique conserved region, the SAP (saf/acinus/protein inhibitor of activated signal transducer and activator of transcription) box, a motif that is found in proteins that are involved in DNA binding, chromatin remodeling, and/or RNA processing (12,13). We have demonstrated that DEK is capable of binding to the TG-rich *pets* site in the human immunodeficiency virus type 2 promoter, where it acts as a transcriptional repressor (14,15). There is sequence similarity between the *pets* site and the Y box in some class II major histocompatibility complex promoters, in particular, HLA–DQA1*0501; DEK appears to bind in an allele-specific manner at this locus (16), which may be a risk factor for the development of oligoarticular-onset JIA in northern European populations (17).

In addition to its DNA binding properties, DEK has been found in association with messenger RNA (mRNA) splicing and export factors, as well as with spliced transcripts, in which it has been shown to influence 3′ splice fidelity (18–20). DEK also appears to play an active role in maintaining higher-order chromatin architecture (21). Intense posttranslational modification of DEK by phosphorylation (22), acetylation (23), and poly(ADP-ribosyl)ation (24) points to the potential importance of these posttranslational modifications for the multiple functions of DEK (22,25). Although the monomeric molecular size of DEK is ~50 kd, as observed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), it can multimerize in a phosphorylation-

dependent manner; a 35-kd form of DEK lacking part of the N-terminal domain has also been described (26).

Although DEK is a nuclear protein that is primarily associated with chromatin throughout the cell cycle (27), we have recently identified 2 independent pathways that lead to the presence of DEK in the extracellular space. The first of these pathways involves non–classical secretion of DEK by activated human monocyte-derived macrophages (MDMs) both in a free form and in exosomes (28). In the second pathway, passive release of poly(ADP-ribosyl)ated, hyperphosphorylated DEK by apoptotic T lymphocytes may occur as a result of Fas ligand– or stress-mediated apoptosis (24). In elucidating these pathways, we have shown that interleukin-8–induced DEK secretion acts as a chemoattractant of peripheral blood leukocytes (28). Identification of DEK in the synovial fluid (SF) of patients with JIA suggests that DEK-induced leukocyte accumulation in the extracellular compartment may well result in subsequent joint inflammation (28).

In this study, we demonstrated secretion of DEK by synovial macrophages purified from the SF of patients with JIA. In addition, we were able to demonstrate purification of IgG2 antibodies from the SF of 10 different patients with JIA; these antibodies can interact with complement, and preferentially recognize the C-terminal half of the DEK protein. The presence of DEK in immune complexes (ICs) purified from the SF of patients with active synovitis also suggests that the interaction of DEK with DEK autoantibodies contributes to the inflammation in the joint. With the use of nano–liquid chromatography tandem mass spectrometry (nano–LC-MS/MS), we mapped the acetylation and phosphorylation patterns on individual amino acids of DEK in extracts derived from primary human MDMs and HeLa cells in order to test the effect of DEK phosphorylation and acetylation on its antigenicity, and our results showed that SF autoantibodies from patients with JIA preferentially recognize acetylated DEK species. Taken together, our results confirm that DEK is secreted by synovial macrophages and demonstrate that DEK forms ICs in the presence of DEK-specific autoantibodies that preferentially recognize acetylated DEK. These findings support the notion that the DEK protein and DEK autoantibodies play an active role in the chronic joint inflammation of JIA.

PATIENTS AND METHODS

Antibodies and expression of recombinant DEK. DEK-specific polyclonal antibodies and expression and purification of His-tagged full-length DEK, as well as His-tagged

DEK fragments (1–187, 187–375, 310–375, 270–350, and 1–350), have been previously described (12,22). Monoclonal and goat polyclonal CD81-specific antibodies were purchased from Santa Cruz Biotechnology.

Collection of SF and cell isolation. SF samples were obtained from patients receiving clinical care in the Pediatric and Adult Rheumatology Clinics at the University of Michigan and from the Pediatric Rheumatology Tissue Repository at Cincinnati Children's Hospital Medical Center, under a protocol approved by our Institutional Review Board. The SF was diluted (1:1) in phosphate buffered saline (PBS), centrifuged at 200g for 30 minutes to separate cells, and frozen at −70°C. DEK-specific autoantibodies from the SF were purified by affinity-column chromatography, as previously described (22). Synovial macrophages were purified from the SF by adherence (details available from the corresponding author upon request) and were grown under conditions as described below.

Isolation of exosomes. To purify exosomes from the synovial macrophages, cells were grown in 10% human serum in RPMI 1640 for 12 days, washed, and incubated in serum-free medium for 48 hours. At the end of the culture period, cell viability was still maintained at 80–90%, as determined by staining with 7-aminoactinomycin D (BD Biosciences) and MTT assay (28). Cell culture supernatants, comprising 2.5×10^7 cells, were collected, and exosomes were isolated as described previously (28,29). All pellet fractions and supernatants were solubilized using standard SDS-PAGE loading buffer and were further analyzed by immunoblotting.

Purification of exosomes using anti-CD81 antibody–labeled magnetic beads. Dynal M-500 beads coated with anti-CD81 antibodies were used to isolate exosomes, as described previously (30). Briefly, Dynal beads were incubated overnight with unconjugated mouse IgG1 anti-goat linker antibody (10 μg antibody/2×10^7 beads; Sigma), washed thoroughly with PBS/0.1% bovine serum albumin (BSA), pH 7.4, followed by a 24-hour incubation in 0.2M Tris/0.1% BSA, pH 8.5 at 4°C. Linker-coated Dynal beads were then incubated overnight at 4°C with 10 μg goat anti-CD81 antibody/1×10^7 beads (Santa Cruz Biotechnology), followed by washing with PBS/BSA. Purified exosomes from the 70,000g fraction were added to the coated beads (100 μg protein per 1×10^7 coated beads) in Dynal buffer A (PBS, pH 7.4/2 mM EDTA/5% BSA) and incubated overnight at 4°C. After washing in PBS, beads were isolated, and purified proteins were resolved by SDS-PAGE for subsequent immunoblot analysis.

Enzyme-linked immunosorbent assay (ELISA) for IgG1 and IgG2 antibodies, and purification of ICs. Isotype characterization of human DEK IgG antibodies, purified from 9 different SF samples from patients with JIA, was performed using ELISA (details available from the corresponding author upon request). ICs were purified from the patients' SF using protein G columns (Pierce Chemical), as described previously (31), and were analyzed by immunoblotting using DEK or C1q-specific antibodies (Santa Cruz Biotechnology) and secondary horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch).

Mass spectrometry analyses and peptide database search for the DEK tryptic digest, and in vitro modification of recombinant His-DEK. Phosphorylation and acetylation sites in recombinant His-DEK, modified in vitro using MDM or HeLa cell extracts, were mapped using nano–LC-MS/MS (details available from the corresponding author upon re-

quest). Preparation of cell extracts from HeLa S3 cells or MDMs and the dephosphorylation procedure for His-DEK have been described previously (22).
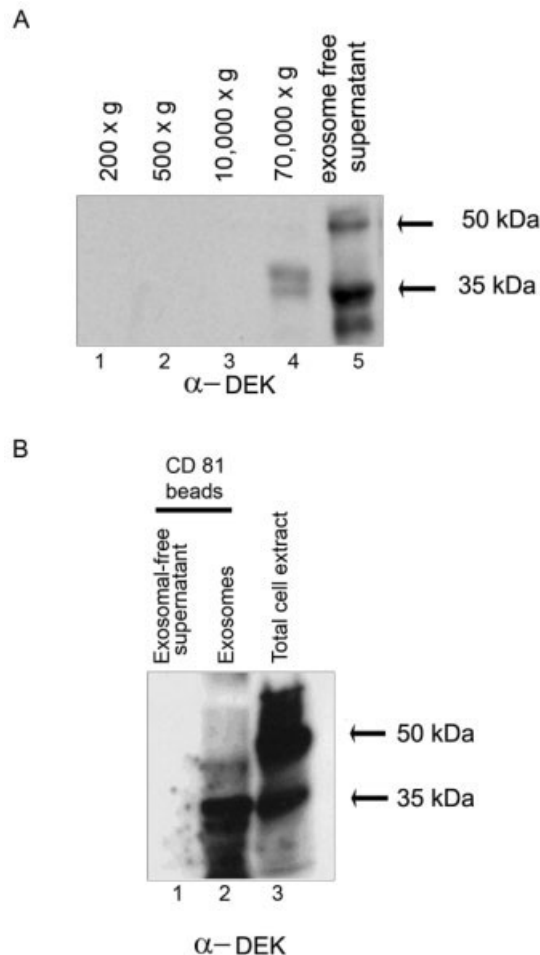

Figure 1. DEK is secreted by synovial macrophages in a free form and via exosomes. **A,** Macrophages purified from the synovial fluid of a patient with pauciarticular juvenile idiopathic arthritis were grown in 10% human serum. Twelve-day macrophages were placed in serum-free medium overnight, and supernatants were subsequently centrifuged at 200g, 500g, 10,000g, and 70,000g. The resulting pellets were analyzed by immunoblotting using monoclonal DEK-specific antibodies. For comparison, an exosome-free fraction is shown. **B,** Magnetic beads coated with antibodies to CD81 were used to further purify exosomes from the exosome-enriched fraction. The recovered fraction was analyzed by immunoblotting using polyclonal DEK antiserum. Lane 1, Negative control: CD81-coated magnetic beads incubated with concentrated protein from the exosome-free supernatant (same as that shown in **A** [lane 5]). Lane 2, Protein recovered from the CD81-coated magnetic beads incubated with 45 μg of protein from the exosome-enriched supernatant fraction obtained by 70,000g centrifugation (same as that shown in **A** [lane 4]). Lane 2 contains ~10-fold less protein than that loaded in lane 1. Lane 3, Intracellular DEK. The 35-kd form of DEK detected may represent a breakdown product (as described in refs. 3, 15, 19, and 26).

Recombinant His-DEK (1 μg/reaction) was modified in reactions containing 300 μg total protein derived from HeLa cell or MDM cell lysates, 1 μM ATP (Roche), 500 μM of acetyl-coenzyme A (acetyl-CoA; Sigma), and protease inhibitors (EDTA-free) (Roche) in the presence of either phosphatase inhibitors (5 mM NaF and 0.5 mM sodium vanadate) or histone deacetylase inhibitors (20 mM sodium butyrate and 50 ng/ml trichostatin A [TSA]) for 10 minutes at 37°C. Reactions were stopped on ice with either 10 mM Tris HCl, 800 mM NaCl, 0.5% Nonidet P40 (NP40), 5 mM imidazole (Sigma), 20 mM NaF, 1 mM sodium vanadate for phosphorylation reactions, or 10 mM Tris HCl, 800 mM NaCl, 0.5% NP40, 5 mM imidazole, 20 mM sodium butyrate, 50 ng/ml TSA for acetylation reactions, or a mixture of both. The modified His-DEK was purified by incubation with Ni^{2+}–nitrilotriacetic acid agarose (Qiagen) for 1 hour at 4°C, followed by 6 wash steps with ice-cold PBS containing either 150 mM NaCl or 300 mM NaCl on Mini columns (Bio-Rad). Elutions were carried out with 200 μl of 2% SDS at 37°C for 15 minutes, and eluted proteins were concentrated as described previously (32). The modified DEK species (250 ng/lane) were further analyzed by immunoblotting with anti-His antibodies or antibodies from the SF of patients with JIA.

RESULTS

Secretion of DEK by synovial macrophages. Based on our previous study showing that DEK is a secreted chemotactic factor (28), we hypothesized that accumulation of DEK in the joints of patients with JIA is likely to be due to active secretion of DEK by synovial monocytic cells, a predominant cell type in inflammatory arthritis. On this assumption, we purified macrophages from the SF of patients with active JIA who were positive for antinuclear antibodies (ANAs), and cultured the macrophages for 12 days in the presence of 10% human serum, as previously described for MDMs; 88% of these cells were positive for the macrophage marker CD11b (further details available from the corresponding author upon request). Supernatants from the cells were collected and exosomes were isolated in consecutive centrifugation steps (28). DEK in the exosome-enriched fraction was detected as proteins of ~45 kd and ~35 kd (Figure 1A, lane 4). DEK in the concentrated exosome-free supernatant was detected as 50-kd and 35-kd proteins (Figure 1A, lane 5), consistent with our previous observations (28).

To confirm the presence of DEK in exosomes from synovial macrophages, the exosome-enriched fraction at 70,000g, as shown in Figure 1A, was also subjected to immunoprecipitation with magnetic beads that
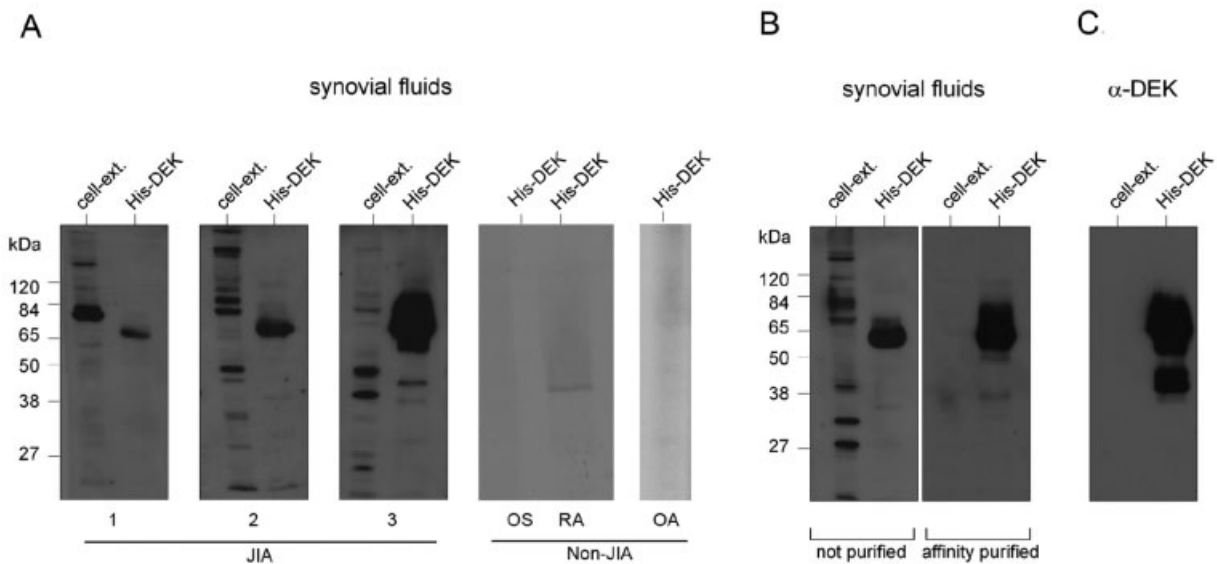

Figure 2. DEK autoantibodies are detected in the synovial fluid (SF) of patients with juvenile idiopathic arthritis (JIA). **A,** Proteins from mock-infected insect cell extracts (cell-ext.; negative control) or recombinant histidine-tagged DEK (His-DEK) expressed and purified from High Five insect cells (1 μg per lane) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to Western immunoblotting with a 1:200 dilution of crude SF, using SF from 3 different patients with JIA who tested positive for DEK-specific autoantibodies (representative of 10 of 13 SF samples screened) or SF from 3 non-JIA patients in whom no to very-low levels of DEK-specific autoantibodies were present (representative sample from a patient with a noninflammatory condition who underwent orthopedic surgery [OS], a patient with rheumatoid arthritis [RA], and a patient with osteoarthritis [OA]). **B,** Samples were also probed with SF that had been affinity purified using SulfoLink Sepharose columns covalently conjugated with recombinant His-DEK, in comparison with non–affinity-purified controls. **C,** Insect cell extracts and recombinant His-DEK were probed with a monoclonal antibody to DEK as a positive control.
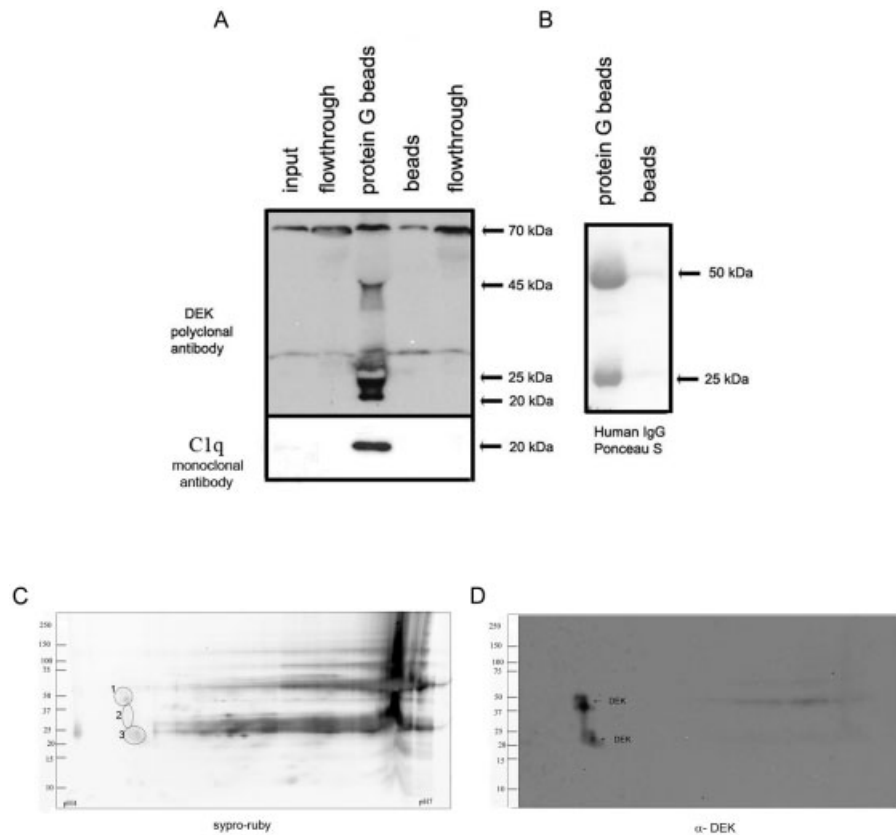
Figure 3. DEK is detected in synovial fluid (SF) immune complexes (ICs). **A,** Protein G–coupled beads or beads alone were used to pull down ICs from the SF of patients with juvenile idiopathic arthritis. Top, DEK proteins in ICs were analyzed by immunoblotting using DEK-specific polyclonal antibodies. DEK antibodies detected a 45-kd band (identified DEK protein size), 70-kd band (possibly nonspecific), and 20-kd band (most likely a breakdown product [36] that is still to be characterized). The flow-through lane represents all of the proteins that did not bind protein G beads. Bottom, C1q was detected in the ICs using monoclonal antibodies to C1q. **B,** The pulled-down proteins were assessed by staining with ponceau S for total protein, with the heavy and light chain of the human Ig pulled down from the SF by the protein G beads, in contrast to that with beads alone. **C** and **D,** Total DEK proteins were detected by staining with Sypro ruby after separation on a 2-dimensional (2-D) gel (**C**), followed by immunoblotting of the 2-D gel with DEK monospecific polyclonal antibodies (**D**). In **C,** corresponding DEK proteins are marked in circles labeled 1, 2, and 3.

were coated with antibodies specific for the exosomal marker CD81 (33) (Figure 1B). Bands corresponding to DEK, representing proteins of ~35 kd and ~45 kd, were found to be specifically associated with the CD81-positive exosome-enriched fraction. No DEK was detected in the exosome-free fraction subjected to purification with CD81 beads (Figure 1B), thus supporting the specificity of the DEK–exosome association. Taken together, our findings demonstrate that synovial macrophages derived from patients with JIA secrete DEK, both in a free form and in exosomes (Figures 1A and B).

Presence of DEK-specific antibodies and DEK protein in the SF of patients with JIA. Although several clinical studies have revealed DEK autoreactivity in the sera of patients with JIA as well as in patients with other autoimmune diseases (2,3,26), the presence of DEK antibodies in inflamed joints has not yet been described. In this study, we detected DEK-specific antibodies in crude SF by probing recombinant DEK protein (expressed in the baculovirus system) or a control mock protein preparation (High Five insect cell extracts) with SF from patients with active JIA (Figure 2A). The presence of DEK-specific antibodies was seen in SF samples from 10 of 13 patients with active JIA, including patients who were negative for ANAs. Very-low to no reactivity to DEK was detected in the SF from non-JIA patients, such as patients with osteoarthritis, those with rheumatoid arthritis, or those with noninflamma-
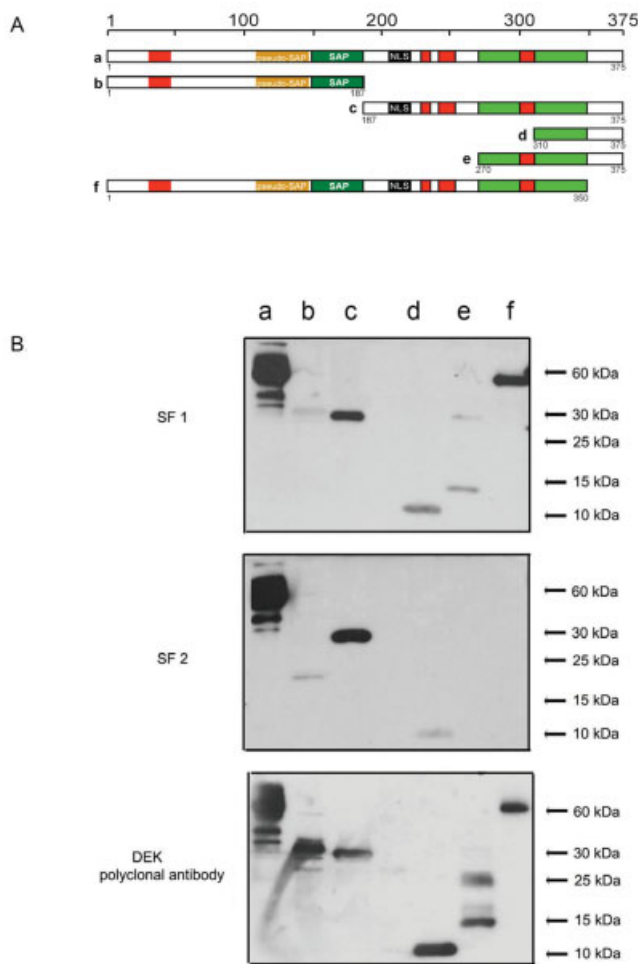
Figure 4. Antibodies from the synovial fluid (SF) of patients with juvenile idiopathic arthritis (JIA) primarily recognize the C-terminal half of the DEK protein. **A,** The illustration shows the recombinant full-length DEK (lane a) and recombinant His-DEK fragments, including the N-terminal half (lane b), C-terminal half (lane c), C-terminal 65–amino acid fragment (lane d), 105–amino acid fragment (lane e), and full-length DEK lacking the C-terminal 25 amino acids (lane f), utilized in these experiments. The functional domains and predicted functional domains are as follows: acidic regions (in red), pseudo–SAP box (13) (in yellow), SAP box (in dark green), second DNA binding domain (12,13) (in light green), and the putative nuclear localization signal (NLS) (in black). **B,** The full-length DEK and DEK fragments (corresponding to lanes a–f in **A**) were expressed and purified from insect cells, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (30 pmoles), and detected with immunoblotting using a 1:200 dilution of crude SF collected from 8 patients with JIA (2 representative samples, SF1 and SF2, are shown) or with DEK-specific polyclonal antibodies as control.

tory conditions having undergone orthopedic surgery (Figure 2A).

Purification of crude SF by DEK-specific affinity-column chromatography resulted in increased antibody sensitivity and specificity (Figure 2B). The column-

purified antibody isolated from the SF was comparable in sensitivity and specificity to a monoclonal DEK-specific antibody (Figure 2C).

Since the physiologic function of any antibody is dependent on its isotype, we analyzed the IgG subclass of the DEK-specific antibodies in the SF from patients with JIA. DEK-specific antibodies from the SF of 9 patients with JIA were purified by affinity-column chromatography prior to being subjected to ELISA and immunoblot analyses. We found that all 9 SF samples contained IgG2 DEK antibodies, while 5 of the 9 samples contained a mixed population of IgG1 and IgG2 DEK antibodies. Some IgG4 reactivity was detected in 2 of the 9 distinct SF samples, but there was no IgG3 or IgA anti-DEK detected in the patients' SF (details available from the corresponding author upon request).

Detection of DEK in ICs in the SF of patients with JIA. The presence of both DEK protein and abundant IgG1 and IgG2 DEK antibodies in the SF of patients with JIA suggested that the presence of DEK–anti-DEK ICs in the synovial space was likely. Antigen–antibody ICs have previously been found in the blood and SF of patients with JIA, although the contribution of ICs to the pathogenesis of JIA remains controversial (34,35). To determine whether the presence of extracellular DEK and DEK antibodies in the SF leads to ICs, we used protein G–coated beads (31) to pull down immunoglobulins from the patients' SF. DEK was detected as ~50-kd and ~20-kd proteins among the IC proteins assessed using DEK-specific polyclonal antibodies (Figure 3A); the immunoglobulin heavy and light chains were identified by ponceau S staining for total protein (Figure 3B).

To confirm that we had detected ICs, we probed for the C1q protein, a component of active ICs. With the use of C1q-specific monoclonal antibodies, we detected a ~20-kd protein in the DEK-containing sample (Figure 3A), supporting the association of DEK with ICs in the SF of patients with JIA. The specificity of the antibodies, and hence these results, was further confirmed by excluding the possibility of nonspecific reactivity using secondary anti-rabbit or anti-mouse antibodies (details available from the corresponding author upon request).

Detection of the ~20-kd DEK protein has been previously described by another group as being associated with the exon junction complex that is involved in mRNA processing (36). To confirm the specificity of these bands, we separated DEK from the other proteins and antibodies in the ICs using 2-dimensional (2-D) gel electrophoresis. Proteins corresponding to molecular weights of ~50 kd, 35 kd, and ~20 kd (Figure 3C, labeled as 1, 2, and 3, respectively) were detected by monospecific DEK antibodies (Figure 3D).
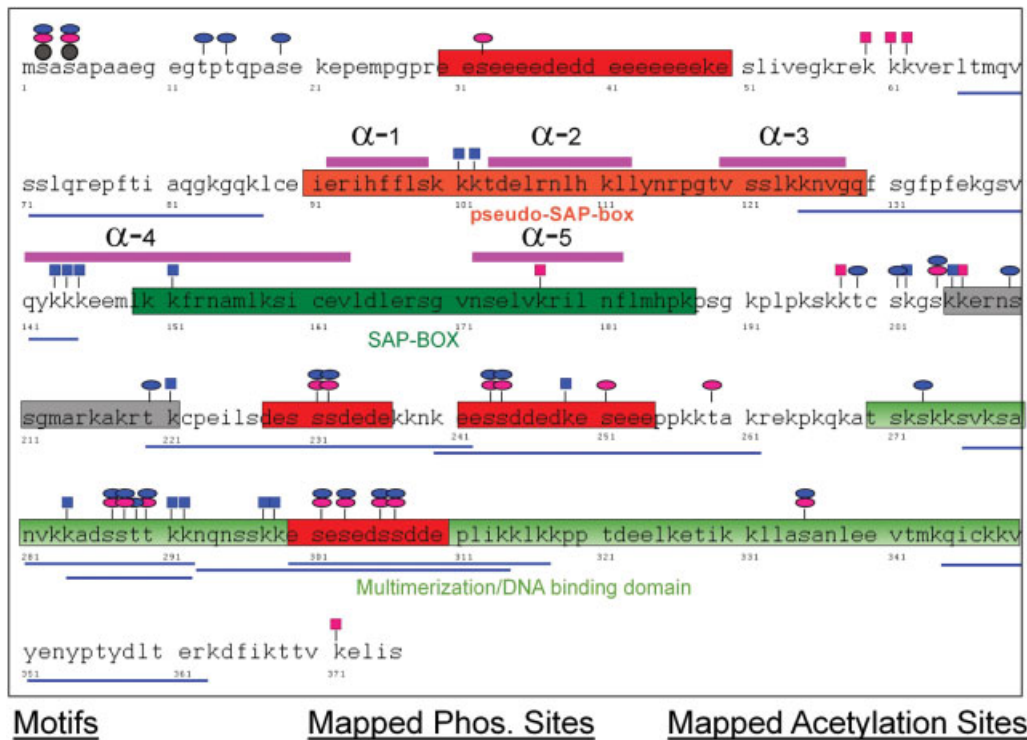
Figure 5. Schematic overview of the acetylation and phosphorylation (Phos.) sites mapped in recombinant His-DEK after treatment with human monocyte-derived macrophages (MDMs) or HeLa cell extracts, indicating the sites of posttranslational modification of the DEK molecule as identified by nano–liquid chromatography tandem mass spectrometry. Recombinant DEK was reacted with extracts from fully differentiated MDMs or HeLa cells in vitro under conditions that favor either acetylation or phosphorylation. Phosphorylated sites are marked by ovals and acetylated sites by rectangles (in blue for MDMs, and in red for HeLa). Highlighted are the functional domains described in Figure 4. Positions of α-helices, as revealed by nuclear magnetic resonance imaging (13), are indicated in pink. Phosphorylation of serine 2 and serine 4 was resistant to treatment with phosphatase, and therefore these represent background. NLS = nuclear localization signal.

Immunogenicity of the C-terminal domain of DEK. Because little is known about autoreactive domains of DEK, our identification of DEK antibodies in the SF led us to investigate which epitopes of DEK may be immunogenic. Using SF from 8 different patients with JIA as a source of DEK antibodies, we probed recombinant full-length DEK and 5 recombinant DEK fragments, including the N-terminal half, C-terminal half, C-terminal 65–amino acid fragment, 105–amino acid fragment, and full-length DEK lacking only the C-terminal 25 amino acids (Figure 4A). All of the SF samples reacted with the C-terminal half of DEK and none showed any reactivity to the N-terminal part of

DEK (Figure 4B). Five of 8 SF samples failed to recognize DEK in the absence of the last 25 amino acids (for example, SF sample 2 in Figure 4B, lane f), suggesting that this epitope may represent the most immunogenic region of DEK.

Posttranslational modification pattern of DEK. As our results showed, DEK can be detected in the SF in several different forms (see Figures 1 and 3D). Posttranslational modifications of DEK, including acetylation and phosphorylation, have been shown to regulate the DNA binding characteristics, nuclear localization, and secretion of DEK (22,23,28), and may account for the different forms of DEK noted in the experiments
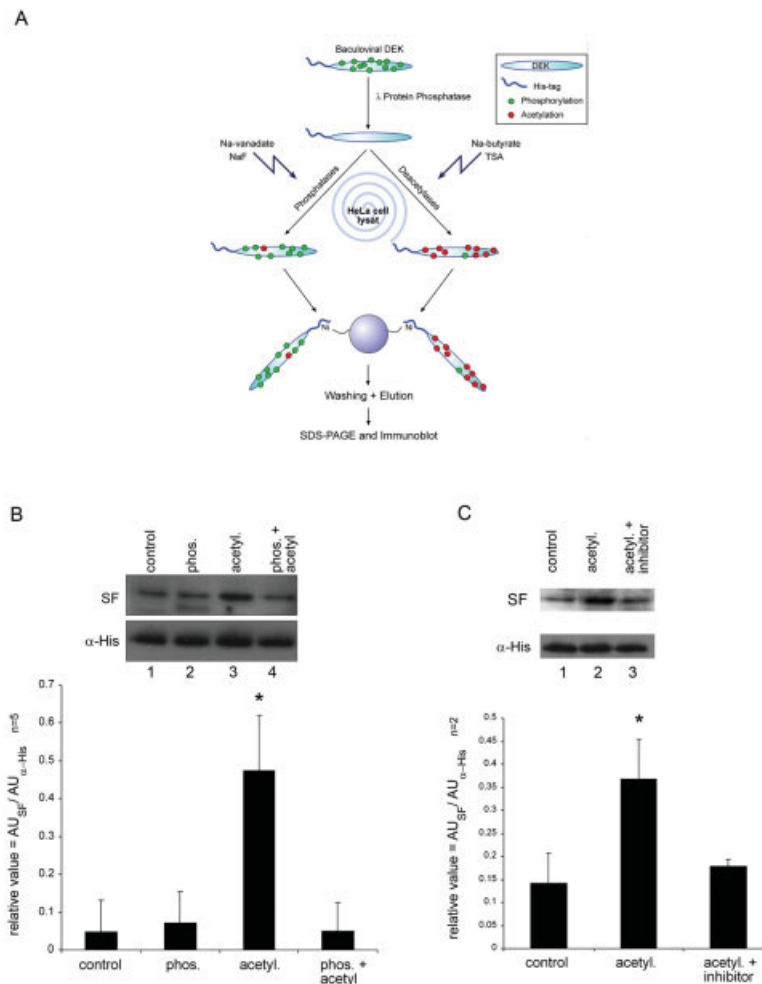
Figure 6. Acetylation increases recognition of DEK by juvenile idiopathic arthritis (JIA) synovial fluid (SF) autoantibodies. **A,** In vitro modification of His-DEK is shown in a schematic overview. SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TSA = trichostatin A. **B** and **C,** Dephosphorylated His-DEK was incubated with protein extract, serving as an enzyme source, along with inhibitors to favor either acetylation or phosphorylation. **B** (top), His-DEK was modified to enhance phosphorylation (phos.) (lane 2), acetylation (acetyl.) (lane 3), or both (lane 4). As a control (lane 1), samples were treated under conditions used to induce phosphorylation and acetylation. Immunogenicity of the modified DEK was detected by immunoblotting using crude JIA SF or His-tag–specific monoclonal antibodies as a loading control. **B** (bottom), The intensity of the bands detected was quantified by densitometry, with results expressed as the mean ± SD of 5 different experiments. ∗ = $P = 0.0052$ versus control, by Student's t-test. **C** (top), Acetylation of His-DEK was inhibited by the acetylase inhibitor anarcardic acid (lane 3). DEK was detected as described in **B**. **C** (bottom), The intensity of the bands was quantified by densitometry, with results expressed as the mean ± SD of 2 different experiments. ∗ = $P = 0.045$ versus control. Densitometry was performed using ImageJ, with results calculated as a relative value (in arbitrary units [AU]), in which the AU values calculated from the SF detection were divided by the AU values of the anti-His loading control.

described above. For that reason, we created a modification map of DEK to identify potential phosphorylation and acetylation sites in recombinant His-DEK,

subjected to incubation with extracts derived from HeLa cells and MDMs. His-DEK was dephosphorylated by λ-phosphatase prior to incubation with the cellular

extracts. The modified protein was run on a gel and was subjected to in-gel tryptic digest, prior to analysis by nano–LC-MS/MS, as previously described (22).

We identified 25 phosphorylation sites and an apparently unprecedented 22 acetylated lysines in the DEK molecule (Figure 5). Serine- and threonine-specific phosphorylation residues were found to be overlapping in the MDM and HeLa cell extracts. Fifteen of the 67 lysine residues of DEK were found to be acetylated in reactions of DEK with MDM cell extracts, as compared with just 7 acetylated lysines identified in reactions of DEK with HeLa cell extracts. Most strikingly, comparison of acetylated residues in the MDM extracts with those in the HeLa extracts showed no overlapping residues, suggesting that acetylation plays an essential role in DEK functions. (Further details on the specific phosphorylation and acetylation sites mapped are available from the corresponding author upon request.)

Induced antibody recognition of DEK by acetylation of DEK in the SF of patients with JIA. Other groups have previously demonstrated that posttranslational modification of proteins can elicit autoimmune responses (37–39). For example, increased citrullination of proteins and the presence of antibodies to citrullinated proteins have recently been found in patients with rheumatoid arthritis (37). Because we noted a striking lack of overlap between DEK acetylation in MDM and HeLa cell extracts, we chose to investigate whether acetylation of DEK might modify its antigenicity. To test this idea, we incubated dephosphorylated His-DEK protein with HeLa cell extracts in the presence of either phosphatase inhibitors or deacetylase inhibitors with acetyl-CoA and ATP to enrich overall phosphorylation and acetylation, respectively (Figure 6A). Control and modified DEK species were analyzed for recognition by DEK-specific antibodies in the SF from a patient with JIA.

As shown in Figure 6B, enhancing phosphorylation of DEK (top panel, lane 2) had no effect on the recognition of DEK by autoantibodies, whereas increased acetylation of His-DEK (top panel, lane 3) favorably enhanced recognition by the patients' autoantibodies as compared with that in control cultures (top panel, lane 1). Addition of anarcardic acid, a potent inhibitor of the acetyltransferases p300 and P/CAF, to the HeLa cell extract prior to modification of DEK reduced the recognition of DEK by the patients' autoantibodies, to the levels found in control cultures (Figure 6C). Enhancing phosphorylation and acetylation together led to loss of the increased immunogenicity seen with acetylation alone, suggesting that there is

interaction between acetylation and phosphorylation of DEK. Taken together, these findings demonstrate that acetylation of DEK potentially enhances its effect as an autoantigen.

DISCUSSION

Juvenile arthritis is a disease that affects children mainly between the ages of 2 years and 16 years (1), leading to common arthritis-related complications, such as limitations in range of motion and function, significant changes in joint architecture that can predispose to the development of early-onset osteoarthritis, and uveitis, a condition that frequently leads to decreased visual acuity and an increased potential for blindness (40). There are no specific diagnostic tests at the present time that can distinguish JIA from other chronic arthritides associated with psoriasis, systemic lupus erythematosus, sarcoidosis, or inflammatory bowel disease. Although both genetic and environmental factors have been implicated in the pathogenesis of JIA (1), its cause is still poorly understood. With the development of new biologic drugs, such as anticytokine agents, there has been substantial progress in disease management. However, none of the available drugs have curative potential, and most have significant side effects. For these reasons, improved understanding of the immunologic mechanisms involved in inciting and perpetuating inflammatory joint disease is necessary for the development of novel, more specific treatments for JIA.

In this study, we have shown that human DEK protein is present in ICs in the SF from the inflamed joints of patients with JIA, and that its presence can be explained by secretion of DEK from local synovial macrophages, both in a free form and via exosomes (Figure 1). These results are in accordance with those from our previous study in which we used MDMs as an experimental model (28). The presence of DEK in exosomes also suggests a mechanism by which DEK may become an autoantigen (41,42).

It is well known that ~40–60% of all patients with JIA have circulating DEK autoantibodies (2), which have been detected mainly in children with oligoarticular JIA and which are nearly omnipresent in children with JIA-associated iridocyclitis (3). In the present study, we provide evidence for the occurrence of both DEK protein and DEK-specific antibodies in the SF from children with active JIA, suggesting a direct role for DEK in the pathogenesis of this disease. We purified DEK-specific antibodies, which are primarily of the IgG2 isotype or a mixed population of IgG1 and IgG2 iso-

types, from the SF of 9 different JIA patients with active synovitis. While IgG1 antibodies can potentially bind CD16 (Fcγ receptor) and CR3 (complement receptor), autoantibodies of the IgG2 isotype predominantly bind complement receptors (43), suggesting that DEK-specific antibodies can be associated with ICs and can stimulate the complement system via the classical pathway. Serum levels of complement degradation products are elevated in patients with JIA (44–46) and are correlated with disease activity. In addition, C1q, a soluble component of the complement defense mechanism that activates the classical pathway primarily through interactions with ICs, has been found in ICs in the sera and SF of patients with JIA (46). We were able to identify DEK, together with C1q, in ICs purified from the SF (Figure 3), further supporting a DEK-specific association with ICs.

To improve our understanding of the nature of DEK-specific antibodies, we screened SF samples from 8 patients with active JIA for their reactivity to specific regions of DEK. We found that DEK-specific antibodies recognize the C-terminal half of the DEK protein, and that loss of the C-terminal 25 amino acids of DEK appears to curtail its recognition by autoantibodies, suggesting that this 25–amino acid domain contributes significantly to the antigenicity of DEK. Enhanced understanding of this region of DEK is likely to contribute to a more complete understanding of how DEK-specific antibodies develop and how they may contribute to the pathogenesis of JIA.

Posttranslational modification of potential autoantigens has recently been found to be an important factor in the induction of autoimmune diseases, including increased citrullination of proteins and production of antibodies to citrullinated proteins in ~60% of adult patients with rheumatoid arthritis (37–39). We have previously demonstrated that the cellular localization of DEK is regulated by posttranslational modifications, including acetylation, phosphorylation, and poly(ADP-ribosyl)ation (13,22–24). In addition, we have shown that SF from patients with JIA efficiently recognize poly(ADP-ribosyl)ated DEK forms, which are released by apoptotic T cells (24). Herein, we showed that multiple forms of DEK are associated with ICs (Figures 3C and D), suggesting that posttranslationally modified forms of DEK are found in the SF. Similar results demonstrating variation in DEK phosphorylation patterns were also shown in a study by Tabbert et al using 2-D gels (47).

We have extended the results from these studies by creating a comprehensive map of posttranslational modifications to DEK. We used HeLa cell extracts, a system in which DEK has been extensively studied, and compared these with activated MDM cell extracts (as a model of chronic inflammation). By assessing the phosphorylation or acetylation state of each individual amino acid of DEK using a highly sensitive molecular approach (nano–LC-MS/MS), we identified 22 acetylated lysines in the DEK molecule, suggesting that these amino acids might be also acetylated in vivo. (Studies are under way to determine the specific lysines necessary for autoantibody recognition.) These results are consistent with our previous findings (23) and further support the idea that acetylation of DEK can account for its antigenicity.

Although most of our current findings are consistent with previous observations regarding the phosphorylation sites of DEK (22) and consistent with the phosphorylation sites listed in the Phosida database, we also identified several novel phosphorylation sites that fully overlapped in cultures of DEK modified by either MDM or HeLa cell extracts. In contrast, the acetylation pattern of DEK in MDMs was distinctly different from that in HeLa cell extracts (Figure 5 and results not shown). Our studies demonstrated that acetylation of DEK is involved in shaping the antigenicity of DEK, as the recognition of DEK by SF autoantibodies was enhanced ~10-fold upon acetylation (Figure 6) and could be blocked by the acetylase inhibitor anarcardic acid (Figure 6B), which inhibits the activity of P/CAF and p300, although the effects were less specific at the concentration used herein. For this reason, we can conclude that the antigenicity of DEK is likely to be mediated by acetylases such as P/CAF or CBP, or by other indirect acetylation of a mediator protein, as has been previously shown for acetylated autoantigens of histone H4 from lupus mice (48). Our results also imply that there is interaction between phosphorylation and acetylation, as phosphorylation alone had no effect on DEK antigenicity but eliminated the preferential recognition of acetylated DEK. This is similar to the phosphorylation/acetylation interdependence seen with histone H3 and p53 (49,50).

In summary, this study demonstrated both DEK protein and DEK-specific antibodies in the SF of patients with JIA. Free-form DEK can be found in the synovium as a result of active release by synovial macrophages and/or apoptosis of T cells. The mechanisms by which DEK may provoke local inflammation include chemoattraction of neutrophils and T cells into the joint (28) and formation of ICs with DEK antibodies or other IgG. DEK-specific antibodies in the SF recognize the C-terminal half of DEK, and acetylation appears to

enhance DEK recognition by SF autoantibodies, suggesting that this posttranslational modification of DEK can induce the generation of DEK autoantibodies and/or enhance IC formation in the inflamed joints of patients with JIA. Taken together, the findings presented herein further elucidate the biology of the DEK autoantigen and its role in the pathogenesis 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. Dr. Mor-Vaknin 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. Mor-Vaknin, Kappes, Legendre, Ferrando-May, Adams, Markovitz.
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Analysis and interpretation of data. Mor-Vaknin, Kappes, Dick, Legendre, Damoc, Teitz-Tennenbaum, Kwok, Markovitz.

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