

# DcR3 Mutations in Patients with Juvenile-onset Systemic Lupus Erythematosus Lead to Enhanced Lymphocyte Proliferation

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**ABSTRACT. Objective.** Previous studies suggested a role for the death decoy receptor 3 (DcR3) in the pathogenesis of adult systemic lupus erythematosus (SLE). We investigated the role of DcR3 in juvenile-onset SLE, to identify polymorphisms that might alter the function of this protein.

**Methods.** DcR3 was measured in the serum of 61 patients with juvenile SLE. The coding region of the *DcR3* gene was sequenced in 100 juvenile and 103 adult patients with SLE, together with 500 healthy controls.

**Results.** DcR3 was elevated in the serum of juvenile patients with active SLE disease ( $440.8 \pm 169.1$  pg/ml), compared to patients with inactive disease ( $122.6 \pm 28.05$  pg/ml;  $p = 0.0014$ ) and controls ( $69.27 \pm 20.23$  pg/ml;  $p = 0.0009$ ). DNA sequencing identified 2 novel missense mutations: c.C167T (p.T56I) in an adult SLE patient and c.C364T (p.H122Y) in a juvenile patient. Recombinant proteins containing these mutations exhibited altered binding kinetics to FasL and they significantly increased lymphocyte proliferation, compared to the wild-type protein ( $p < 0.05$ ). The adult patient with SLE carrying the p.T56I mutation had significantly increased lymphocyte proliferation compared to 3 SLE controls matched for age, sex, and disease severity.

**Conclusion.** DcR3 may play an etiologic role in SLE through either elevated serum levels of wild-type DcR3 or normal levels of gain-of-function DcR3 proteins that increase lymphocyte proliferation. (J Rheumatol First Release June 1 2013; doi:10.3899/jrheum.121285)

## Key Indexing Terms:

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Systemic lupus erythematosus (SLE) is a multifactorial autoimmune disorder with aberrant autoantibody production leading to abnormalities in multiple organs. Many genes have been found to play roles in pathogenesis<sup>1</sup>, but these do not fully account for the pathogenesis, indicating that other, unidentified genes or other factors are likely to be involved.

Death decoy receptor 3 (DcR3) is member 6B of the tumor necrosis factor receptor superfamily (TNFRSF6B). It is a secreted molecule, lacking a transmembrane domain<sup>2</sup>, and it functions as a decoy, by neutralizing the biological effects of at least 3 members of the TNF superfamily, including FasL<sup>2</sup>, TL1A<sup>3</sup>, and LIGHT<sup>4</sup>. These ligands can, among other effects, induce apoptosis in various cell types<sup>3,5,6</sup>; thus one important role of DcR3 is to prevent cell death under certain circumstances. DcR3 is expressed by various tumors<sup>2,7</sup>, providing one possible mechanism for such tumors to escape immune surveillance. In addition, DcR3 is secreted by activated T cells<sup>8</sup> to suppress activation-induced cell death (AICD) in T cells<sup>2,9</sup>. DcR3 also stimulates lymphocyte proliferation<sup>9,10,11</sup>, thereby fine-tuning homeostasis of T cell expansion.

Several lines of evidence suggest a pathogenic role of DcR3 in SLE. For example, transgenic mice expressing human DcR3 exhibited an SLE-like syndrome<sup>12</sup>, and adult patients with SLE had increased serum DcR3 levels, compared to patients with rheumatoid arthritis and healthy controls<sup>9,13</sup>. DcR3 can enhance proliferation and inhibit AICD in T cells<sup>2,9,11</sup>, and T cells from patients with SLE had enhanced reactivity to DcR3 stimulation compared to those from healthy controls<sup>9</sup>.

While there are some similarities in the symptoms of adult SLE and juvenile-onset SLE, the patterns of disease and organ involvement are quite different<sup>14,15,16</sup>. Juvenile patients usually have more severe and frequent renal, hematologic, and central nervous system involvement, while adult patients more commonly present with arthritis, photosensitivity, and discoid rash<sup>17</sup>. Thus, it is possible that the genes and associated pathways involved in the pathogenesis of both SLE types might be different.

The aim of our study was to investigate the role of DcR3 in juvenile-onset SLE and to identify polymorphisms that may alter the function of this protein. While we identified significantly elevated levels of this molecule in the sera of patients with active (but not inactive) disease, we also found 2 missense mutations in the *DcR3* gene of 2 patients. Moreover, recombinant proteins containing these mutations altered binding affinity for FasL and significantly enhanced lymphocyte proliferation. These novel data point to gain-of-function mutations in *DcR3* that have significant roles in disease pathogenesis.

## MATERIALS AND METHODS

*Patients and controls.* We collected blood samples of 100 juvenile and 103 adult patients with SLE. Juvenile-onset SLE is defined by age at diagnosis

< 16 years. Sera samples were collected from 61 of these 100 patients and 28 healthy controls matched for age and ethnicity. The mean age of these 61 patients was 11.1 years when they were first diagnosed and 14.8 years when their sera samples were taken. The SLE Disease Activity Index (SLEDAI) of each patient was measured at the time of serum collection. Patients with SLEDAI scores  $\geq 10$  and  $< 10$  were classified as active ( $n = 12$ , all females) and inactive SLE ( $n = 49$ , 44 females and 5 males), respectively. Clinical manifestations of these 61 patients are summarized in the Appendix. Genomic DNA was extracted from leukocytes of 203 SLE patients and 500 healthy controls using the QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen).

These patients had been treated in accord with standard guidelines<sup>18,19,20</sup>. Informed consent was obtained from each patient and control, and this study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University.

*ELISA for serum DcR3 levels.* Sera from 61 juvenile patients with SLE (56 females, 5 males) and 28 unaffected controls matched for age and ethnicity (17 females, 11 males) were analyzed for levels of DcR3 using an ELISA kit with lower limit of detection of 7 pg/ml (BMS2031, Bender MedSystems) according to the manufacturer's instructions. Absorbance values were measured at 450 nm using a Biotrak II Plate Reader (Biosciences).

*Mutation analysis of DcR3 gene.* All coding regions of the *DcR3* (*TNFRSF6B*) gene were amplified by PCR in 203 patients with SLE. Briefly, PCR reactions were carried out in a 20  $\mu$ l volume containing 50 ng genomic DNA, 1  $\times$  PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM deoxyribonucleoside triphosphate, 0.2 mM of each primer, 5% dimethylsulfoxide, and 0.5 U Taq polymerase (Fermentas, Thermo Scientific). Primers and PCR conditions are shown in Table 1. PCR products were treated with ExoSAP-IT according to the manufacturer's instructions (USP Corp.) and sent for direct sequencing (Macrogen Inc.). Sequence data were analyzed using Sequencher (version 4.2; Gene Codes Corp.).

*PCR-restriction fragment-length polymorphism (PCR-RFLP) to identify DNA mutations.* PCR amplification covering nucleotides 167 and 364 (relative to the transcriptional start site of *DcR3*) was performed in 500 unaffected ethnicity-matched controls. RFLP was performed in a 20  $\mu$ l volume of PCR products using *Ban*I (New England Biolabs) for c.C167T and *Rsa*I (New England Biolabs) for c.C364T. The resulting products were further analyzed by 2% agarose gel electrophoresis.

*Protein sequence analysis.* For protein sequence comparison, DcR3 orthologs were obtained by searching for the *TNFRSF6B* gene in all species available from the Ensembl Website (European Bioinformatics Institute-Wellcome Trust Sanger Institute; Website: [www.ensembl.org/index.html](http://www.ensembl.org/index.html)). The DcR3 protein sequences from 14 vertebrate species were aligned by ClustalX2 software, version 2.0.11. PolyPhen (<http://genetics.bwh.harvard.edu/pph/>) and SIFT ([http://sift.jcvi.org/www/SIFT\\_enst\\_submit.html](http://sift.jcvi.org/www/SIFT_enst_submit.html)) online software was used to predict the possible influence of both missense mutations on protein function.

*Cloning, mutagenesis, expression, and purification of DcR3.* The pRK5 vector engineered to express *DcR3-Fc* was kindly provided by Genentech. Two variants of this vector, expressing either the c.C167T or c.C364T mutation, were introduced into the pRK5-wild type *DcR3-Fc* using the QuikChange Lighting Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene). Recombinant proteins were obtained in 2 ways. First, plasmids encoding either the wild-type or mutant proteins were transfected into the COS7 cell line with Lipofectamine<sup>TM</sup>2000 reagent, according to the manufacturer's instructions (Invitrogen). Second, in experiments designed to obtain stably-transfected cell lines, pRK5-wild-type, -c.C167T, and -c.C364T *DcR3-Fc* were subcloned to the pIRES2-EGFP expression vector (BD Biosciences, Clontech) and transfected into COS-1 or COS-7 cells with Lipofectamine<sup>TM</sup>2000 reagent according to the manufacturer's instructions (Invitrogen). Transfection efficiency was determined by measurement of GFP-expressing cells using flow cytometry (Guava EasyCyte Plus;

Table 1. Primers and PCR conditions.

Names	Primers	Annealing Temperature, °C	PCR Size, bp
Exon1-2-F	5' CAC CCT TGG ACT GAG CTC T3'	64	1112
Exon1-2-R	5' GGC ATG CCT CAG GCT AGA TG3'		
Exon3-F	5' AGC TCT CTG ACC GAA GGC TC3'	60	536
Exon3-R	5' CCT CTT TCA GTG CAA GTG GG3'		
c.C167T-F	5' AGT GGC AGA AAC ACC CAC CTA CC3'	60	316
c.C167T-R	5' AGG TGG ACA CGA TGC GTG CTC C3'		
c.C364T-F	5' GAG TGG CAG AAA CAC CCA CC3'	63	443
c.C364T-R	5' AAC TGG TGT CCT AGC TCA GG3'		

Millipore) and the expression of all transgenes was examined in cell culture supernatants by immunoblotting. For protein purification, cell culture supernatants were dialyzed against 20 mM sodium phosphate buffer, pH 7.0, and applied to HiTrap Protein G HP columns (GE Healthcare). The column was then washed with 20 mM sodium phosphate buffer, pH 7.0, and bound proteins were subsequently eluted with 100 mM glycine-HCl buffer, pH 2.7. Each fraction was neutralized with 1.0 M Tris-HCl, pH 9.0, to preserve protein stability. The purified fractions were dialyzed against phosphate buffered saline (PBS), protein concentrations measured using the Micro BCA Protein Assay according to manufacturer's instructions (Thermo Scientific), and the proteins were used in further functional analysis experiments.

**Surface plasmon resonance (SPR) binding assay.** Protein binding assays were performed by SPR (Autolab Esprit; Eco Chemie). Experiments were performed at 25°C, using gold-coated SPR sensor chip bearing poly (acrylic acid) brushes at 50% graft density for FasL-FLAG (F4428; Sigma) immobilization, which was performed by an amine coupling reaction. The wild-type, p.T56I, and p.H122Y DcR3-Fc proteins were injected over the sensor chip at concentrations ranging from 10.8 to 346 nM.

**Apoptosis assay.** Jurkat cells ( $1 \times 10^6$  cells/ml) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin). They were treated with 7.27 ng/ml FasL-FLAG (F4428; Sigma) and then crosslinked with 0.25 µg/ml anti-FLAG in the absence or presence of the wild-type DcR3-Fc, the DcR3-Fc mutants p.T56I and p.H122Y, and human Fc (10 µg/ml) as a control at 37°C for 16 h. Apoptosis was then determined by AnnexinV/PI staining; lymphocytes were isolated from peripheral blood using Lymphoprep (Axis-Shield) and apoptosis was performed as described<sup>9</sup>. Purified lymphocytes ( $2 \times 10^5$  cells/well in a 96-well plate) were stimulated with 1 µg/ml phytohemagglutinin (PHA) for 24 h and cultured in the presence of 1 ng/ml interleukin 2 (IL-2) for 5 days at 37°C in RPMI-1640 supplemented with 10% FBS and penicillin/streptomycin. Activated lymphocytes were then restimulated with plate-bound anti-CD3, prepared by coating wells with 1 µg/ml anti-CD3 (MAB100; R&D Systems) in PBS. Cells were then incubated in the absence or presence of wild-type, p.T56I, and p.H122Y DcR3-Fc or human Fc (10 µg/ml) as a control for 16 h. Apoptosis was then determined by AnnexinV/PI staining. Statistical analyses were evaluated by Student t test and p values < 0.05 were considered statistically significant.

**Lymphocyte proliferation assay.** Lymphocytes were isolated from peripheral blood from healthy controls and patients with SLE using Lymphoprep. For the proliferation assay, lymphocytes isolated from 4 healthy controls ( $2 \times 10^5$  cells/well) were cultured for 72 h in 96-well plates precoated with suboptimal anti-CD3 concentrations (50 ng/ml anti-CD3 in PBS; MAB100, R&D Systems) in the presence or absence of the wild-type, p.T56I, and p.H122Y DcR3-Fc. Human Fc (10 µg/ml) and anti-CD28 (1

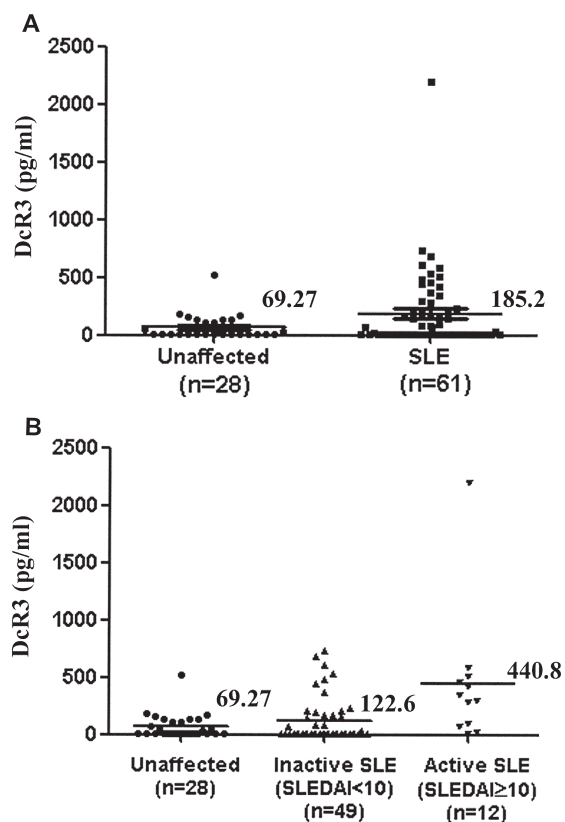
µg/ml) were used as negative and positive controls, respectively. After 72 h incubation, the cultures were pulsed with [<sup>3</sup>H]-thymidine (1.0 µCi/well) for 16 h before cell harvesting. [<sup>3</sup>H]-thymidine incorporation was then measured using a multipurpose scintillation counter (LS6500; Beckman). The value most distant from the mean was considered an outlier and was excluded from the statistical analysis.

A proliferation assay was performed on lymphocytes isolated from an adult patient with SLE who was carrying the c.C167T DcR3 mutation and compared with lymphocytes from 3 SLE controls with wild-type DcR3. Cells ( $2 \times 10^5$  cells/well) were cultured 72 h in 96-well plates with soluble anti-CD3 (MAB100; R&D Systems) at concentrations of 50, 500, and 1000 ng/ml. After 72 h incubation, cultures were pulsed with [<sup>3</sup>H]-thymidine (1.0 µCi/well) for 16 h before cell harvesting. [<sup>3</sup>H]-thymidine incorporation was then measured using a multipurpose scintillation counter (LS6500; Beckman). The experiments from each preparation of cells were performed in triplicate.

**Statistical analyses.** ELISA data are represented as mean ± standard error of the mean (SEM) and median. Differences in mean DcR3 levels between SLE groups and unaffected controls were evaluated with a nonparametric Mann-Whitney rank-sum test using Prism 5 (GraphPad software). A p value < 0.05 was considered statistically significant. In SPR experiments, the protein binding constant values (Kd) indicate best-fit values ± SE, as determined by Kinetic Evaluation V.5.3 (Autolab Esprit software) assuming the monophasic association model. The results of cell apoptosis and lymphocyte proliferation assays were analyzed by a Student t test using Prism 5 (GraphPad software). A p value < 0.05 was considered statistically significant.

## RESULTS

**Increased levels of DcR3 in juvenile patients with SLE.** Previous work has implicated DcR3 in the pathogenesis of adult SLE, and so our first experiments measured levels of this molecule in the sera of patients with juvenile-onset SLE, compared to healthy controls. Levels of DcR3 were increased in serum of 61 juvenile patients with SLE ( $185.2 \pm 42.47$  pg/ml) compared to 28 controls ( $69.27 \pm 20.23$  pg/ml), but this increase did not reach statistical significance ( $p = 0.1930$ ; Figure 1A). However, when the patient data were analyzed according to disease severity status at the time of serum collection (Figure 1B), it was found that those with active disease had significantly higher serum DcR3 levels (mean  $440.8 \pm 169.1$  pg/ml; median 323.6 pg/ml) than patients with inactive disease ( $122.6 \pm 28.05$  pg/ml, median



**Figure 1.** Increased serum levels of death decoy receptor 3 (DcR3) in patients with juvenile systemic lupus erythematosus (SLE). (A) Serum DcR3 levels from 61 patients and 28 controls. (B) Serum DcR3 levels in patients with active (SLEDAI  $\geq 10$ ;  $n = 12$ ) and inactive disease (SLEDAI  $< 10$ ;  $n = 49$ ). Horizontal line indicates mean serum DcR3 value. SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.

$\leq 7$  pg/ml;  $p = 0.0014$ ) or healthy controls ( $69.27 \pm 20.23$  pg/ml, median  $\leq 7$  pg/ml;  $p = 0.0009$ ). Of note, 30 of the 61 patient samples analyzed had undetectable serum DcR3 levels ( $\leq 7$  pg/ml), and of these, 29 (96.7%) were from patients with inactive disease. These data show that DcR3 levels are elevated in serum of juvenile patients with SLE, but only in those patients with active disease (Appendix).

**DcR3 gene mutations in patients with SLE.** We then sequenced the entire coding regions of the *DcR3* gene in 203 patients with SLE (100 with juvenile-onset SLE and 103 adult patients). We identified 2 heterozygous missense mutations in 2 unrelated patients with SLE. A c.C364T (p.H122Y) mutation was found in a juvenile patient (Figure 2A), while a c.C167T (p.T56I) mutation was identified in an adult patient (Figure 2C). We then designed an RFLP assay based on these mutations: the c.C364T mutation resulted in the generation of an *RsaI* restriction site (Figure 2E), whereas the c.C167T mutation destroys a *BanI* restriction site (Figure 2F). These assays were therefore used to screen 500 healthy control samples for the presence of either of these mutations. While the c.C167T mutation was absent,

the c.C364T mutation was detected in 1 of these 500 control DNA samples. These 2 missense mutations have never been reported previously.

The juvenile patient with SLE (Patient 46; in Appendix) presented with generalized petechiae and purpura at 8 years of age. She was followed regularly, with initial diagnosis of idiopathic thrombocytopenic purpura. At age 16 years, she developed oral ulcers, malar rash, discoid rash, and lupus nephritis. She was also positive for antinuclear antibodies and anti-dsDNA antibodies. She was in a quiescent disease phase (SLEDAI = 0) when her serum sample was taken and measured for DcR3 levels. Her serum DcR3 level was 172.55 pg/ml, slightly lower than the mean of serum DcR3 levels ( $185.2 \pm 42.47$  pg/ml) in juvenile patients with SLE (Figure 1A; Appendix).

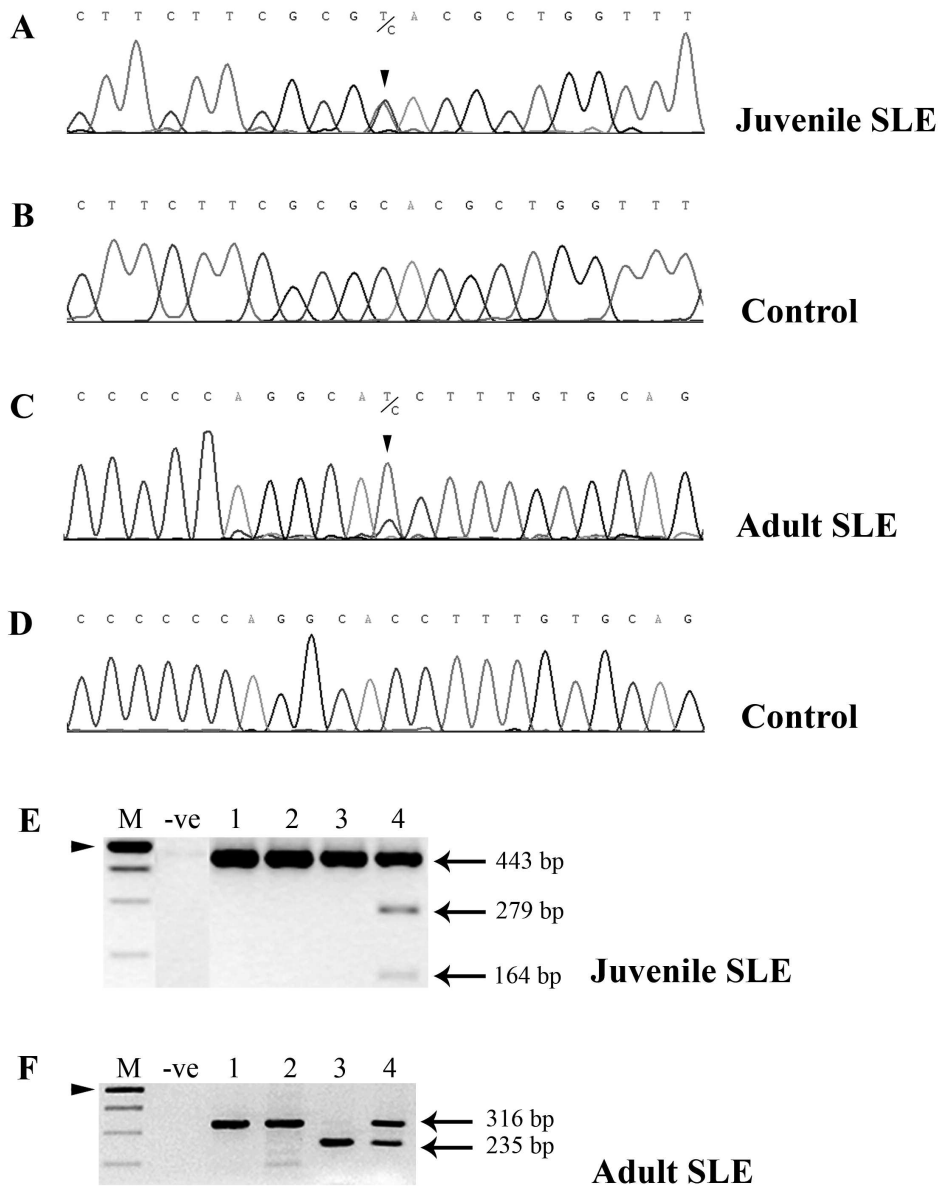
The adult patient with SLE was diagnosed with discoid rash lupus erythematosus (DLE) when she was 25 years old. A year later, she had oral ulcers, malar rash, photosensitive skin, and lupus nephritis. Her serum DcR3 was undetectable.

None of the family members of these 2 patients have been diagnosed with SLE. Familial studies indicated that the mutations in both patients were inherited from their mothers, neither of whom had symptoms of SLE.

**Effect of mutations on protein structure.** The human *DcR3* comprises 3 exons (Figure 3A), with the c.C167T and c.C364T mutations located in exon 1. The c.C167T mutation is predicted to result in a change in amino acid 56 from threonine (polar) to isoleucine (nonpolar), whereas the c.C364T mutation is predicted to change amino acid 122 from histidine (polar) to tyrosine (polar). Both these amino acids are located within cysteine-rich regions of *DcR3* that are associated with the binding domains for FasL, LIGHT, and TLA1. Database analysis revealed that threonine 56 is conserved across 14 diverse species (Figure 3B), while histidine 122 is conserved in 5 mammalian species (Figure 3C). Modeling software was used to predict the likely consequences of these mutations on the 3D structure and ligand-binding activity of *DcR3*. PolyPhen predicted that the p.T56I and p.H122Y mutants were “probably” and “possibly” damaging, respectively, while SIFT predicted that the p.T56I and p.H122Y mutants were “deleterious” and “tolerated,” respectively.

**Effects of mutations on DcR3 functions.**

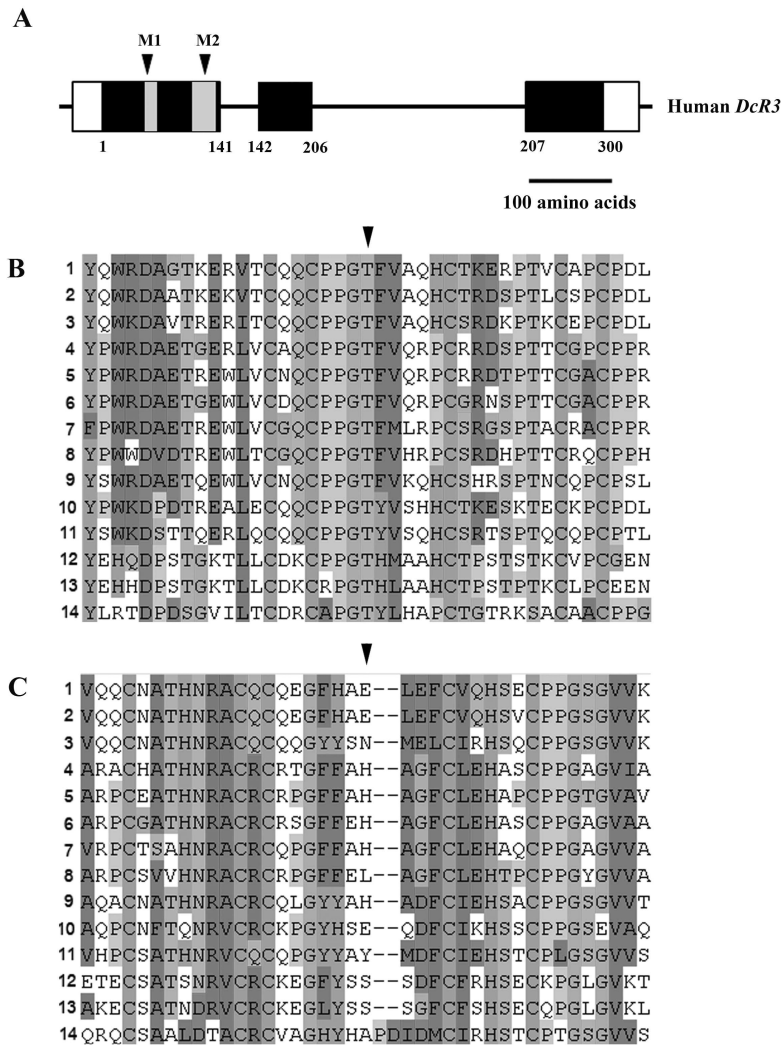
1. Binding affinity to FasL. Recombinant proteins for the wild-type *DcR3* and the p.T56I and p.H122Y mutants were generated as Fc chimeras to allow purification. Surface plasmon resonance was then performed to determine the binding affinities for these proteins to FasL (Figure 4). Wild-type *DcR3*-Fc had a binding constant ( $K_d$ ) of  $128 \pm 1.1$  nM under these conditions (best-fit value  $\pm$  SE, number of concentrations = 5,  $B_{max} = 708.6 \pm 53.82$ ,  $R^2 = 0.9895$ ). However, the T56I mutant had a 2.4-fold higher binding constant of  $52.8 \pm 0.3$  (best-fit value  $\pm$  SE, number of



**Figure 2.** Mutation analysis. Chromatograms show death decoy receptor 3 (*DcR3*) sequences in (A) juvenile SLE patient; c.C364T; (B) control; wild-type; (C) adult SLE patient; c.C167T; and (D) control; wild-type. PCR-RFLP confirmed the mutations in (E) juvenile SLE; c.C364T; and (F) adult SLE; c.C167T. Arrowheads indicate 500 bp markers. M: 100 bp markers; -ve: negative controls; Lane 1: PCR from unaffected control; Lane 2: PCR from SLE patient; Lane 3: RFLP from unaffected control; Lane 4: RFLP from SLE patient. The c.C364T mutation generates an *RsaI* restriction site. RFLP analysis of the juvenile SLE patient revealed digested products of 279 and 164 bp (from the mutant allele), plus a 443 band amplified from the nonmutated allele (lane 4), while the wild-type fragment from the control was undigested by *RsaI*, showing a single product of 443 bp (Lane 3). The c.C167T mutation destroys a *BanI* site. Thus 1 allele of the adult SLE was undigested by *BanI*, revealing a 316 bp product, while the nonmutated allele was digested to 235 bp plus 81 bp (not detected on the gel; Lane 4). Nonmutated wild-type PCR products from the control revealed a digested product of 235 bp (Lane 3). SLE: systemic lupus erythematosus; PCR-RFLP: PCR-restriction fragment-length polymorphism.

concentrations = 5,  $B_{\max} = 676.6 \pm 26.94$ ,  $R^2 = 0.9911$ ), whereas the H122Y mutant had a 0.7-fold lower binding constant of  $176.0 \pm 2.5$  nM (best-fit value  $\pm$  SE, number of concentrations = 5,  $B_{\max} = 488.0 \pm 21.25$ ,  $R^2 = 0.9977$ ).

2. Apoptosis. In view of the altered binding affinity of the mutant *DcR3* proteins to FasL, we then investigated their effects on apoptosis of either cultured Jurkat cells or human lymphocytes. Jurkat cells were incubated with FasL-FLAG



**Figure 3.** The death decoy receptor 3 (*DcR3*) gene structure and alignment. (A) Black and gray boxes indicate coding regions of *DcR3*. Gray boxes are FasL-binding domains. M1 and M2 indicate the position of the mutations in adult (c.C167T; p.T56I) and juvenile (c.C364T; p.H122Y) patients with SLE, respectively. Numbers below each box indicate the first and last amino acid residue of each exon. (B) Alignment centering around residue 56 (p.T56I, adult SLE patient) and (C) residue 122 (p.H122Y, juvenile SLE patient) of *DcR3* orthologs from 14 vertebrate species including 1: *Gallus gallus* (chicken); 2: *Meleagris gallopavo* (turkey); 3: *Taeniopygia guttata* (Zebra finch); 4: human; 5: *Ailuropoda melanoleuca* (panda); 6: *Bos taurus* (cow); 7: *Loxodonta africana* (elephant); 8: *Myotis lucifugus* (little brown bat); 9: *Sarcophilus harrisii* (Tasmanian devil); 10: *Anolis carolinensis* (anole lizard); 11: *Ornithorhynchus anatinus* (platypus); 12: *Takifugu rubripes* (puffer fish); 13: *Tetraodon nigroviridis* (green spotted puffer fish); and 14: *Gadus morhua* (Atlantic cod). SLE: systemic lupus erythematosus.

and then crosslinked with anti-FLAG antibodies to induce apoptosis. Experiments were performed in the absence (control) and presence of increasing concentrations of the wild-type or mutant *DcR3*-Fc proteins. Wild-type *DcR3*-Fc proteins blocked FasL-induced apoptosis in a dose-dependent manner, with 50% inhibition of apoptosis observed at about 125  $\mu\text{g/ml}$  of *DcR3* (Figure 5A). However, the mutant proteins had effects on FasL-induced apoptosis very similar to those of the wild-type protein, under these experimental

conditions. Similar experiments were then performed measuring activation-induced apoptosis (PHA, IL-2, followed by anti-CD3 ligation) of purified human lymphocytes. Wild-type *DcR3*-Fc blocked activation-induced apoptosis (Figure 5B), but the mutant proteins were equally effective as the wild-type protein.

3. Lymphocyte proliferation. Reports have shown that *DcR3* can induce T cell activation through costimulation<sup>9,11</sup>. Consequently, purified human T cells were suboptimally

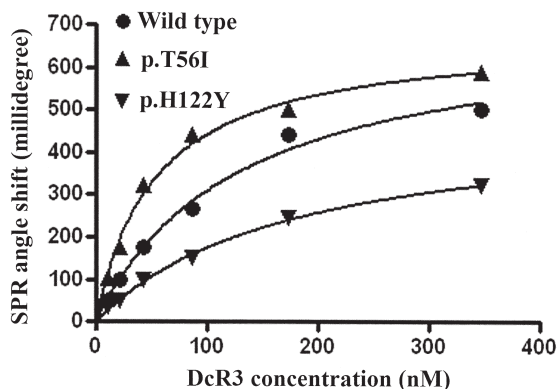


Figure 4. Surface plasmon resonance (SPR) signal response of the wild-type DcR3-Fc, p.T56I and p.H122Y DcR3-Fc mutants to immobilized FasL, as a function of ligand concentration. The binding constants (Kd) of FasL to the wild type, p.T56I, and p.H122Y DcR3-Fc were  $128 \pm 1.1$ ,  $52.8 \pm 0.3$ , and  $176 \pm 2.5$  nM (Best-fit value  $\pm$  SE), respectively. DcR3: death decoy receptor 3.

stimulated by CD3 ligation and then incubated with the wild-type or mutant DcR3-Fc. Lymphocyte activation was determined by [ $^3$ H]-thymidine incorporation, and human Fc and anti-CD28 antibodies were used as negative and positive controls, respectively. DcR3 proteins were added to these cultures at concentrations of  $20 \mu\text{g/ml}$  (Figure 6A) and  $40 \mu\text{g/ml}$  (Figure 6B). All DcR3 proteins (wild-type and mutant) at both concentrations used were able to stimulate lymphocyte proliferation, in a dose-dependent manner. However, when used at  $40 \mu\text{g/ml}$ , both mutants stimulated significantly greater levels of lymphocyte proliferation compared to the wild-type control, with  $p = 0.045$  and  $0.035$  for the p.T56I and p.H122Y, respectively (Figure 6B).

Proliferation assays were then performed on lymphocytes isolated from the adult SLE patient carrying the c.C167T DcR3 mutation and compared to lymphocytes from 3 age- and sex-matched SLE controls with similar disease activity. Rates of lymphocyte proliferation (expressed as mean  $\Delta\text{cpm} \pm \text{SD}$ ,  $n = 3$ ) were as follows: adult SLE with mutation,  $56,560 \pm 8,863$ ,  $52,120 \pm 4,163$ ,

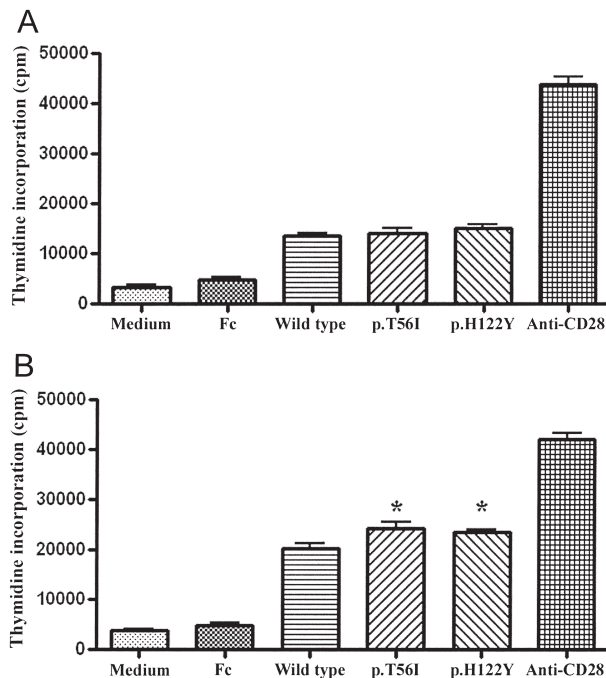


Figure 6. Wild-type, p.T56I, and p.H122Y DcR3-Fc enhanced lymphocyte proliferation under suboptimal anti-CD3 stimulation. (A) Wild-type and mutants at  $20 \mu\text{g/ml}$  concentration; (B) wild-type and mutants at  $40 \mu\text{g/ml}$  concentration. Human Fc ( $10 \mu\text{g/ml}$ ) and anti-CD28 ( $1 \mu\text{g/ml}$ ) were used as negative and positive controls, respectively. At  $40 \mu\text{g/ml}$  concentration, both p.T56I and p.H122Y DcR3-Fc mutants significantly enhanced lymphocyte proliferation compared to wild-type DcR3-Fc ( $p = 0.045$  and  $0.035$ , respectively). Values are means  $\pm$  standard deviation (SD) of counts per minute (cpm) from lymphocytes from 4 healthy controls. Student t test was used to compare means between wild-type and mutants;  $p < 0.05$  considered statistically significant. DcR3: death decoy receptor 3.

and  $54,220 \pm 2,087$ ; SLE controls,  $24,290 \pm 7,232$ ,  $25,030 \pm 5,942$ , and  $22,270 \pm 6,250$  after stimulation with 50, 500, and  $1000 \text{ ng/ml}$  anti-CD3, respectively (Figure 7). The adult SLE patient with the mutation had significantly increased lymphocyte proliferation compared to the other patients, after stimulation with 50 ( $p = 0.0238$ ), 500 ( $p = 0.0491$ ), and  $1000$  ( $p = 0.0284$ )  $\text{ng/ml}$  anti-CD3.

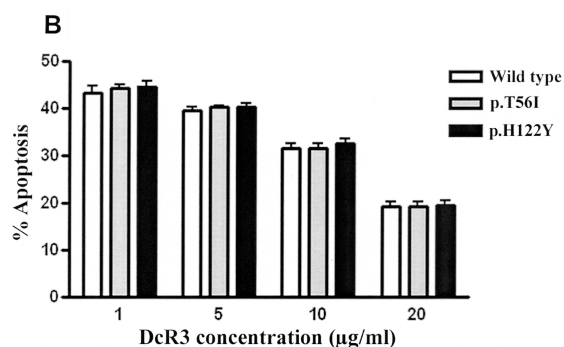
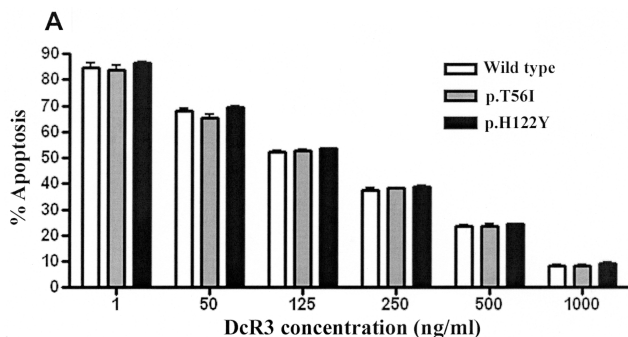
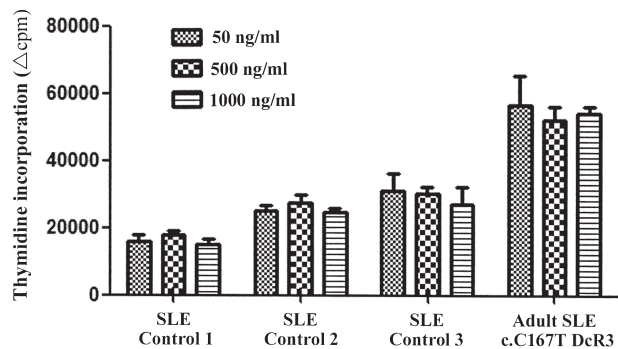


Figure 5. Effects of wild-type, p.T56I, and p.H122Y DcR3-Fc on apoptosis. (A) FasL-induced Jurkat cell apoptosis (values are means  $\pm$  SD,  $n = 3$ ). (B) Activation-induced cell death in human lymphocytes (values are means  $\pm$  SD,  $n = 5$ ). DcR3: death decoy receptor 3.



**Figure 7.** The adult patient with SLE with c.C167T *DcR3* had increased lymphocyte proliferation via anti-CD3 stimulation. Lymphocytes from SLE controls 1, 2, and 3 with wild-type *DcR3* and the adult SLE patient with c.C167T *DcR3* were stimulated with 50, 500, and 1000 ng/ml anti-CD3. All SLE patients were matched for age and sex and had inactive disease (SLEDAI < 10) when lymphocytes were isolated. Unstimulated lymphocytes, as control, were cultured in medium without anti-CD3 and baseline rates of [<sup>3</sup>H]-thymidine incorporation were subtracted from rates obtained for stimulated cells. Values are mean ± standard deviation (SD) of counts per minute (cpm) for [<sup>3</sup>H]-thymidine after subtraction of baseline controls. Student t test was used to compare means between the adult patient with the mutation and SLE controls; *p* < 0.05 considered statistically significant. SLE: systemic lupus erythematosus; *DcR3*: death decoy receptor 3; SLEDAI: SLE Disease Activity Index.

## DISCUSSION

We report 3 novel findings. First, we show that serum levels of *DcR3* are elevated in juvenile patients with active SLE disease. Second, we have identified 2 novel missense mutations in the *DcR3* gene. Third, these mutant forms of *DcR3* encode proteins that have altered function with respect to FasL binding and ability to enhance lymphocyte proliferation. These new data add further support for the role of *DcR3* in the pathogenesis of SLE.

While previous work has shown elevated *DcR3* protein levels in the serum of adult patients with SLE, we investigated whether patients with the juvenile form of this disease also displayed elevated serum levels. Although serum levels of *DcR3* were increased in the juvenile patients with SLE (185.2 ± 42.27 pg/ml) compared to controls (69.27 ± 20.23 pg/ml), this difference did not reach statistical significance. This may be due to large variations in serum levels between individuals, particularly in the patient group (Figure 1). However, when we subgrouped the patients into those with active disease (SLEDAI ≥ 10) and those with inactive disease (SLEDAI ≤ 10), a significant difference was observed: in those with active disease, levels were 440.8 ± 169.1 pg/ml, while in those with inactive disease, the value was 185.2 ± 42.27 pg/ml (*p* = 0.0014). Interestingly, of the 30 patient samples with undetectable *DcR3* serum levels, 29 were from patients with inactive disease. These data point to a role for elevated *DcR3* levels as a disease activity marker.

In addition to changes in the absolute levels of a particular protein in disease etiology, loss- or gain-of-func-

tion, as a result of gene mutation, can also have a profound effect on protein activity and hence a role in pathogenesis. We sequenced the entire coding region of the *DcR3* gene in 203 unrelated patients with SLE (100 with juvenile onset disease and 103 with adult SLE). We identified 2 new heterozygous missense mutations in this gene: p.H122Y in a juvenile patient and p.T56I in an adult patient. Both mutations resulted in changes in the polarity of the amino acids in the protein. In the p.T56I mutation, the uncharged (at neutral pH) polar amino acid threonine is substituted for the nonpolar amino acid isoleucine. In the p.H122Y mutant, the polar histidine (slightly positively charged at neutral pH) is substituted by tyrosine (uncharged at neutral pH), which may be modified by phosphorylation. Both these mutations lie in the FasL-binding domain, implying that these substituted amino acids could alter the binding properties of *DcR3* toward its ligands, and hence its functional properties. The importance of residues 56 and 122 in *DcR3* function is demonstrated by the fact that the former is conserved in all 14 vertebrate species available in the Ensembl database, while the latter residue is conserved in all 5 mammalian species (Figure 3, Table 2). Prediction software predicted that both mutations might result in some functional changes of the protein. Moreover, the c.C167T was absent in all 500 controls. These observations suggest that the mutations might be pathogenic.

We then investigated whether these mutations had any consequences for the function of *DcR3*. Using SPR, we found that the mutant proteins had altered binding affinity for FasL, one of the 3 known ligands for this decoy receptor. We found that both mutations affected the binding affinity of *DcR3*, but with opposing effects. The p.T56I mutant displayed increased binding affinity, whereas the p.H122Y mutant had lower binding affinity. Changes in binding affinity may result in dysregulated apoptosis, which is implicated in the pathogenesis of SLE, and is one mechanism that could result in the generation/exposure of self-antigens and autoantibody production<sup>21,22,23</sup>. Alternatively, lower rates of apoptosis could provide a mechanism for nonresolving inflammation and persistence of activated cells, such as activated T lymphocytes<sup>9,24,25</sup>. We therefore measured the ability of the wild-type and mutant *DcR3* to protect Jurkat cells from FasL-induced apoptosis, or human lymphocytes from activation-induced apoptosis (Figure 5). While we could demonstrate effective, dose-dependent inhibition of apoptosis by recombinant *DcR3*, we could not identify any significant difference in efficiency of apoptosis protection with either of the mutant proteins, compared to control *DcR3*.

*DcR3* has also been shown to induce T cell activation through costimulation, although the mechanism for this is incompletely defined<sup>9,10,11</sup>. Thus, our next experiments were designed to determine whether the mutant proteins acted identically to the wild-type *DcR3* in this activity.



Table 2. Characteristics of patients with mutations in *DcR3*.

Characteristic	Patient 1, Adult SLE	Patient 2, Juvenile SLE
Clinical features	Malar rash, discoid rash, photosensitive skin, oral ulcers, and lupus nephritis	Malar rash, discoid rash, oral ulcer, lupus nephritis, positive anti-dsDNA, and positive antinuclear antibody
Mutation	Heterozygous c.C167T	Heterozygous c.C364T
Protein	p.T56I	p.H122Y
Functional domain	FasL, LIGHT, and TL1A binding domains	FasL, LIGHT, and TL1A binding domains
Evolutionary conservation	Conserved in 14 vertebrate species	Conserved among 5 mammalian species
Parents	Presence in the mother	Presence in the mother
Controls	Absence	1 per 1000 alleles
PolyPhen	Probably damaging	Possibly damaging
SIFT	Deleterious	Tolerated

DcR3: death decoy receptor 3; SLE: systemic lupus erythematosus.

Human lymphocytes were suboptimally stimulated by CD3 ligation and then incubated with the wild-type or mutant DcR3 at 2 different concentrations. These proteins were effective in enhancing T cell proliferation in partially activated T cells, and this effect was dose-dependent (Figure 6). However, at the higher concentration (40  $\mu\text{g/ml}$ ), the mutant proteins induced a small but significantly increased rate of lymphocyte proliferation, compared to the wild-type DcR3. Both mutant proteins displayed enhanced proliferative activity, even though binding experiments revealed that the p.T56I mutant had increased affinity for FasL, whereas the p.H122Y mutant had decreased affinity for this ligand (Figure 4). The reasons for this are unknown, but it should be borne in mind that FasL is only 1 of 3 known ligands that bind DcR3, the others being LIGHT and TL1A. While DcR3 has been shown to be effective in blocking FasL-induced apoptosis<sup>2,12,26</sup>, LIGHT, but not FasL, was found to block DcR3-mediated T cell costimulation<sup>9</sup>. These observations indicate that DcR3-LIGHT interactions may be responsible for T cell costimulation, perhaps by LIGHT acting as a receptor, transducing intracellular signals, as well as a ligand for DcR3 and other receptors<sup>11</sup>. In our experiments measuring T cell costimulation (Figure 6), it is therefore likely that DcR3 was interacting with LIGHT to enhance proliferation. In these experiments, both mutant proteins stimulated higher levels of lymphocyte activation than the wild-type protein. Further studies are required to determine the binding affinities of the mutant proteins to LIGHT. Moreover, lymphocyte proliferation in the adult SLE patient with c.C167T (p.T56I) *DcR3* was also increased compared to SLE controls with wild-type *DcR3*, supporting the role of DcR3 in SLE pathogenesis.

There was no detectable link between mutations and the serum levels of DcR3. Our findings suggest that DcR3 could be involved in SLE pathogenesis either quantitatively or qualitatively. Some patients may develop SLE by increased

serum levels of wild-type DcR3, or alternatively by a normal serum level of gain-of-function DcR3, which increases lymphocyte proliferation. Therefore, the absence of a correlation between mutations and the serum levels of DcR3 is not unexpected.

There are some SLE patients without *DcR3* mutations who are clinically similar to the patient with the C364T mutation in *DcR3* (Patients 4, 39, and 45, Appendix). They had some similar clinical observations, including oral ulcers, blood disorder, immunologic disorder, and positive antinuclear antibodies. SLE is a heterogeneous disorder and the molecular processes responsible for pathogenesis in patients with similar clinical manifestations may not be identical. The fact that out of 203 patients we found only 2 with mutations in *DcR3* suggests mutant DcR3 is involved in only a small proportion of patients with SLE.

In addition to the detection of these mutations in SLE patients, we also found the p.H122Y allele in 1 out of 500 healthy controls (1/1000 alleles). Further, family studies indicated that the 2 mutant alleles were maternally inherited, but the mothers were not diagnosed with SLE. Therefore, these mutations *per se* are unlikely to be sufficient to result in the development of SLE. It is likely that other genetic or environmental factors are required for the manifestation of SLE.

Our study represents the first identification of 2 novel mutations in *DcR3* that affect lymphocyte proliferation and an association with juvenile-onset and adult SLE.

#### ACKNOWLEDGMENT

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**Appendix 1.** Characteristics of the 61 juvenile SLE patients with serum DcR3 measurements.

Patient	Age at Diagnosis, yrs	Clinical Characteristics											At Time of Serum Collection			
		Malar Rash	Discoid Rash	Photosensitivity	Oral Ulcer	Arthritis	Serositis	Kidney Disorder	Neurological Disorder	Blood Disorder	Immunologic Disorder	ANA	Sex	Age, yrs	SLEDAI	Serum DcR3, pg/ml
1	10					+		+		+		+	F	11	0	213.65
2	13	+		+				+			+	+	F	13	16	296.00
3	10		+					+			+	+	F	12	4	0
4	13		+		+					+	+	+	F	17	0	0
5	11				+	+		+		+		+	F	15	6	142.80
6	11	+						+		+		+	F	14	0	0
7	10	+		+							+	+	F	12	0	0
8	11						+	+			+	+	F	21	0	738.25
9	11			+				+		+		+	F	12	0	166.50
10	13							+		+	+	+	F	14	16	419.60
11	12						+	+		+	+	+	F	23	0	376.35
12	10					+				+	+	+	F	13	2	201.75
13	10		+	+				+			+	+	F	16	2	83.45
14	15	+			+			+			+	+	F	20	2	0
15	6				+			+		+	+	+	F	8	8	686.44
16	11		+	+	+			+		+	+	+	F	16	4	0
17	9						+	+		+	+	+	F	12	4	156.90
18	10						+	+	+	+	+		F	10	28	504.45
19	11			+	+			+		+	+	+	M	13	9	446.10
20	12					+				+	+	+	F	15	2	0
21	6	+			+					+	+	+	F	16	12	26.98
22	7	+	+	+	+			+		+	+	+	M	13	2	0
23	12						+	+		+	+	+	M	12	2	0
24	14	+	+					+		+	+	+	F	14	6	539.20
25	12		+			+		+		+		+	F	12	10	0
26	14	+		+	+	+		+		+		+	F	15	6	0
27	8					+				+	+	+	F	11	0	0
28	10	+	+			+		+		+	+	+	F	13	11	455.45
29	6						+			+	+	+	F	18	0	0
30	12			+	+			+		+	+	+	F	13	24	2195.75
31	11	+			+						+	+	F	13	0	0
32	11				+		+	+			+	+	F	12	8	207.15
33	12	+		+	+			+		+	+	+	F	21	2	0
34	12	+		+		+					+	+	F	20	2	613.98
35	13				+			+		+		+	F	14	7	236.30
36	9	+			+			+		+	+	+	F	10	12	351.15
37	10		+		+	+		+		+	+	+	F	12	6	0
38	10	+						+		+	+	+	F	11	10	69.96
39	10	+	+		+				+	+	+	+	M	15	0	34.98
40	8		+					+		+	+	+	F	12	0	0
41	15	+			+	+		+		+	+	+	F	16	6	0
42	11	+						+		+	+	+	M	18	4	0
43	13							+		+	+	+	F	13	10	288.26
44	13							+		+	+	+	F	14	0	0
45	12	+		+	+					+	+	+	F	13	6	70.50
46	8	+	+		+					+	+	+	F	29	0	172.55
47	15							+		+	+	+	F	24	2	0
48	7				+					+	+	+	F	12	0	0
49	13	+						+	+		+	+	F	17	6	0
50	12	+			+						+	+	F	18	0	0
51	10	+		+				+		+	+	+	F	14	0	0
52	11							+		+	+	+	F	20	0	146.60
53	13	+								+	+	+	F	19	0	87.95
54	10	+				+		+		+	+	+	F	11	0	0
55	14	+			+						+	+	F	17	4	0
56	11	+			+	+		+	+	+		+	F	14	0	0
57	14			+	+			+		+	+	+	F	20	0	480.60
58	12		+						+	+	+	+	F	13	29	582.50
59	9				+	+		+		+	+	+	F	12	0	0
60	14	+		+		+		+		+	+	+	F	14	0	0
61	12							+	+	+	+	+	F	12	16	92.50

DcR3: death decoy receptor 3; ANA: antinuclear antibodies; SLEDAI: SLE Disease Activity Index; SLE: systemic lupus erythematosus.

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