A variant of death-receptor 3 associated with rheumatoid arthritis interferes with apoptosis-induction of T cell

Rheumatoid arthritis (RA) is a chronic polyarthritis of unknown etiology. To unravel the molecular mechanisms in RA, we performed targeted DNA sequencing analysis of patients with RA. This analysis identified a variant of the death receptor 3 (DR3) gene, a member of the family of apoptosis-inducing Fas genes, which contains four single-nucleotide polymorphisms (SNPs) and a 14-nucleotide deletion within exon 5 and intron 5. We found that the deletion causes the binding of splicing regulatory proteins to DR3 pre-mRNA intron 5, resulting in a portion of intron 5 becoming part of the coding sequence, thereby generating a premature stop codon. We also found that this truncated DR3 protein product lacks the death domain and forms a heterotrimer complex with wildtype DR3 that dominantly negatively inhibits ligand-induced apoptosis in lymphocytes. Myelocytes from transgenic mice expressing the human DR3 variant produced soluble truncated DR3, forming a complex with TNF-like ligand 1A (TL1A), which inhibited apoptosis induction. In summary, our results reveal that a DR3 splice variant that interferes with ligand-induced T cell responses and apoptosis may contribute to RA pathogenesis.

The death receptor 3 (DR3), also named WSL-1, Apo3, TNF-receptor-related apoptosis-mediating protein, lymphocyte-associated receptor of death (LARD), TR3, or tumor-necrosis factor receptor superfamily member 25 (TNFRSF25) (1), is a member of the tumor necrosis factor receptor (TNFR) superfamily that includes Fas, TNFR1, DR4, DR5, and DR6 (2–5). It contains four extracellular cysteine-rich motifs and a characteristic intracellular death domain capable of mediating either cellular apoptosis via TNFR-associated death domain protein (TRADD) followed by association with Fas-associated death domain or NFκB activation via TRADD followed by interaction with TNFR-associated factor 2 (TRAF2) (6, 7). The DR3 is expressed primarily in lymphocytes, upon activation with immune complex, Toll-like receptor, or inflammatory cytokines such as TNF or IL1β (2–5, 8–14). The ligand for DR3 is the TNF-like ligand 1A (TL1A), also known as TNF ligand superfamily member 15 (TNFSF15) (15). Studies have shown that TL1A expression correlates with the presence and severity of mucosal tissue inflammation of patients with ulcerative colitis and Crohn’s disease (9, 10, 16–22). Studies also suggested the importance of TL1A signaling in the pathogenesis of autoimmune or inflammatory diseases such as rheumatoid arthritis (8, 12, 13, 23–26), psoriasis (27, 28), primary biliary cirrhosis (29, 30), ankylosing spondylitis (31, 32), and allergic lung inflammation (33, 34), however, the disease mechanisms involving TL1A and its receptor DR3 especially in humans remain elusive.

In the present study, we investigated the role of DR3 by directly sequencing the entire DR3 genome to identify a novel genetic variant of DR3 encoding a truncated DR3 that inhibits ligand-induced apoptosis in a dominant-negative fashion in patients with rheumatoid arthritis (RA). We studied the expression of the variant DR3, composition of death receptor trimer, and the contribution of variant DR3 to the induction of apoptosis and arthritis.

Results

Variant of DR3

We directly sequenced the entire DR3 genome of patients with RA, and found a variant of the DR3 gene that contains four single nucleotide polymorphisms (SNPs) and one 14-nucleotide deletion, which we termed polymorphisms a, c, d, e, and b (Fig. 1A). SNPd, rs3138155; SNPc, rs3138156; SNPb, rs3138153; SNPa, rs3138158; and SNPs, rs3138156, as registered GenBankTM.© 2018 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.

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In nature, the majority of DR3-positive PBLs are T cells (Fig. S1), and the DR3 gene splice variants as expressed in the lymphocytes of Caucasians typically skip between exons 4 and 5 (designated LARD4, -5, and -6) (4). Thus, we studied the expression of variant DR3 by constructing a DR3 minigene encompassing exons 4–7 (Fig. 1B), and transfected it into Jurkat cells to detect mRNA for 4 splice variants using reverse transcription-PCR (RT-PCR) followed by Southern blotting (Fig. 1C). Product 1 includes intron 6 in its coding sequence, leading to a premature stop codon (asterisks) emerging at exon 7 (designated LARD2) (5). According to Screaton et al. (5), this LARD2 protein was not detectable in Western blots of human lymphocytes. We also did not detect this using RT-PCR, and thus, this variant was considered sterile. Products 3 and 4 contained a portion of intron 5, g.2636–g.2792 in the coding sequence, leading to a premature stop codon (asterisks) and the generation of a truncated DR3 molecule. The inserted segment of intron 5 could potentially function as an exon and polymorphism d was responsible for this insertion (Fig. 1C). Indeed, RT-PCR analysis showed that this segment of intron 5 could be

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**Figure 1. Polymorphisms of DR3 in lymphocytes.** A, location of polymorphisms in relationship to transmembrane (TM) and death domains (DD) found in patients with RA (n = 50). Numbered boxes represent exons. The polymorphisms were: a, g.1775A>G (rs11800462); b, g.2457_2382delT14; c, g.2531C>T; and e, g.2826A>G (rs3138156) from the first nucleotide of ATG sequence. These polymorphisms corresponded to the previous nomenclature, numbered from the first base of exon 1 as follows: nt 564 (A → G); Asp-159 → Gly, nt 630 + 622 (del 14), nt 631–538 (C → T), nt 631–391 (A → T), and nt 631–243 (A → G), respectively. GenBank accession numbers for DR3 cDNA, authentic genomic DR3, and variant genomic DR3 are U746116, AB051850.1, and AB051851.1.

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accession No. AB051850.1 to DR3 and AB051851.1 to variant DR3.

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Detected as a putative exon in lymphocytes from patients having variant DR3 genotype after stimulation with phorbol myristate acetate (PMA, 20 ng/ml) and phytohemagglutinin (PHA, 1 μg/ml) for 48 h (Fig. 1D). The DR3 variant was amplified from the lymphocytes of patients carrying the variant genotype. DR3 mRNA variants such as LARD3–5 (5) were difficult to detect by Northern blotting and other methods (35), but can be detected by RT-PCR (5). RT-PCR primers encompassing exon 4 and intron 5 were used to detect the putative exon encoded by intron 5 that is induced upon stimulation (Fig. 1, E and F). As shown in Fig. 1G, quantitative PCR specific for the putative exon encoded by intron 5 showed significantly higher amounts of variant DR3 mRNA (products 3 and 4 of Fig. 1C) in lymphocytes from patients with variant DR3 as compared with normal. In addition, cell lysates from patients’ lymphocytes were subjected to Western blotting using anti-DR3 (SS) antibody and anti-DR3 (CT) antibody. Because SS antibody could recognize the extracellular portion of DR3, the truncated variant DR3 was detected as a 26-kDa protein in individuals with the variant DR3 genotype (Fig. 1H).

Splicing regulatory protein binding to the variant DR3 pre-mRNA

The 5’-end of intron 5 (putative exon) contains the nucleotide sequence “tttagta,” where the first “tag” corresponds to a 3’ consensus splice sequence (GenBank accession AB051851.1). A consensus polypyrimidine tract also exists in the upstream of the tag sequence, suggesting that insertion of sequences from intron 5 into the variant DR3 mRNA could be due to the involvement of splicing modulators. Such modulators have been shown to alter gene expression in Drosophila by binding to sequences(594,195),(741,216) in a putative exon (36). Therefore, we analyzed nuclear proteins bound to DR3 pre-mRNA intron 5. We first compared the amounts of nuclear proteins bound to variant intron 5 and wildtype intron 5 pre-mRNA by silver staining. We detected the proteins of 100, 70, and 60 kDa that bound more strongly to exon 2 pre-mRNA than to wildtype intron 5. These same proteins also bound more strongly to variant intron 5 pre-mRNA than to wildtype intron 5. The amounts of nuclear proteins bound to wildtype intron 5 and SNAPd were comparable (Fig. 2A). We next analyzed the nuclear proteins bound to variant intron 5 pre-mRNA by using mass spectrometry. We identified 100-, 70-, and 60-kDa nuclear proteins as three splicing factors: proline- and glutamine-rich (SFPQ), the heterogeneous nuclear ribonucleoprotein L (hnRNP L), and the non-POU domain-containing octamer-binding protein (NONO), selectively bound to the variant intron 5 versus wildtype intron 5 (p < 0.05) encompassing SNAPd (Fig. 2B). This would suggest a model in which mutation in the DR3 sequence lead to abnormal binding of splicing regulatory proteins to DR3 intron 5, thereby inducing pre-mRNA expression and insertion of a portion of intron 5 into the resulting mRNA product.

Molecular assembly of DR3

We next examined assembly of DR3 complexes containing variant and wildtype DR3 by expressing in 293T cells. Immunoblot analysis showed that His-tagged wildtype DR3 (His-DR3) co-immunoprecipitated with EGFP-DR3D159G (wildtype DR3 with a D159G substitution, i.e. polymorphism a) (Fig. 3A, lane 3). His-DR3 also co-immunoprecipitated with the variant DR3 gene product, regardless of whether it contained the D159G substitution (EGFP-Var DR3-D159G) or not (EGFP-VarΔDR3) (Fig. 3A, lanes 4 and 5). Experiments in stably transformed BHK cells indicated that the variant DR3 specifically co-immunoprecipitated with wildtype DR3 (Fig. 3B). Immunofluorescent microscopy showed that truncated EGFP-Var DR3-D159G was co-expressed with wildtype His-DR3 on the cell surface (Fig. 3C). Thus, based on the mutations described above, truncated DR3 molecule lacking both death domain and transmembrane portion did assemble with the wildtype DR3 molecule to make a heterozygous trimmer complex.

Defect in apoptosis induction

With reference to prior discovery on the contribution of apoptosis defects to the pathogenesis of autoimmunity or arthritis (36–43), we studied the effect of the variant DR3 on death signaling (44). We transfected 293T cells with the vectors expressing wildtype DR3 and TRADD in combination with increasing amounts of variant DR3 (EGFP-Var DR3-D159G). We observed that His-DR3 co-immunoprecipitated with FLAG-tagged TRADD (FLAG-TRADD) (Fig. 4A, lane 2). The amount of TRADD co-precipitating with His-DR3 gradually decreased with increasing amounts of EGFP-Var DR3-D159G added (Fig. 4A, lanes 3 and 4), indicating that the variant DR3 dominantly interfered with the assembly of wildtype DR3 and TRADD, thereby inhibiting apoptotic signal transduction. This is consistent with the finding that truncated DR3, which lacks the normal death domain, does not bind TRADD (3). To verify the effect of Var DR3-D159G on apoptosis induction, we transfected 293T cells with wildtype DR3 and Var DR3-D159G, tagged with GFP. Cells transfected with wildtype DR3 displayed a morphological alteration typical of cells undergoing apoptosis (Fig. 4B, top). However, in the case of transfection with Var DR3-D159G, cells did not display any apoptotic changes (Fig. 4B, bottom), whereas a small number of apoptotic cells existed in the case of co-transfection with wildtype and Var DR3-D159G (Fig. 4B, middle, arrowheads). Caspase-8 activity was decreased as increasing amounts of variant DR3-D159G were transfected (Fig. 4B, right). We also observed that ligand-induced apoptosis by TL1A (15) was defective in the lymphocytes of patients having the variant DR3 genotype, as shown by annexin V-staining (Fig. 4C) and Western blotting for time-dependent caspase 8 cleavage (Fig. 4D). Furthermore, apoptosis induction in lymphocytes by an agonistic anti-DR3 (SS) antibody was defective in patients harboring variant DR3 (n = 8) as compared with healthy controls (n = 20) (Fig. 4E).

Studies of transgenic mice expressing variant type human DR3 gene

We generated the transgenic (TG) mice expressing a variant type human DR3 gene (vhDR3 mice) (Fig. 5, A and B). Splenocytes from TG or littermate mice were treated with cycloheximide (10 μg) for 1 h, followed by treatment with 300 ng/ml of human recombinant TL1A overnight. Because cleavage of Caspase 3 and poly(ADP-ribose) polymerase (PARP) is considered hallmarks of apoptosis (44), they were determined by Western blots. As shown in Fig. 5C (left), splenocytes from TG mice did not
show cleavages of both Caspase 3 and PARP, whereas cleaved substrates were clearly detected in littermate’s splenocytes. Furthermore, because DR3 is also able to induce NFκB activation via TRADD, we studied TL1A-induced NFκB activation to find that although NFκB degradation and nuclear translocation of NFκB p65 were operated minutely in wildtype mice, those were disturbed in TG splenocyte (Fig. 5C, right).

Next, myelocytes from vhDR3-TG mice and littermate control mice were cultured in vitro, and subjected to immunoblotting with anti-human DR3 antibodies. The vhDR3 molecule was specifically detected as a truncated protein of 28 kDa (Fig. 5D, left upper). When supernatants were subjected to immunoprecipitation with anti-TL1A Abs, soluble vhDR3 could be detected in the vhDR3-TG supernatants, indicating the formation of a complex between vhDR3 and human TL1A (Fig. 5D, left lower). To further examine the effect of vhDR3 on TL1A-induced signaling for cell death, TF-1 cells were stimulated overnight with 300 ng/ml of TL1A in the presence of culture supernatants from TG or littermates myelocytes. As sown in Fig. 5D (right), the culture supernatants from TG mice did block the TL1A-induced cleavage of PARP dose-dependently.

Finally, type II collagen-induced experimental arthritis was induced in vhDR3-TG mice (n = 12) and littermates (n = 10), and the arthritis score of vhDR3-TG mice was significantly higher than those of littermates on days 6–10 (p < 0.01) (Fig. 5D). We found strong inflammatory changes, including layered thickening of synovial cells and massive invasion of lymphocytes, and up-regulated expression of MMP3 in the joint of TG mice, whereas TUNNEL positive cells were not detected, indicating that apoptosis-induction was disturbed by vhDR3 (Fig. 5E).

**Variant DR3 gene in rheumatoid arthritis patients**

The variant DR3 gene as examined by the presence of the polymorphism d was found in 15 of 611 (2.45%) anti-citrullinated protein antibody (ACPA)-positive patients with RA, 15 of 538 (2.79%) ACPA- and rheumatoid factor-positive patients with RA, and 1 of 138 (0.72%) ACPA-negative patients with RA. The DR3 variant gene was found in 2 of 547
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Figure 3. Assembly of variant and authentic DR3 molecules in a transient expression system. A, assembly of variant and authentic DR3 molecules in a transient expression system. Lysates of 293T cells were immunoprecipitated (IP) and subjected to Western blotting (WB) with anti-His monoclonal antibody and anti-GFP antibody. His-tagged wildtype DR3 (His-DR3) could assemble with DR3 having an amino acid substitution due to polymorphism a (DR3-D159G), and with variant DR3 with (Var DR3-D159G) or without (Var DR3) this amino acid substitution. B, assembly of truncated V5-tagged variant DR3-D159G (V5-VarDR3-D159G) with DR3, DR4, or DR5 expressed in stable BHK transformants. Lysates were immunoprecipitated with anti-V5 monochlonal antibody and blotted with an anti-DR3 (CT) antibody specifically recognizing the death domain, anti-DR4 antibody, anti-DR5 antibody, or an anti-V5 monochlonal antibody recognizing V5-Var DR3-D159G. C, immunofluorescent microscopy showing assembly of variant DR3 (EGFP-Var DR3-D159G; green) with wildtype DR3 (His-DR3; red) on the cell surface of 293 T cells (yellow).

(0.37%) healthy control individuals. The odd’s ratio and relative risk as compared with healthy controls were 6.86 and 6.71 for ACPA-positive patients with RA, and those were 7.82 and 7.63 for ACPA- and rheumatoid factor-positive patients with RA. When the patients who underwent joint replacement surgery were retrospectively compared, the frequency of variant DR3 was 8 of 42 (19.0%) and 0 of 59 (0%) in patients with RA and osteoarthritis, respectively (p = 0.00058), suggesting that variant DR3 may predispose to progressive joint destruction.

Discussion

We identified a DR3 polymorphism that is over-represented in patients with RA. The DR3 variant, containing four SNPs and one 14-nucleotide deletion within exon 5 and intron 5, resulted in the insertion of a portion of intron 5 into the coding sequence, thereby generating a premature stop codon. We showed that several splicing factors SFQ, hnRNP L, and NONO selectively bound to the mutated DR3 intron 5 pre-mRNA versus wildtype intron 5. SFQ is a factor required early in splicingosome formation (45, 46), and the SFQ–NONO splicing complex is involved in the binding of U4/U5/U6 tri-small nuclear ribonucleoprotein (47). The hnRNP L regulates the splicing pathway by binding to CAR motifs and activation-responsive sequence motifs (48, 49). Because genetic mutations near the splice sites can alter splicing patterns (50, 51) and splicing enhancers in introns can promote exon insertion (52), we envisioned that these splicing regulatory proteins bound to the mutated DR3 intron 5 pre-mRNA might promote the insertion of a portion of intron 5 into the final mRNA to generate the variant DR3. A very similar splice variant of the Fas gene that contains a portion of intron 5 as a putative exon has been reported in cutaneous T cell lymphoma (53).

Previous studies have shown that the death receptor containing DR3 efficiently transmits the death signal via complex formation with TRADD and Fas-associated death domain (3, 54). Borysenko et al. (56) have pointed out in patients with RA that one polymorphism in the DR3 variant, that we named ‘polymorphism a,” i.e. g.1755A→G; Asp-159 → Gly (GenBank accession numbers AB051851.1 and AB308321.1), occurs in a region critical for the structural integrity of ligand–receptor complexes (55). This could lead to destabilization of the variant DR3. We now show that a DR3 variant devoid of its own death domain can assemble with authentic DR3 to inhibit the apoptosis induction in the lymphocytes of rheumatoid patients. This is consistent with a previous finding showing that a truncated DR3 lacking the death domain did not bind TRADD (3). We in fact found that the lymphocytes of patients expressing the variant DR3 were highly resistant to ligand-induced apoptosis (Fig. 4E). Furthermore, in experiments, the myelocytes of mice over-expressing the human truncated DR3 molecule produced a soluble protein (vhDR3) that inhibited apoptosis induction in vitro in a similar fashion to Fas (57, 58), and experimental collagen-induced arthritis was enhanced in vivo in transgenic mice expressing vhDR3. Thus, whereas DR3 is characterized by the existence of plenty of splice variants (1, 3, 5), a novel mechanism of DR3 splice variants that interferes with ligand-induced apoptosis seems to operate in the disease process in RA. Furthermore, the following findings may add evidence to the contribution of genetic or functional defects of DR3 to the pathogenesis of RA. DR3 gene duplication was over-represented in RA (59). The CpG islands in the promoter region of the DR3 gene were hypermethylated, where inverse correlation existed in the levels of serum DR3 protein and promoter CpG methylation in rheumatoid synovia (60). In relationship to DR3, TRAIL-induced apoptosis has been shown to be defective in the monocytes of RA patients (60), and the decoy receptor 3 that protects cells from Fas-induced apoptosis was also over-represented in RA (61, 62).

Experimental procedures

Patients and samples

Peripheral blood samples from patients with RA and healthy controls were obtained from the Konan-Kakogawa hospital,
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Kobe University Hospital, with written informed consents, based on the approval by the institutional review board of Kobe University.

DNA sequencing

Genomic DNA, obtained from PBL of patients with RA and healthy controls, was directly sequenced or sequenced after cloning into pT7Blue vector (Promega, Madison, WI).

Minigene experiment

A portion of the DR3 genome encompassing exons 4–7 was amplified, subcloned into the pcDNA3.1 vector (Promega), and transfected into Jurkat cells (Riken, Tokyo, Japan). Total RNA was extracted from cells stimulated with PMA (20 ng/ml) and PHA (1 μg/ml) for 48 h, amplified, and analyzed by RT-PCR and Southern blotting. DNA was transferred to Hybon-N nylon membrane (Amersham Biosciences), and hybridized with the exon 4 probe 5′-GCCAGGCTGGTTTGTGGA-3′ or the intron 5 probe 5′-CTCATGCTGTAATCCCAGC-3′. For site-directed mutagenesis, genomic DNA was amplified by AmpliTaq DNA polymerase using the primers 5′-GGGGTACCATCCGCTTCCTGCCCCAGGCTGGTTTGTGGA-GTGC-3′ (forward) and 5′-CCGCTCGAGGGGCCACCTCCAGTGCCAGTGGCGGTATGTGTAGGTCAGG-3′ (reverse), and the products were subcloned into pcDNA3.1 (Promega).

Site-directed mutagenesis to mutations a, c, d, and e was performed in a 25-μl reaction containing 40 μM of each dNTP, reaction buffer, 1.25 units of Pfu Turbo DNA polymerase (Stratagene), 62.5 ng each of the sense and antisense primers, and 50 ng of the wildtype vector. Primers were: 5′-GGTTC-
CCGCAGAGGTACTGACTGTGGGA-3' (sense) and 5'-H11032-TCC-3
CACAGTCAGTACCTCTGCGGGAACC-3' (antisense).

Mutation “a” was introduced at underlined bases. Other mutations, c, d, and e, were introduced likewise.

Statistical analysis
Data are expressed as the mean ± S.D., and Student’s t test and Fisher’s exact test were used for statistical analyses. All statistical tests were two-sided and were performed at an α level of 0.05.

Study of splicing regulatory proteins
Wildtype (wt) intron 5, variant type (var) intron 5 having mutations of SNP b, c, d, and e (Var intron 5) and wt exon 2 in

Figure 5. Studies of TG mice expressing variant-type human DR3 gene. A, the construction of genes injected into C57BL/6 mice. B, RT-PCR of variant human DR3 mRNA (vhDR3) in organs from TG or littermate (ctr) mice. C, Western blot of Caspase 3, PARP, IκBα, and NF-κB p65 after TL1A (300 ng/ml) stimulation overnight followed by cycloheximide (10 μg/ml) treatment for 1 h. D, functional add-back assay of vhDR3. Left upper, detection of vhDR3 protein. A truncated DR3 molecule was specifically detected in vhDR3-TG myelocytes. Lower left, interaction between soluble vhDR3 and TL1A. TL1A-bounded soluble vhDR3 molecule from culture supernatants of TG myelocytes were analyzed by Western blotting after immunoprecipitation using anti-TL1A Abs. Right, inhibition of PARP cleavage by soluble vhDR3. TF-1 cells were stimulated overnight with TL1A (300 ng/ml) and culture supernatant containing soluble vhDR3 from TG or littermate myelocytes. E, evaluation of collagen-induced arthritis. Assessment of arthritis was made until 14 days after the 2nd immunization (score range: 0–12, * p < 0.01). F, histological evaluation of collagen-induced arthritis joints including HE staining, immunohistochemistry for MMP3, and TUNNEL staining (day 14). Positive control for MMP3 and TUNNEL were mouse spleen and rat thymus, respectively.
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The human DR3 gene were amplified from PBL of a patient with RA by RT-PCR. Amplified products used as templates to generate RNA using ScriptMAX™ Thermo T7 Transcription Kit (TOYOBO CO., Osaka, Japan). RNA-binding nuclear proteins were isolated using the synthesized RNA (400 pmol) and the 3'-biotinylated single strand (ss) oligonucleotide (500 pmol, Sigma) using the MACS™ Streptavidine Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). RNA-binding nuclear proteins were resolved by SDS-PAGE, visualized using the Silver StainII Kit from Wako (Wako Pure Chemical Industries, Osaka, Japan).

Antibodies used for Western blotting were: mouse anti-SFPQ monoclonal Ab, mouse anti-hnRNP L monoclonal Ab, and rabbit anti-NONO polyclonal Ab were purchased from Santa Cruz, CA, and ProteinTech Group (Chicago, IL), respectively. The levels of SFPQ, hnRNP L, and NONO proteins bound to RNA were determined by semiquantification of digitally captured image using the public domain NIH Image program. Values were normalized to the level of nuclear protein bound to wt intron 5 pre-mRNA.

mRNA analysis

For RT-PCR, total RNA was extracted from lymphocytes (1 × 10⁶ ml⁻¹) after stimulation with PMA (20 ng/ml) and PHA (1 μg/ml) for 48 h. For quantitative PCR, total RNA was extracted from lymphocytes (1 × 10⁶ ml⁻¹) after stimulation with PHA (1 μg/ml) and recombinant human interleukin-2 (rhIL-2, 20 ng ml⁻¹) for 48 h and then with rhIL-2 (20 ng ml⁻¹) for 11 days.

Probes used for RT-PCR were: 5'-CCCGTGTACCTCACA-AGAAG-3' (forward, exon 2) and 5'-GTTCATAGAGCAGG-3' (reverse, exon 5). For authentic and variant DR3 mRNA: the latter probe was 5'-TCGAGATGTACCTCTTCT-3' (forward, exon 4, for 1st PCR), 5'-ACTGCCAACACCA-TGCCATTAGAC-3' (forward, exon 4 for 2nd PCR), and 5'-CATCTTGGCTACACGGT-3' (reverse, intron 5, for 1st and 2nd PCR). TaqMan probes and primers for quantitative RT-PCR were 5'-TCTGGGCCCCACGGAC-3' (exons 5–6), 5'-TGTTCCCGAGGATACTGACT-3' (forward, exons 4–5) and 5'-ACCCAGAACATCTGCTCT-3' (reverse, exons 6–7) encompassing exons 4–7 for wildtype DR3; 5'-TGCTGCTGGCTTCTATGAACAT-3' (exon 5), 5'-TG-TTCCCGAGGATACTGACT-5' (forward, exons 4–5) and 5'-AACACGTTGAAACCCGCCT-3' (reverse, intron 5) encompassing exon 4 to intron 5 for mutant DR3. Glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control.

Study of DR3 assembly

Western blots were probed with anti-DR3 (SS) or anti-DR3 (CT; Imgenex, San Diego, CA) antibody. Anti-DR3 (SS) antibody, raised by multiple antigen technology (Amersham Biosciences) against N-terminal 25–46 amino acids, specifically bound DR3 and did not without cross-react against DR4, DR5, DR1, DR2, or Fas in BHK and WR19L transfectants.

Variant and wildtype DR3 cDNA was subcloned into the pT7Blue vector (Promega) and integrated into the pCDNA3.1/His vector (Invitrogen) to synthesize His-tagged DR3 (His-DR3). Likewise, enhanced green fluorescent protein (EGFP)-tagged authentic DR3, EGFP-tagged variant DR3 with (Var DR3-D159G) or without (Var DR3) amino acid substitution, and V5–Var DR3-D159G were synthesized. We transfected 293T or BHK cells expressing DR3, DR4, or DR5 with these vectors using Lipofectamine Plus reagent (Invitrogen). Lysates were immunoprecipitated with anti-His (Invitrogen) or anti-V5 (Invitrogen) monoclonal antibody, and reacted with antibodies to GFP (Santa Cruz Biotechnology), His, FLAG (Sigma), V5, DR4 (Santa Cruz), DR5 (Sigma), and DR3 (SS). Morphological changes and manner of molecular assembly of cells were detected by phase-contrast and fluorescent microscopy.

In Fig. 3C, wildtype DR3 (His DR3) was stained with tetramethylrhodamine isomer R (TRITC)-conjugated anti-rabbit antibody, and variant DR3 (EGFP-Var DR3-D159G) was stained with anti-GFP antibody. In Fig. 4B, both wildtype DR3 and variant DR3 were tagged by EGFP and stained with anti-GFP antibody, as referred to phase-contrast images.

Induction of apoptosis

The TRADD cDNA was amplified using primers 5'-CGAGGCGGCCAAGGTTG-3' (forward) and 5'-GGTTCAGCATAGGCCGAGAC-3' (reverse), and subcloned into pT7Blue (Promega), then integrated into the pCMV-Tag 2 vector (Stratagene) to synthesize FLAG-tagged TRADD. We transfected 293T cells with EGFP-Var DR3-D159G and/or His-Var DR3, and observed apoptosis by immunofluorescence microscope.

The 293T cells were also transfected with His-DR3 and/or Var DR3-D159G, and cell lysates were assessed for caspase 8 activity and DR3 protein simultaneously after 24 h. Lysates were reacted with Ac-IETD-AMC substrate (Peptide Institute, Edmonton), and caspase activity was determined by the fluorescence of released AMC in a CytoFluor 4000 (ABI) fluorescence spectrophotometer. Arbitrary fluorescence units (ΔAFU) were calculated as: (caspase activity of DR3 vector) – (caspase activity of empty vector).

Lymphocytes (1 × 10⁶/ml) were stimulated with TL1A (200 ng/ml) for 16 h and observed for surface annexin V (Trevigen, Gaithersburg, MD) expression by flow cytometry. Lysates from stimulated lymphocytes pretreated with cycloheximide were incubated with TL1A (200 ng/ml: R&D Systems) for 8–16 h and subjected to SDS-PAGE, followed by Western blotting with anti-caspase 8 antibody (Cell Signaling Technology, Danvers, MA). Human lymphocytes were treated with agonistic anti-DR3 (SS) antibody, anti-Fas monoclonal antibody (agonistic antibody CH-11; MBL, Nagoya, Japan), or anti-Fas polyclonal antibody (neutralize antibody HPA027444; Funakoshi, Tokyo, Japan) for 48 h, and the percent of cell death was calculated.

DR3 transgenic mouse

C57BL/6 mice were obtained from SLC (Hamamatsu, Japan). Mutant-type human DR3 cDNA (consisted of exon 1 to 5 with a point mutation of g.1755A→G; Asp-159, an intron 5 segment including stop codon) were ligated into downstream of the mouse H-2 promoter. The construct was injected into fertilized eggs of C57BL/6 mice.
Splenocyte and TF-1 cells were lysed by hypotonic buffer (25 mM Tris, pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 1.5 mM EGTA, 0.5% sodium deoxycholate, 1 mM PMSF, and 10 mM sodium orthovanadate). After the cytoplasmic protein was clarified by centrifugation at 15,000 rpm for 10 min, nucleus protein samples were obtained from the centrifuged pellet. The precipitation was lysed with buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM PMSF, and 10 mM sodium orthovanadate. Samples were probed with antibodies to Caspase 3, PARP (Cell Signaling Technology), IkBa, NFkB/p65 (BD Bioscience), and TL1A (Santa Cruz).

Myelocytes were obtained from mice femurs and cultured in RPMI 1640 with 10% FBS. After overnight culture, cells were incubated with 25 ng/ml of mouse GM-CSF (Wako) for 72 h in serum-free medium (COSMEDIUM; Cosmo Bio, Tokyo, Japan) and subjected to Western blots probed with anti-DR3 (SS). The culture supernatants of mice myelocytes were concentrated using Centriplus concentrators (Millipore, Bedford, MA), followed by immunoprecipitation with anti-TL1A Abs (R&D Systems) and Western blot with anti-DR3 Abs (B65; Neomarkers Inc., Fremont, CA). TF-1A cells were stimulated with TL1A (300 ng ml⁻¹) and culture supernatants of myelocytes from TG or littermate control mice overnight. Western blot of TF-1A cells were probed with anti-total PARP, and anti-cleaved PARP (Cell Signaling Technology).

For induction of CIA, bovine type II collagen (Koken, Tokyo, Japan) was dissolved in 0.1M acetic acid solution and emulsified for 2 weeks with complete Freund’s adjuvant (CFA) (Difco, Detroit, MI). 7-Week-old DBA1/B6 mice, generated by backcross of C57BL/B6 mice toward DBA1/J mice until F6, were immunized by 200 μl of the emulsion. 21 days after primary immunization, an additional injection was performed as a booster immunization. The arthritis score was evaluated by each paw according to the following grading scale: 0 = normal joint, 1 = 1 or 2 swollen joints or slight swelling of front and hind paws, 2 = more than 3 swollen joints or significant swelling of front and hind paws, 3 = extreme swelling of the entire paw. An arthritis score (range 0–12) was assigned to each mouse by summing the scores of each paw. For histological studies, anti-MMP3 antibody (ab35015, Abcam) was used for immunohistochemistry and the Apo-1 Peroxidase In Situ Apoptosis Detection Kit (S7100, Millipore) was for TUNEL staining.

Author contributions—A. H. performed and analyzed most of the experiments and wrote the manuscript. Y. K., K. M., and H. K. performed DNA sequencing, genotyping, and study of DR3 assembly. K. Y. and M. M. performed study of splicing regulatory proteins. K. T., T. S., and H. K. assisted with the study of apoptosis and analysis of results. K. T. performed study of DR3 transgenic mice. K. K. performed genotyping. T. K., H. Y., and K. S. collected human samples. S. S. conceived the idea for the project, supervised the experiments, and wrote the manuscript. All authors contributed to the final manuscript.

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