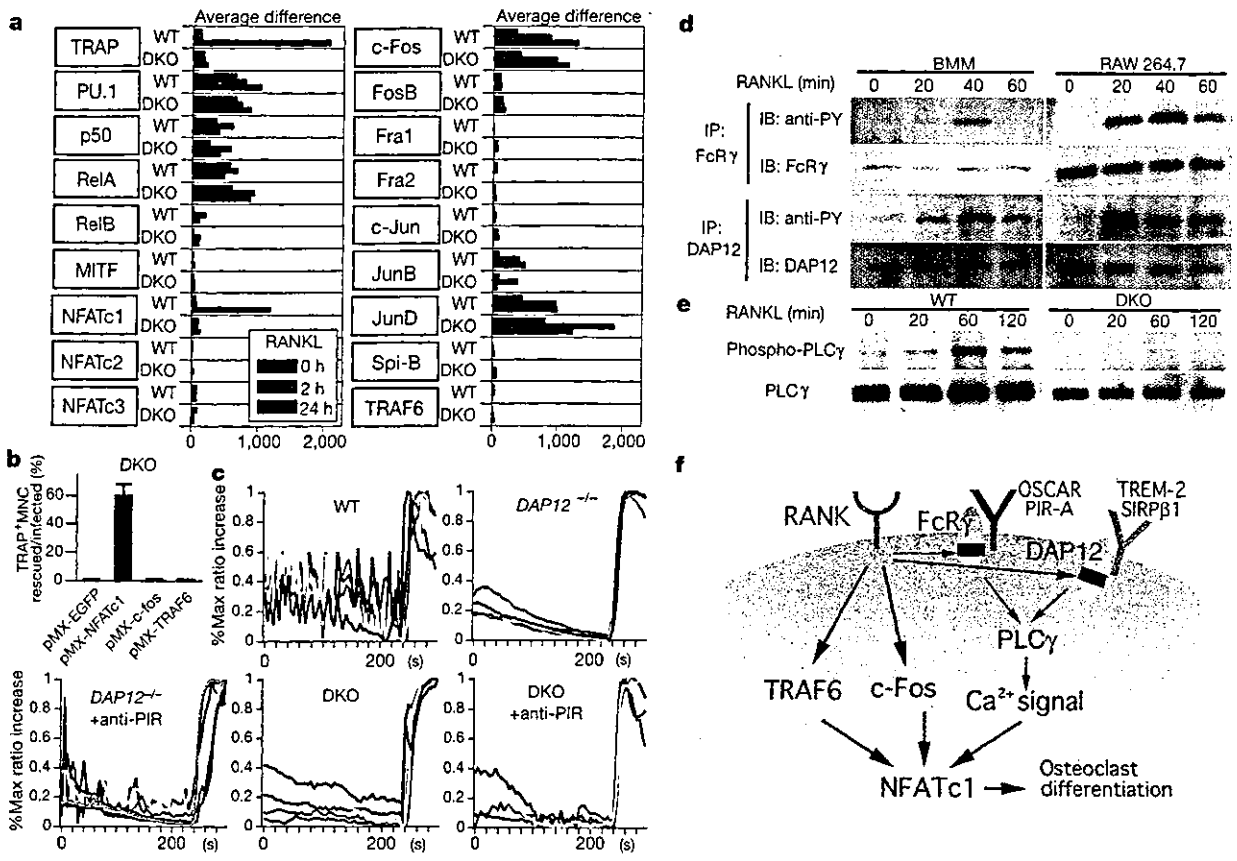

Figure 4 ITAM-harbouring adaptors are essential for RANKL induction of calcium signalling and NFATc1. **a**, GeneChip analysis of mRNA expression of transcription factors and effector molecules involved in RANKL signalling. NFATc1 induction is most strongly impaired in *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ (DKO) cells. **b**, Introduction of NFATc1 by pMX retrovirus vector (pMX-NFATc1) into the *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ precursor cells efficiently rescued the osteoclastogenesis (with bone-resorbing activity; not shown) in the presence of RANKL and M-CSF. **c**, Calcium signalling in osteoclast precursor cells stimulated with RANKL and M-CSF for 24 h. Calcium oscillation was not observed in *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ or *DAP12*$^{-/-}$ cells. Stimulation with plate-bound anti-PIR antibody rescued the calcium signalling in *DAP12*$^{-/-}$ cells, but not in *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ cells. **d**, Phosphorylation of FcRγ and DAP12 by RANKL. Phosphorylation of FcRγ and DAP12 induced by RANKL in BMMs as well as in RAW 264.7 cells. PY, phosphotyrosine. **e**, Impaired phosphorylation of PLCγ1 by RANKL in *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ cells. **f**, A schematic model of ITAM-mediated costimulatory signal in RANKL-stimulated induction of osteoclast differentiation. Phosphorylation of ITAM stimulated by immunoreceptors and RANKL–RANK interaction results in the recruitment of Syk family kinases, leading to the activation of PLCγ and calcium signalling, which is critical for NFATc1 induction. NFATc1 induction is also dependent on c-Fos and TRAF6, both of which are activated by RANKL. RANKL may also contribute to efficient ITAM signalling through the induction of immunoreceptors or their putative ligands.

osteoclast precursor cells derived from wild-type and *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ mice. Among the transcription factors and effector molecules involved in RANKL signalling[6], the induction of nuclear factor of activated T cells c1 (NFATc1) is most strongly suppressed in *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ cells (Fig. 4a). We analysed the protein expression of essential molecules for osteoclastogenesis[2,4,6] including NFATc1, c-Fos and TRAF6, and revealed that NFATc1 expression in *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ cells stimulated with RANKL is barely detectable, but the expression of c-Fos or TRAF6 is still observed under the same conditions (Supplementary Fig. 4a). To confirm the crucial role of NFATc1 as a downstream target, we examined whether ectopic expression of NFATc1, c-Fos or TRAF6 by retrovirus-mediated gene transfer rescues the differentiation block of osteoclasts in *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ cells. Ectopic expression of NFATc1, but not c-Fos or TRAF6, resulted in efficient osteoclast formation even in *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ cells (Fig. 4b).

RANKL-induced calcium signalling is essential for autoamplification of the *NFATc1* gene during osteoclastogenesis[6], whereas immunoglobulin-like receptors activate phospholipase Cγ (PLCγ) and calcium signalling through ITAM in immune cells[13,21]. We therefore examined calcium signalling in RANKL-stimulated osteoclast precursor cells derived from *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ mice. As shown in Fig. 4c, calcium oscillation induced by RANKL is not significantly observed in *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ cells, which suggests that the inhibition of this calcium signalling explains the impaired induction of NFATc1. Furthermore, stimulation with the anti-PIR antibody rescued the defect in calcium signalling and NFATc1 expression in *DAP12*$^{-/-}$ cells, suggesting that the stimulation of an immunoreceptor is required for RANKL-induced activation of the calcium signal leading to NFATc1 induction and osteoclastogenesis (Fig. 4c and Supplementary Fig. 4b). These findings suggest that FcRγ and DAP12 are required for the induction of NFATc1, the crucial step in the RANKL-induced osteoclast differentiation programme, through activation of calcium signalling.

To gain further insight into the mechanism linking RANKL and ITAM-mediated calcium signalling, we analysed the phosphorylation events induced by RANKL. Our analysis revealed that RANKL induces the phosphorylation of both FcRγ and DAP12 in BMMs as well as in RAW 264.7 cells (Fig. 4d). We further demonstrated that RANKL-induced phosphorylation of PLCγ is impaired in *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ cells (Fig. 4e), but RANKL-induced phosphorylation of p38, JNK and IκB is not affected (Supplementary Fig. 4c). This suggests that FcRγ and DAP12 are specifically

involved in the PLCγ–calcium pathway. In addition, piceatannol, the inhibitor of Syk kinase, which is recruited to ITAM in immune cells, has an inhibitory effect on osteoclastogenesis (Supplementary Fig. 4d).

Our results show that the ITAM-harbouring adaptors FcRγ and DAP12 deliver essential signals, in concert with RANK-induced signalling cascades, for terminal differentiation of osteoclasts. Our study establishes the importance of the ITAM-mediated costimulatory signal in RANKL-induced osteoclast differentiation (Fig. 4f), which is analogous to the role of costimulatory signals in the immune system[13,21]. Although most of the ligands for the immunoreceptors remain to be identified, putative ligands are provided by distinct cell types: osteoclast precursor cells themselves stimulate exclusively DAP12-associated receptors, but osteoblasts also stimulate FcRγ-associated receptors. The defective osteoblast function in *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ mice suggests that the osteoclast–osteoblast communication through these receptors may couple bone resorption to formation. It remains to be elucidated whether inhibitory receptors such as FcγRIIB and PIR-B (Supplementary Fig. 3a) contribute to counterbalancing the ITAM signal in osteoclast differentiation. It is notable that osteoclastogenesis is enhanced in mice lacking phosphatases such as SHP-1 or SHIP-1, which counterbalance the ITAM signal in the immune system[23,24]. The mutations of the ITAM-harbouring adaptors and their associating receptors are related to pathological conditions in the skeletal system[25–27], suggesting that the regulation of costimulatory signals may provide a novel strategy for the treatment of skeletal disorders. □

Methods

Mice and analysis of bone phenotype

DAP12$^{-/-}$ *FcRγ*$^{-/-}$ mice were generated by crossing between *DAP12*$^{+/-}$ *FcRγ*$^{+/-}$ males and females obtained by mating of *DAP12*$^{-/-}$ mice in the 129/SvJ and C57BL/6 (B6) hybrid background[16] with *FcRγ*$^{-/-}$ mice in B6 background[20]. *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ mice grow normally and are fertile, without gross abnormalities. *FcγRIII*$^{-/-}$ mice have been described previously[28]. All experiments were performed with appropriate littermate controls. Histological, histomorphometric and microradiographic examinations were performed using essentially the same method as described previously[29]. Statistical analysis was performed using the Student's *t*-test (#$P < 0.05$, *$P < 0.01$, **$P < 0.001$). All mice were born and kept under pathogen-free conditions.

Flow cytometry and immunoprecipitation

Complementary DNAs for *FcRγ*, *DAP12*, *DAP10* and *RIIB–OSCAR* chimaera coding for the extracellular domain of FcγRIIB and the transmembrane and cytoplasmic portion of OSCAR were inserted into the pIRES-puro vector (BD Biosciences). 293T cells were transfected transiently with these vectors, harvested after 48 h, stained with PE-conjugated anti-FcγRIIB/III antibody (2.4G2) and monitored by flow cytometry. For immunoprecipitation, cells were lysed with digitonin buffer (1% digitonin, 13.6 mM triethanolamine, 150 mM NaCl, 1 mM EDTA, 10 mM iodoacetamide, protease inhibitors, pH 7.8). Cell lysates were incubated with 2.4G2 (ATCC), anti-OSCAR, anti-TREM-2 (6E9) (prepared by T. Takai), anti-PIR (6C1, a gift from H. Kubagawa), anti-NKG2D (Santa Cruz) and anti-SIRPβ1 (prepared by T. M.) antibodies and Protein A Sepharose. The specificity of newly prepared antibodies was determined using flow cytometry, and their agonistic effects on ITAM-mediated signalling events were confirmed. Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotted with anti-FcRγ or -DAP12 antibodies (prepared by T. Takai).

In vitro osteoclastogenesis

We have described our method of *in vitro* osteoclastogenesis previously[17,29]. Briefly, after depleting adherent cells, non-adherent bone marrow cells were cultured in α-MEM (Gibco BRL) with 10% FBS (Sigma) containing 10 ng ml^{-1} M-CSF (Genzyme). After two days, adherent cells were used as BMMs. Monocyte/macrophage progenitor cells of *DAP12*$^{-/-}$ *FcRγ*$^{-/-}$ mice were derived from the spleen and similarly cultured with M-CSF for 2 days. These osteoclast precursor cells were further cultured in the presence of 100 ng ml^{-1} soluble RANKL (Peprotech) and 10 ng ml^{-1} M-CSF to generate osteoclasts (RANKL/M-CSF system). RANKL/M-CSF were used at these concentrations throughout the paper unless otherwise described. To examine the *in vitro* osteoclastogenesis from *DAP12*$^{-/-}$ BMMs, the contamination of stromal/osteoblastic cells should be strictly avoided[17]. These culture conditions are different from those previously described[16], in which osteoclast formation was not completely blocked. The co-culture of osteoclast precursor cells and osteoblasts derived from calvarial cells was performed in the presence of 10^{-8} M 1, 25 $(OH)_2$ vitamin D_3 and 10^{-6} M dexamethasone in the absence of recombinant RANKL and M-CSF as described previously[19]. Three to five days later, TRAP$^+$ multinucleated (more than three nuclei) cells were counted. All data are expressed as mean ± s.e.m. ($n = 6$). TRAP$^+$ MNCs were characterized by examining the bone-resorbing activity on dentine slices as described previously[6]. For crosslinking experiments, culture plates were preincubated with PBS containing 5 μg ml^{-1} antibody at 4 °C for 24 h before seeding precursor cells.

Retroviral gene transduction

Retroviral vectors pMX-DAP12, pMX-DAP12Y65F, pMX-FcRγ and pMX- FcRγY65F were constructed by inserting *DAP12* or *FcRγ* cDNA, and their mutant cDNAs generated by PCR-directed mutagenesis into pMX-IRES-EGFP vector[17]. Other vectors such as pMX-NFATc1, pMX-c-fos and pMX-TRAF6 have been described previously, and packaging was performed as described elsewhere[6,29]. Two days after inoculation, BMMs were cultured with RANKL and M-CSF. After four days, osteoclastogenesis was evaluated by TRAP staining. The rescuing effect was normalized by measuring infection efficiency assessed by GFP expression as previously described[6,29].

GeneChip analysis

RNA extraction was previously described[6]. Total RNA (15 μg) was used for cDNA synthesis by reverse transcription followed by synthesis of biotinated cRNA through *in vitro* transcription. After cRNA fragmentation, hybridization with mouse U74Av2 or A430 GeneChip (Affymetrix) was performed and analysed according to the manufacturer's protocol. GeneChip analysis was repeated several times and yielded similar results; a representative set of data is shown.

Calcium measurement

Osteoclast precursor cells were incubated with RANKL in the presence of M-CSF for 24 h and subjected to calcium measurement as previously described[6]. RANKL does not induce a calcium spike immediately in the osteoclast precursor cells, although it does so in the mature osteoclasts[6,30].

Phosphorylation of FcRγ/DAP12 and PLCγ

Osteoclast precursor cells or RAW 264.7 cells were stimulated by 100 ng ml^{-1} soluble RANKL after 6 h of serum starvation. After various time periods, cell extracts were harvested from the cells using TNE buffer containing 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 2 mM Na_3VO_4, 10 mM NaF and 10 μg ml^{-1} aprotinin. Cell extracts were incubated with 1 μg of anti-DAP12 or anti-FcRγ antibodies for 1 h at 4 °C. Immune complexes were recovered with Protein A Sepharose, subjected to SDS-PAGE and blotted with anti-phosphotyrosine antibody (4G10, Upstate) or the indicated antibodies. Activation of PLCγ was detected using anti-PLCγ1 and anti-phospho-PLCγ1 antibodies (Santa Cruz).

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A Splice Variant of the TCR ζ mRNA Lacking Exon 7 Leads to the Down-Regulation of TCR ζ, the TCR/CD3 Complex, and IL-2 Production in Systemic Lupus Erythematosus T Cells[1]

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The reduction or absence of TCR ζ-chain (ζ) expression in patients with systemic lupus erythematosus (SLE) is thought to be a factor in the pathogenesis of SLE. We previously reported a splice variant of ζ mRNA that lacks the 36-bp exon 7 (ζ mRNA/exon 7(−)) and is accompanied by the down-regulation of ζ protein in T cells from SLE patients. In this study, we show that EX7− mutants (MA5.8 cells deficient in ζ protein that have been transfected with ζ mRNA/exon 7(−)) exhibit a reduction in the expression of TCR/CD3 complex and ζ protein on their cell surface as well as a reduction in the production of IL-2 after stimulation with anti-CD3 Ab, compared with that in wild-type (WT) mutants (MA5.8 cells transfected with the WT ζ mRNA). Furthermore, real-time PCR analyses demonstrated that ζ mRNA/exon 7(−) in EX7− mutants was easily degraded compared with ζ mRNA by the WT mutants. Pulse-chase experiment showed ζ protein produced by this EX7− mutants was more rapidly decreased compared with the WT mutants. Thus, the lower stability of ζ mRNA/exon 7(−) might also be responsible for the reduced expression of the TCR/CD3 complex, including ζ protein, in SLE T cells. ***The Journal of Immunology,*** **2005, 174: 3518–3525.**

Systemic lupus erythematosus (SLE)[3] is an autoimmune disease of unknown etiology (1–3). The disease is characterized by a large number of immunological abnormalities that appear to result from defects in T cells, B cells, and monocytes (4, 5). T cells are considered to be central to the pathogenesis of SLE because a dysfunction in their regulatory action may be responsible for the altered immune responses and overproduction of pathogenic autoantibodies (6). Abnormalities in peripheral blood T cells (PBTs) from SLE patients include T lymphocytopenia, low proliferative responses to lectin, anti-CD3 and anti-CD2 stimulation (7, 8), and a lower production of Th-1 type cytokines, such as IL-2 (9–11). Although the costimulatory pathway is up-regulated, the TCR/CD3 pathway appears to be down-regulated (12, 13). We and other groups have reported that a reduction in tyrosine phosphorylation and the diminished expression of TCR ζ protein (ζ) play crucial roles in the pathogenesis of SLE (14–16). Clinically, a reduction in ζ expression is not correlated with either the disease activity of SLE or the dose of prednisolone (17). In contrast, we and other groups have reported alterations in the ζ mRNA open reading frame (ORF) or the 3′-untranslated region of ζ mRNA in T cells from SLE patients (18–22). ζ has crucial roles in signal transduction through the TCR/CD3 complex (23–25) and in the efficient transport of the assembled TCR complexes to the cell surface (26). ζ is composed of three ITAM domains that are sufficient to couple chimeric receptors to early and late signaling events (24, 25, 27–32). The mutation of tyrosines within the ITAM or the nonphosphorylated and monophosphorylated motif abrogates the signal transduction ability (32, 33), suggesting that these tyrosines and their phosphorylation have crucial roles in protein function. Furthermore, ζ contains the GTP/GDP binding site, which is located immediately before the third ITAM (34). We have previously reported that 14 of 21 patients with SLE had decreased expression of ζ in PBTs. And 2 of the 14 SLE patients were lacking of the exon 7 portion of ζ mRNA (14). Exon 7 of ζ mRNA spans the GTP/GDP binding site proximal to the third ITAM. Thus, the aberrant ζ protein lacking exon 7 may result in skewed signal transduction, without efficient interaction with Shc and/or GTP/GDP. However, the involvement of ζ mRNA with an altered ORF, specifically in exon 7, in the decreased expression of ζ in SLE T cells has not been previously reported. To investigate the effect of ζ mRNA lacking exon 7 on the intracellular and cell surface expression of the ζ protein and TCR/CD3 complex, ζ cDNA lacking exon 7 (ζ cDNA/exon 7(−)) or wild-type (WT) ζ cDNA were transfected using a recombinant retrovirus system into murine T cell hybridomas (MA5.8) (35) deficient for ζ expression. In this study, we report that not only the expression of ζ protein, but also the expression of the TCR/CD3 complex, was down-regulated on the cell surface of the MA5.8 mutant cells expressing ζ mRNA/exon 7(−) because of a reduction in ζ mRNA stability.

Materials and Methods

Cell lines and inhibition of RNA synthesis

The MA5.8 cells (lacking endogenous ζ expression) were kindly provided by Prof. Takashi Saito (Chiba University, Chiba, Japan), and the RetroPackPT67 (BD Clontech) was used as the dualtropic packaging cell line.

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; Ct, threshold cycle; ER, endoplasmic reticulum; IP, immunoprecipitation; ORF, open reading frame; PBT, peripheral blood T cell; PVDF, polyvinylidene difluoride; WT, wild type; ζ, TCR ζ protein.

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For experiments involving the inhibition of RNA synthesis, cell cultures were incubated with 4 μg/ml actinomycin D in the culture medium. Samples were collected for up to 48 h after drug exposure.

DNA transfection and infection

The DNA transfection and infection protocols have been previously described (36). Full-length WT human ζ cDNA (ζ cDNA; +136~+1627 (1492 bp)) and human ζ cDNA lacking exon 7 (ζ cDNA/exon 7(−): +136~+564, +600~+1627 (1456 bp)) were amplified from the PBTs of a normal healthy control and an SLE patient (KS), respectively, using RT-PCR (Fig. 1). Each cDNA was ligated into a *SalI*-cut pDON-AI (Takara Bio), and each of the pDON-AI construct was sequenced in both directions to verify the nucleotide sequences. Purified pDON-AI containing the WT ζ cDNA insert, the ζ cDNA/exon 7(−) insert, or without any DNA insert were then transfected into 5.0×10^6 RetroPackPT67 cells using a cationic liposome kit (TransFast Transfection Reagent: Promega). Forty-eight hours after transfection, 10 ml of DMEM was added to the cells, and supernatant containing the same amount of the vector retrovirus was subsequently used to infect 1.0×10^7 MA5.8 cells in the presence of 8 μg/ml polybrene. After 24 h of incubation, G418 was added to select the infected cells, and 30 random colonies were selected and cultured together to construct MA5.8 mutants (WT, EX7−, and NEG, respectively).

RT-PCR

Whole mRNA was isolated from the cell samples and the mRNA was converted to whole cDNA by reverse transcriptase, according to a previously described method (18). Using 5 μl of the whole cDNA as the template, specific cDNA was amplified by PCR using specific primers and TaqDNA polymerase (Applied Biosystems). The PCR conditions were as follows: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, for a total of 35 cycles. The primers for amplifying the human full-length ζ cDNA were arranged upstream of the ORF 5′-TCAGCCTCTGCCTCCCAGCCTCTTTCT-3′ (+136 to +162) and 3′ end of exon 8 5′-GCAGAGCAGAGAGCGTTTTCCATCCAT-3′ (+1627 to +1601) of human ζ mRNA (37). To amplify the murine CD3ε cDNA (25), specific primers were arranged as follows: 5′-ATCCTGTGC CTCAGCCTCCTAGCTGT-3′ (+25 to +50) and 5′-ATGGGCTCAT AGTCTGGGTTGGGAA-3′ (+494 to +488). As a positive control, murine β-actin cDNA was amplified by PCR using the following primers: 5′-GGCCAACCGTGAAAAGATGA-3′ (+419 to +438) and 5′-CACGC TCGGTCAGGATCTTC-3′ (+669 to +650). PBTs were isolated from whole blood according to a previously described method (18).

Real-time PCR

The primers for human ζ were located in two different exons of each gene to avoid the amplification of any contaminating genomic DNA (37): the forward primer was 5′-TGCTGGATCCCAAACTCTGC-3′ (+254 to +272) (exon 3) and the reverse primer was 5′-CCCGGCCACGTC TCTTG-3′ (+434 to +449) (exon 5). The TaqMan probe was 5′-ATG GAATCCTCTTCATCTATGGTGTCATTCTCAC-3′ (+284 to +317) and had a fluorescent reporter dye (FAM) covalently linked to its 5′-end and a downstream quencher dye (TAMRA) linked to its 3′-end. The primers for murine CD3ε cDNA were located in two different exons of each gene (38): the forward primer was 5′-GGACAGTGGCTACTACGTCT GCTA-3′ (+307 to +330) (exon 4) and the reverse primer was 5′-TGAT GATTATGGCTACTGCTGTCA-3′ (+423 to +400) (exon 7). The TaqMan probe was 5′-CACCTCCACACAGTACTCACACACTCGA-3′ (+400 to +373). In addition, the forward primer of 5′-GGCCAACCGT GAAAAGATGA-3′ (+419 to +438) and the reverse primer of 5′-CACGCTCGGTCAGGATCTTC-3′ (+669 to +650) for the murine β-actin cDNA were designed in exon 3 and exon 4, respectively. The TaqMan probe for the murine β-actin cDNA was 5′-TTTGAGACCTTCAACAC CCCAGCCA-3′ (+450 to +474).

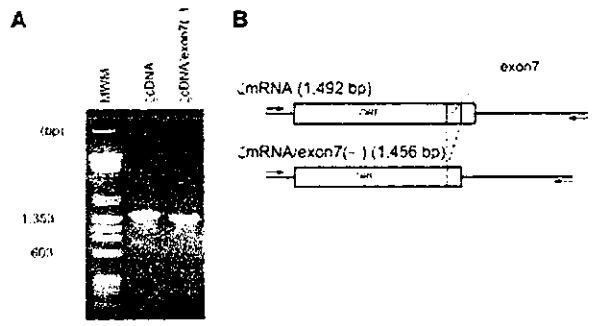

FIGURE 1. RT-PCR of human ζ cDNA lacking exon 7. *A*, WT human full-length ζ cDNA (ζ cDNA) (1492 bp) and human ζ cDNA lacking exon 7 (ζ cDNA/exon 7(−)) (1456 bp) were amplified by RT-PCR from PBTs obtained from a healthy normal control and an SLE patient (KS), respectively, and electrophoresed on an agarose gel (1.0%). *B*, ζ mRNA/exon 7(−) is lacking the 36-bp region corresponding to exon 7. The arrows indicate the specific primers used to amplify the full-length ζ cDNA.

Amplification and detection of specific products were performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems) according to a previously described amplification protocol (36). To prepare a template DNA standard, a target DNA fragment was amplified by PCR and was fused into pCRII vector by using TA cloning kit (Invitrogen Life Technologies), amplified, and refined. The amount of standard DNA construct per well was adjusted to 10 pg and then serially diluted, yielding samples containing 10, 1, 10^{-1}, 10^{-2}, and 10^{-3} pg, which were then used to construct standard plots.

Cell surface biotinylation, immunoprecipitation (IP), and SDS-PAGE

Cells (1.0×10^7 cells/ml) were biotinylated in bicarbonate buffer to label the cell surface proteins using a previously described method (36). Cells were then lysed, and the cleared lysates were immunoprecipitated for 2 h at 4°C with 2 μg of mouse anti-human ζ mAb (TIA-2) (Coulter Immunology), rabbit anti-mouse TCRα mAb (Santa Cruz Biotechnology), rabbit anti-mouse TCRβ mAb (Santa Cruz Biotechnology), goat anti-mouse CD3ε mAb (Santa Cruz Biotechnology), goat anti-mouse CD3γ mAb (Santa Cruz Biotechnology), or goat anti-mouse CD3δ mAb (Santa Cruz Biotechnology) bound to 15 μl of equilibrated protein G-Sepharose (Amersham Biosciences). The resulting pellets were resuspended in a nonreducing sample buffer and loaded on a 12% SDS-PAGE.

Western blot analysis

Cells were lysed with the lysis buffer and disrupted by sonication according to a previously described method (17). After centrifuging at 10,000 × *g* for 5 min, the supernatant was loaded on a 15% SDS-PAGE gel using a reducing method. The proteins were electrophoretically blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore), and the membranes were soaked at 37°C for 1 h in blocking agents (Blockace; Dainippon Pharmaceuticals). The blots were then probed with a mouse anti-human ζ mAb (TIA-2) at 16°C for 1 h. TIA-2 was visualized using a peroxidase-conjugated anti-mouse IgG (Amersham Biosciences). Biotinylated proteins were detected using streptavidin-peroxidase (Southern Biotechnology Associates). After washing three times, the signals were detected by chemiluminescence-enhancing reagents (Amersham Biosciences). The treated membranes were visualized on ECL x-ray film (Amersham Biosciences). The density of the specific bands was quantified as index by scanning with a Scan Jet II (Hewlett Packard) and National Institutes of Health Image Software (version 1.56).

Flow cytometric analysis

The flow cytometric analysis procedure has been previously described (17). Briefly, MA5.8 or the transfectants were stained with a FITC-conjugated Armenian hamster anti-mouse CD3ε mAb (145-2C11) (Coulter Immunology) or an FITC-conjugated mouse anti-human ζ mAb (TIA-2) (Coulter Immunology). The analysis was performed using a FACScan flow cytometer and consort-30 software. An FITC-conjugated Armenian hamster anti-mouse IgG (Coulter Immunology) and an FITC-conjugated mouse anti-human IgG (Coulter Immunology) were used as negative controls.

Pulse-chase experiment

Cells (3.0×10^7) were collected and washed twice with PBS. Cells were labeled in methionine-free RPMI 1640 medium (Sigma-Aldrich) containing 0.21 mCi of ProMix [^{35}S]methionine in vitro cell labeling mix (Amersham Biosciences). Five hours later, the medium was removed, cells were washed three times with PBS and were chased with RPMI 1640 medium containing methionine for 0, 2, and 4 h. Cells were then washed three times with PBS and incubated for 15 min with 200 μl of lysis buffer. The cell lysates were centrifuged at 10.000 × *g* for 5 min. The supernatant was retained for protein assay using the BCA protein kit (Pierce). Equal amounts of proteins for each condition were then immunoprecipitated. Protein bands were detected by autoradiography using BAS5000 system (Fuji Photo Film).

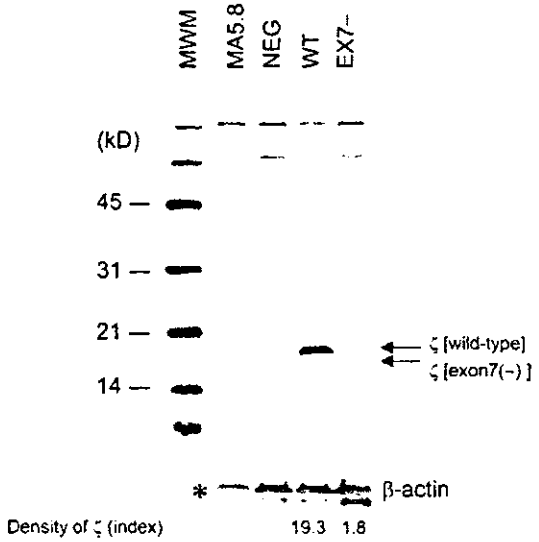

FIGURE 2. Western blot analysis of human ζ expressed by MA5.8 mutants. Cell lysates from MA5.8 and its mutants (NEG, WT, and EX7−) were electrophoresed on 15% SDS-polyacrylamide gels using a reducing method and blotted onto a PVDF membrane. The membranes were then incubated with a mouse anti-human ζ mAb (TIA-2) followed by a peroxidase-conjugated anti-mouse IgG. After treatment with chemiluminescence-enhancing reagents, the membranes were visualized on ECL x-ray films, and the densities of the 18-kDa WT ζ protein (ζ[wild-type]) and the 17-kDa short-form exon 7-deleted ζ protein (ζ[exon 7(−)]) bands (indicated by the arrows) were quantified as index. *, Western blot of the MA5.8 mutants using a hamster anti-mouse β-actin mAb.

Intracellular staining for ζ, CD3ε, and endoplasmic reticulum (ER)

Cells were resuspended in PBS, laid on poly-L-lysine-coated slides for 15 min at 37°C, and fixed for 10 min with 4% paraformaldehyde, and permeabilized for 10 min at room temperature with washing buffer (HEPES-buffered PBS containing 0.1% Triton X-100). Cells were then stained with FITC-conjugated Armenian hamster anti-mouse CD3ε mAb (145-2C11) (BD Pharmingen) in PBS containing 1% BSA, a mouse anti-human ζ mAb (6B10.2) (Santa Cruz Biotechnology), followed by Alexa Fluor 568 goat anti-mouse IgG (H+L) (Molecular Probes), and a rabbit polyclonal Ab to calreticulin (Novus Biologicals) followed by Alexa Fluor 647 $F(ab')_2$ of goat anti-rabbit IgG (H+L) (Molecular Probes). The samples were mounted in DakoCytomation Fluorescent Mounting Medium (DakoCytomation) and were examined using a Leica TCS SP2 confocal microscope (Leica Camera).

Ab stimulation and IL-2 quantification

Anti-mouse CD3 mAb (KT3) (Coulter Immunology) was bound for 16 h to a 24-well, flat-bottom plate in PBS. The wells were rinsed with fresh PBS three times before the addition of the cells. Fifty microliters of transfected cells (1.0×10^6 cells/ml) were added to each well and incubated at 37°C in 7.0% CO_2. Culture supernatants were harvested, and their aliquots were collected and frozen at 1, 2, and 3 days after stimulation. The harvested supernatants were assayed using a standard IL-2 assay. Recombinant murine IL-2 (BD Pharmingen) was used as a standard.

Statistical analysis

Statistical significance was calculated using the Student's t test for unpaired data using Statview software (version 4.5; Abacus). A value of $p < 0.05$ was considered statistically significant.

Results

Western blot analysis of ζ protein in MA5.8 mutants expressing ζ mRNA/exon 7(−)

In a Western blot analysis using an anti-human ζ mAb (TIA-2), the production of the exon 7-deleted ζ (16 kDa) by the EX7− mutants was only 9.3% (index ratio, 1.8:19.3) of the WT ζ (18 kDa) produced by the WT mutants (Fig. 2). Therefore, we concluded that the expression of ζ protein was reduced in mutants containing ζ mRNA/exon 7(−).

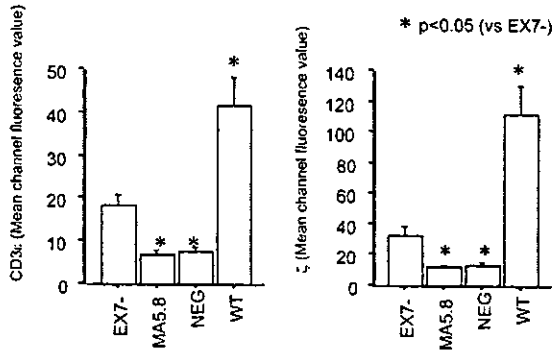

FIGURE 3. Flow cytometric analysis of the MA5.8 mutants. The surface expression of the TCR/CD3 complex and the ζ protein on MA5.8 and its mutants (NEG, WT, and EX7−) was quantified as the mean channel fluorescence value using FITC-conjugated anti-mouse CD3ε mAb (145-2C11) and FITC-conjugated anti-human ζ mAb (TIA-2), respectively. Each experiment was performed in triplicate. The mean channel fluorescence value of the EX7− mutants was compared with that of MA5.8 and the other MA5.8 mutants (MA5.8, NEG, and WT), respectively. Statistical significance was calculated using Student's t test. Bars show the mean ± SD.

Analysis of ζ protein and TCR/CD3 complex on the cell surface of MA5.8 mutants expressing ζ mRNA/exon 7(−) by FACS and IP

To investigate the expression of ζ protein and the TCR/CD3 complex on the cell surface, MA5.8 and its mutants were stained with an FITC-conjugated anti-mouse CD3ε mAb (145-2C11) or an FITC-conjugated anti-human ζ mAb (TIA-2) and analyzed by flow cytometry (Fig. 3). This experiment was performed in triplicate. Although the expression of ζ protein on the cell surface of EX7− mutants (mean channel fluorescence value, 34.48 ± 5.07 (mean ± SD)) was significantly ($p < 0.05$) up-regulated, compared with the MA5.8 cells (12.52 ± 1.50) and the NEG (13.78 ± 1.58) mutants, it was significantly ($p < 0.05$) lower than that of the WT mutants (112.19 ± 18.27). The CD3ε expression level on the EX7− mutants (18.44 ± 2.50) was significantly ($p < 0.05$) higher than those on the MA5.8 cells (7.00 ± 1.00) and the NEG mutants (7.49 ± 1.25). However, it was significantly ($p < 0.05$) lower than that on the WT mutants (41.74 ± 6.51).

To confirm the cell surface expression of ζ protein and the TCR/CD3 complex, we subjected MA5.8 and its mutants to surface biotinylation, IP, SDS-PAGE analysis under nonreducing conditions, and Western blot analysis (Fig. 4). IP of the WT mutants with both hamster anti-mouse CD3ε mAb (145-2C11) and mouse anti-human ζ mAb (TIA-2) yielded the following surface labeled proteins: mature forms of the TCR$\alpha\beta$ heterodimers ($\alpha\beta_m$) (67–95 kDa), a ζ homodimer (34 kDa) (indicated as the open arrowheads), CD3γ (26 kDa), CD3δ (28 kDa), and CD3ε (23 kDa), indicating that TCR/CD3 complex is producing on the WT mutants. The protein bands of the TCR/CD3 components were confirmed by the Western blot and IP of a whole cell lysate of the WT mutants with Abs against each TCR/CD3 component. IP of the WT mutants with nonspecific hamster and mouse IgG did not yield any of these proteins (data not shown). Interestingly, IP of the EX7− mutants with anti-CD3ε mAb or anti-human ζ mAb demonstrated a reduced expression of the cell surface short ζ homodimer (32 kDa) (indicated as the closed arrowheads) as well as a decreased expression of the cell surface CD3γ and CD3ε accompanied with the absence of CD3δ, indicating the down-regulation of TCR/CD3 complex on the cell surface of EX7− mutants.

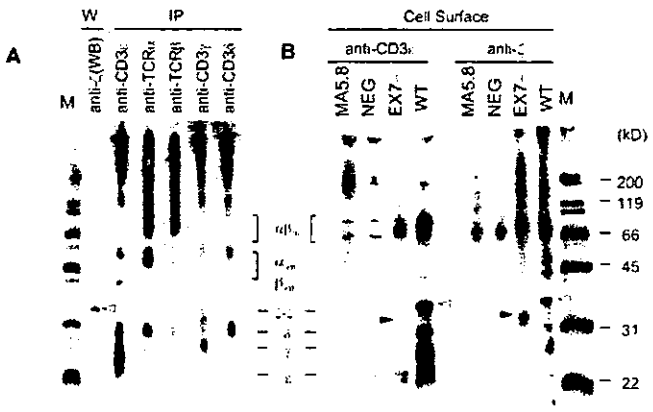

FIGURE 4. *A*, Western blot and IP of TCR/CD3 components in WT mutant. The cell lysates from WT mutants were electrophoresed on 12% SDS-polyacrylamide gels using a nonreducing method and were blotted onto a PVDF membrane. The membranes were then incubated with a mouse anti-human ζ mAb (TIA-2) followed by a peroxidase-conjugated anti-mouse IgG (*lane W*). The cell lysates from WT were immunoprecipitated using a goat anti-mouse CD3ε mAb (145-2C11), rabbit anti-mouse TCRα and TCRβ mAbs, and goat anti-mouse CD3γ and CD3δ mAbs bound to protein G-Sepharose. The pellets were electrophoresed on 12% SDS-polyacrylamide gels using a nonreducing method and were blotted onto a PVDF membrane. The membranes were then incubated with the Ab against each TCR/CD3 component, followed by a peroxidase-conjugated anti-goat or anti-rabbit IgG (*lane IP*). $\alpha\beta_m$, α_{im}, and β_{im} indicate the mature forms of the TCR αβ-chains, the immature forms of TCRα, and the immature forms of the TCR β-chains, respectively. *B*, IP of cell surface TCR/CD3 complexes and ζ protein in MA5.8 and its mutants. MA5.8 and its mutants (NEG, EX7−, and WT) were biotinylated and lysed in a cell lysis buffer. The cell lysates were immunoprecipitated using goat anti-mouse CD3ε mAb (145-2C11) or mouse anti-human ζ mAb (TIA-2) bound to protein G-Sepharose. The pellets were resuspended in a nonreducing sample buffer and loaded on a 12% SDS-PAGE. Biotinylated proteins were blotted onto PVDF membranes and detected using streptavidin-peroxidase. After treatment with chemiluminescence-enhancing reagents, the membranes were visualized on ECL x-ray films. M, The protein molecular markers. The open and closed arrowheads indicate the protein bands of the WT (34-kDa) and the exon 7-deleted short-form (32-kDa) ζ homodimer, respectively.

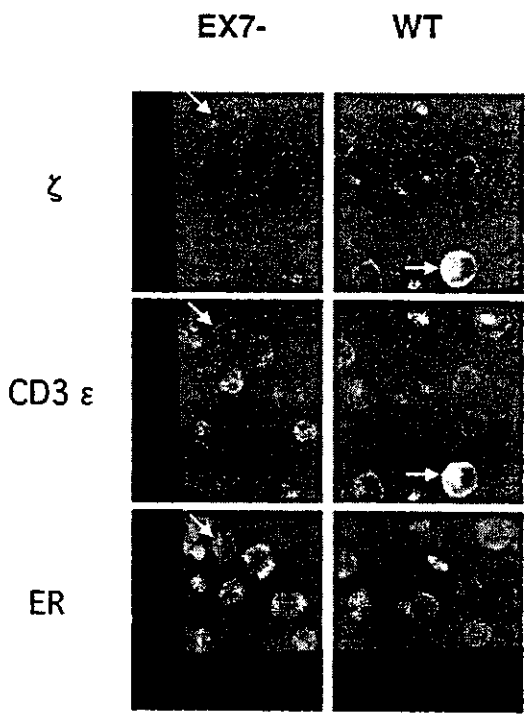

FIGURE 5. Intracellular staining for ζ, CD3ε, and ER. The EX7− and WT mutants were laid on poly-L-lysine-coated slides, fixed with 4% paraformaldehyde, and permeabilized with washing buffer. Cells were then stained with FITC-conjugated Armenian hamster anti-mouse CD3ε mAb (145-2C11) (green color), a mouse anti-human ζ mAb (6B10.2), followed by Alexa Fluor 568 goat anti-mouse IgG (H+L) (red color), and a rabbit polyclonal Ab to calreticulin followed by Alexa Fluor 647 $F(ab')_2$ of goat anti-rabbit IgG (H+L) (blue color). The samples were mounted and were examined using a confocal microscope. White arrows show the ring-shaped pattern of the WT mutants with 6B10.2 and 145-2C11, whereas yellow arrows indicate the cytoplasmic pattern of the EX7− mutants with 6B10.2, 145-2C11, and anti-calreticulin Ab.

Confocal microscopic analysis of MA5.8 cells

To explore the intracellular localization of TCR/CD3 complex including ζ in the MA5.8 mutants, WT and EX7− mutants were fixed, permeabilized, and stained with anti-ζ, anti-CD3ε, and anti-calreticulin Abs, respectively (Fig. 5). The staining of the WT mutant with 6B10.2 (anti-ζ, red in Fig. 5) and 145-2C11 (anti-CD3ε, green in Fig. 5) observed in a confocal microscopy showed the ring-shaped pattern (indicated as the white arrows), indicating the cell surface expression of TCR/CD3 complex including ζ in the WT mutants. In contrast, the staining pattern of the EX7− mutants with 6B10.2 (anti-ζ) or 145-2C11 (anti-CD3ε) was similar to that with anti-calreticulin Ab (indicated as the yellow arrows), indicating the staining of the ER detected by anti-calreticulin (blue in Fig. 5) as well as some weak dots on the cell surface (indicated as the yellow arrow), indicating low expression of the original TCR/CD3 complex of MA5.8 cells. From these observations, most of the TCR/CD3 complex in the EX7− mutants could be retained in the ER.

Decrease in IL-2 production in MA5.8 mutants expressing ζ mRNA/exon 7(−)

To evaluate the effect of exon 7 deletion in ζ mRNA, MA5.8 mutants were stimulated with anti-mouse CD3ε mAb (145-2C11) (Fig. 6). IL-2 production in the WT, NEG, or MA5.8 mutants on day 1, 2, or 3 after stimulation was compared statistically with that in the EX7− mutants. IL-2 production in the EX7− mutants on day 1 (1.50 ± 2.12 ng/ml), day 2 (3.00 ± 4.24 ng/ml), and day 3 (4.50 ± 0.71 ng/ml) was significantly ($p < 0.01$) lower than that in the WT mutants on day 1 (54.00 ± 0.00 ng/ml), day 2 (81.50 ± 3.54 ng/ml), and day 3 (89.50 ± 2.12 ng/ml), respectively. Consequently, IL-2 production in the MA5.8 mutants expressing ζ mRNA/exon 7(−) was lower than that in the MA5.8 mutants expressing WT ζ mRNA.

ζ mRNA stability assay

To evaluate the relationship between the reduction in ζ protein expression and exon 7 deletion, we examined the stability of ζ mRNA. WT and EX7− mutants were exposed to actinomycin D to inhibit transcription. The cell cultures were incubated with 4 μg/ml actinomycin D, and the cells were collected at 0, 6, 12, 24, and 48 h after drug exposure. One microgram of whole mRNA was isolated from the cell samples and converted to whole cDNA by reverse transcriptase. Using 5 μl of the whole cDNA as the template, ζ, CD3ε, and β-actin cDNA in the WT or the EX7− mutants were quantified by real-time PCR. To validate the real-time PCR, the standard curves for human ζ, murine CD3ε, and murine β-actin gene were constructed from the pCRII fused with ζ cDNA (1492 bp), CD3ε cDNA (470 bp), and β-actin cDNA (250 bp), respectively. The critical threshold cycle (Ct) for ζ, CD3ε, and β-actin cDNA was inversely proportional (correlation coefficient of all

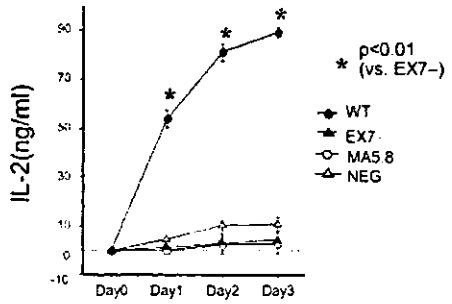

FIGURE 6. IL-2 production in MA5.8 mutants after stimulation with anti-CD3ε Ab. Anti-mouse CD3ε mAb (145-2C11) was bound to a 96-well, flat-bottom plate. MA5.8 and its mutants (NEG, WT, and EX7−) were then added to the wells and incubated. The culture supernatants were collected 1, 2, and 3 days after stimulation and assayed using a standard IL-2 assay. Each experiment was performed in triplicate. IL-2 production in WT, NEG, or MA5.8 on day 1, 2, or 3 after stimulation was compared statistically with that in EX7− mutants by using Student's *t* test. Bars show the mean ± SD.

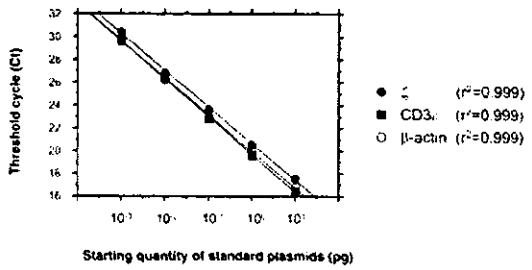

FIGURE 7. Standard curves for quantifying the amount for ζ, CD3ε, and β-actin cDNA. Human ζ ORF cDNA (1492 bp), murine CD3ε cDNA (470 bp), and murine β-actin cDNA (250 bp) were fused with pCRII vector, respectively. Real-time PCR was performed with the serial dilution (10, 1, 10^{-1}, 10^{-2}, and 10^{-3} pg) of the plasmid DNA as the template to estimate the critical Ct.

three genes was 0.999) to the logarithm of the initial amount of the standard template DNA (Fig. 7). Then the Ct for these cDNA were measured by the real-time PCR. These cDNA were measured by three separate experiments, and the statistical significance was calculated using the Student's *t* test. As a result, demonstrated in Table I and Fig. 8*A*, the transcript of CD3ε and β-actin in the two MA5.8 mutants was gradually degraded over time after the treatment of actinomycin D. Decrease in the expression of these mRNA itself was not affected following the transfection of ζ mRNA because there was no observed difference in the protein expression of ζ and CD3ε of the WT or EX7− mutants at 0, 24, and 48 h after the transfection by Western blot (data not shown). However, there seemed to be a difference in the kinetics of mRNA stability between the WT and EX7− mutants because the transcripts of β-actin in the WT mutants were easily degraded compared with the EX7− mutants at 6 h after the actinomycin D treatment. The amount of ζ or CD3ε transcript was evaluated as the relative quantity against β-actin cDNA (Table I and Fig. 8*B*). As a result, the relative amount of ζ mRNA in the EX7− mutants (0.008 ± 0.001) was already significantly ($p < 0.01$) lower than that in the WT mutants (0.014 ± 0.002) before the actinomycin D treatment. And the relative amount of the WT ζ mRNA in the WT mutants increased constantly, while that of the ζ mRNA/exon 7(−) in the EX7− mutants did not change and was significantly ($p < 0.01$) lower than that of the WT ζ transcript over time (Fig. 8*Bi*). In contrast, relative amount of CD3ε mRNA in both MA5.8 mutants was almost the same at 0, 12, and 48 h after the actinomycin D treatment. From these observations, we can conclude that the ζ mRNA/exon 7(−) in the EX7− mutants was less stable than the WT ζ mRNA in the WT mutants. In contrast, the stability of the CD3ε mRNA was similar in the EX7− mutants and the WT mutants (Fig. 8*Bii*).

Pulse-chase experiment using MA5.8 mutants

To confirm whether decreased stability of the ζ mRNA/exon 7(−) in the EX7− mutants could be related to the reduced amount of ζ protein, we compared the production of ζ in the WT and EX7− mutants. After labeling with [^{35}S]methionine in methionine-free medium for 5 h, WT and EX7− mutants were chased with the complete medium for 0, 2, and 4 h. The cell lysate was then incubated with mouse anti-human ζ mAb (TIA-2) bound to protein G-Sepharose. The resulting pellets were resuspended in a nonreducing sample buffer and loaded on a 12% SDS-PAGE followed by the autoradiography. As shown in Fig. 9, short ζ homodimer (32 kDa) produced by the EX7− mutants (indicated as the closed arrowheads) was gradually decreased over time, whereas the expression level of the WT ζ homodimer (34 kDa) produced by the WT mutants (indicated as the open arrowheads) did not change even after 4 h from chasing. From these observations, reduced ζ mRNA/exon 7(−) due to altered transcription and/or mRNA stability could lead to decreased ζ protein formation; as degradation of mRNA occurs, less protein is made.

Table I. *mRNA stability assay for the EX7− or WT mutants*

		0 h	6 h	12 h	24 h	48 h
Amount of cDNA (× 0.01 pg)						
EX7−	ζ	0.605 ± 0.058	0.789 ± 0.291	1.068 ± 0.197	0.471 ± 0.064	0.303 ± 0.052
	CD3ε	3.190 ± 0.356	5.549 ± 0.242	3.778 ± 0.503	0.608 ± 0.023	0.059 ± 0.008
	β-actin	73.292 ± 1.100	85.049 ± 2.462	34.860 ± 2.886	5.103 ± 0.097	1.431 ± 0.190
WT	ζ	1.970 ± 0.243	1.712 ± 0.169	1.067 ± 0.232	0.613 ± 0.026	0.286 ± 0.049
	CD3ε	7.319 ± 0.505	0.997 ± 0.034	0.132 ± 0.004	0.024 ± 0.003	0.005 ± 0.001
	β-actin	138.530 ± 5.495	12.063 ± 0.517	2.138 ± 0.053	0.514 ± 0.041	0.133 ± 0.019
Relative amount of cDNA						
ζ:β-actin ratio	EX7−	0.008 ± 0.001	0.009 ± 0.003	0.031 ± 0.006	0.092 ± 0.013	0.212 ± 0.036
	WT	0.014 ± 0.002 *	0.142 ± 0.014 **	0.499 ± 0.108 **	1.193 ± 0.051 *	2.152 ± 0.365 **
CD3ε:β-actin ratio	EX7−	0.044 ± 0.005	0.065 ± 0.003	0.108 ± 0.014	0.119 ± 0.005	0.041 ± 0.005
	WT	0.053 ± 0.004 N.S.	0.082 ± 0.003 **	0.061 ± 0.002 N.S.	0.047 ± 0.005 *	0.034 ± 0.005 N.S.

*, $p < 0.01$.
**, $p < 0.001$.

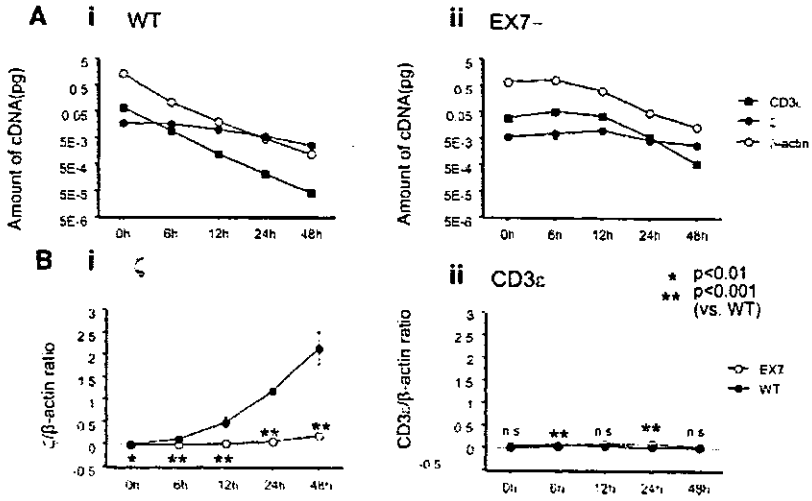

FIGURE 8. Reduction in ζ mRNA stability in the absence of the 36-bp portion of exon 7. MA5.8 mutants (WT and EX7−) were cultured and incubated with 4 μg/ml actinomycin D in the culture medium. Samples were collected at various time points, and the mRNA was subsequently extracted and converted to whole cDNA. *A*, Using 5 μl of the whole cDNA as the template, ζ, CD3ϵ, and β-actin cDNA in the WT (*i*) or the EX7− mutants (*ii*) were quantified by real-time PCR. Each experiment was performed in triplicate. *B*, The amount of ζ (*i*) or CD3ϵ (*ii*) mRNA expression was evaluated as the relative quantity against β-actin cDNA in WT (●) or EX7− mutants (○). Bars show the mean ± SD. *, $p < 0.01$; **, $p < 0.001$ of EX7− vs WT.

Discussion

We previously reported that ζ mRNA/exon 7(−), a splice variant of ζ mRNA, was detected in SLE T cells (14). The cytoplasmic domain of ζ is sufficient for coupling to receptor-associated signal transduction (25). This cytoplasmic domain contains three ITAM domains that, when phosphorylated, serve as docking sites for signaling proteins like ZAP70, actin, PI3K, and Shc. In particular, PI3K preferentially binds to ITAM1 (39), whereas Shc and the actin cytoskeleton interact predominantly with ITAM3 (40, 41). The ITAM sequence alone is sufficient to couple chimeric receptors to early and late signaling events (27). Mutations at tyrosines within the ITAM or nonphosphorylated and monophosphorylated motifs abrogate the signal transduction ability (32), suggesting a crucial role for phosphorylation of tyrosines. The GTP/GDP binding site, a glycine-rich sequence of GxxxxGKGxxGxxxG, is a unique portion that has the capacity to bind GTP/GDP, but not GMP or ATP, and is responsible for the G protein signaling pathway (38). The ζ mRNA mutation of the exon 7 deletion found in SLE patients influenced the ITAM3 domain and the GTP/GDP binding site, two regions that are critical for signal transduction involving the ζ protein. In this study, we attempted to confirm that a reduction in ζ protein expression occurs in cells containing ζ mRNA/exon 7(−) using a recombinant retrovirus system described by Bolliger et al. (42) and Weissman et al. (43).

The down-regulation and smaller size of the ζ protein in EX7−, as confirmed by the Western blot analysis, suggests that the production of the smaller ζ protein is down-regulated when it is translated from ζ mRNA/exon 7(−) because of the exon 7 deletion. These observations were also confirmed by IP and FACS analyses. Reportedly, TCR/CD3 complexes cannot be expressed on the cell surface without binding to the ζ homodimer in the cytoplasm (44–46). Therefore, in the MA5.8 mutants expressing ζ mRNA/exon 7(−), the TCR/CD3 complex might be down-regulated on the cell surface because of the reduction in the expression of ζ homodimer in IP using biotinylated cell surface proteins. Confocal microscopic analysis also revealed reduced cell surface expression of the ζ protein and the retention of TCR/CD3 complex in the cytoplasm of the EX7− mutant. Other groups have shown that the expression of the detergent-insoluble membrane-associated form of ζ was reduced in SLE T cells (47), supporting our results. The reduction in IL-2 production in the EX7− mutants revealed that the signal from the TCR was not transduced into the cytoplasm by anti-CD3ϵ Ab stimulation in this MA5.8 mutant. The results obtained using the MA5.8 mutants in this study may explain the mechanism behind the reduction in ζ protein expression in SLE T cells.

We examined the stability of ζ mRNA to investigate the reduction in ζ protein expression in the MA5.8 mutants expressing ζ mRNA/exon 7(−). From our observations, ζ mRNA/exon 7(−) in the EX7− mutants appeared to be less stable and more easily degraded than the WT ζ mRNA in the WT mutants. In pulse-chase experiment, ζ protein produced by the EX7− mutants was gradually decreased while the expression level of the ζ protein by the WT mutants did not change over time. From these observations, unstable ζ mRNA/exon 7(−) in the EX7− mutants could be related to the reduced amount of ζ protein. Therefore, it is conceivable that a reduction in ζ mRNA/exon 7(−) stability may lead to a reduction in the expression of ζ homodimer, leading to the absence of TCR/CD3 complex expression on the cell surface. The lower basal levels of ζ mRNA/exon 7(−) that were observed in the EX7− mutants before the treatment of actinomycin D, compared with that of the WT ζ mRNA in WT, may also be caused by mRNA instability in the EX7− mutants. Moreover, other reasons for reduced protein including increased degradation by a ubiquitin-proteasome pathway, as shown by Tsokos and colleagues (47), could also contribute to decrease expression of ζ in SLE T cells.

Several reports have been made on the relationship between exon deletion or exon skipping and the down-regulation of protein expression. Leitner et al. (48) reported that exon 3 skipping in (6*R*)-5,6,7,8-tetrahydro-L-biopterin mRNA in human monocytes/macrophages leads to the down-regulation of protein synthesis.

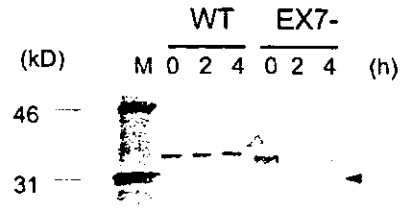

FIGURE 9. Pulse-chase experiment of MA5.8 mutants. A total of 3.0×10^7 of MA5.8 mutants (WT and EX7−) was collected and washed twice with PBS. Cells were labeled in methionine-free RPMI 1640 medium containing ProMix [^{35}S]methionine in vitro cell labeling mix. Five hours later, the medium was removed, and cells were chased with RPMI 1640 medium containing methionine for 0, 2, and 4 h. Cells were then washed with PBS and incubated for 15 min with lysis buffer. The cell lysate was then incubated with mouse anti-human ζ mAb (TIA-2) bound to protein G-Sepharose. The resulting pellets were resuspended in a nonreducing sample buffer and loaded on a 12% SDS-PAGE. Protein bands were detected by autoradiography using BAS5000 system. M, The protein molecular markers of ^{14}C-methylated proteins.

Krummheuer et al. (49) also demonstrated that an alternative splicing pattern in HIV type 1 mRNA, resulting in the production of exon 2 as the leader exon, stimulates protein synthesis in HIV type 1 viruses. Exon-deletion or exon-skipping in mRNA has also been reported to be correlated with mRNA instability. Schwarze et al. (50) reported that frameshift mutations producing a premature termination codon in exon 6, 9, or 27 of type III procollagen mRNA leads to a reduction in both mRNA stability and protein synthesis. Kawamoto (51) demonstrated that the nucleotide region from +62 to +166, representing exon 1, of the nonmuscle myosin H chain-A gene could up-regulate its protein synthesis by affecting the pretranslational steps (transcriptional and mRNA stability). In contrast, parathyroid hormone-related protein mRNA containing exon 7 and exon 8 was reported to be stable, whereas that including exon 9 was unstable (52). From our observations in the present study, the deleted 36-bp portion representing exon 7 in ζ mRNA appears to be critical for ζ mRNA stability and may be correlated with the down-regulation of ζ and the TCR/CD3 complex in SLE T cells. Actually, cell surface expression of TCR/CD3 complex including ζ was reduced in T cells of the two SLE patients (patients HE and KS), who were lacking of exon 7 portion in their ζ mRNA (14). As we have found ζ mRNA/exon 7(−) in only 2 of 21 lupus patients, it will be important to determine how frequent this mutation occurs in the T cells of a large population and how it contributes to abnormal T cell functions, such as cytotoxicity. Previously, we reported that the expressions of ζ and the TCR/CD3 complex were down-regulated on the cell surface of MA5.8 mutant cells expressing ζ mRNA containing an alternatively spliced 3′-untranslated region because of a reduction in ζ mRNA stability (36). SLE T cells bear both the WT and the splice variant of ζ mRNA (22). Thus, it would be interesting to compare the combined effect of transfection of both WT and the splice variant form of ζ mRNA on IL-2 production and cell surface expression of TCR/CD3 complex to explore whether these splice variants of ζ mRNA are more dominant. This project is now underway in our laboratory. Taken together, the reduced stability of ζ mRNA produced by aberrant ζ mRNA forms might be crucial to explaining the reduced expression of ζ seen in SLE T cells.

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Disclosures

The authors have no financial conflict of interest.

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ORIGINAL ARTICLE

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CD154 expression and mRNA stability of activated CD4-positive T cells in patients with systemic lupus erythematosus

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Abstract The expression of CD154 (CD40 ligand) on activated CD4+ T cells is known to be transient and tightly regulated for antigen-specific immune responses, and is increased and prolonged among patients with systemic lupus erythematosus (SLE). We investigated the regulation of CD154 expression by determining the protein and mRNA expression with PMA and ionomycin stimulation in CD4+ T cells, and confirmed their increase and prolongation in SLE T cells. Treatment with actinomycin D, a transcription inhibitor, after PMA and ionomycin stimulation was performed, and the findings revealed that the stability of CD154 mRNA increased significantly in activated SLE T cells compared with that of controls. However, alternations or abnormal sequences were not identified in the 3′ untranslated region, including AU-rich elements and CU-rich sequences, while their partial involvement in the post-transcriptional regulation of CD154 mRNA stability has been reported. With 96 h culture in vitro, the destabilization of CD154 mRNA was demonstrated, resulting in a corresponding decrease and normalization of surface expression on activated SLE T cells. We speculate that the CD154 expression on T cells from SLE patients may be increased and prolonged, with mRNA stabilization being related to a continuous stimulation in vivo.

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Key words CD154 (CD40L) · mRNA stability · Systemic lupus erythematosus (SLE) · 3′ untranslated region

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by various autoantibodies and pathogenic immune complexes containing DNA and antidouble-stranded DNA antibody, which are deposited in the glomeruli, the dermo–epidermal junction of skin lesions, blood vessel walls, and the choroid plexus of SLE patients.[1-3]

CD154, a type II glycoprotein belonging to the TNF-α family, is expressed predominantly on activated CD4+ T cells. Transient expression of CD154 may allow only appropriate antigen-specific B cell activation via CD40 expressed on B cells following immunoglobulin class switching and germinal center formation.[3,4] An increased percentage of peripheral lymphocytes expressing CD154 is reported in patients with SLE.[5,6] The infiltration of CD154-positive mononuclear cells has also been demonstrated in kidney sections of class III or V lupus glomerulonephritis. Furthermore, the blocking of CD154 with monoclonal antibodies (MAb) has been demonstrated to interfere with ongoing lupus nephritis and prolong survival in murine models.[1,7] These findings indicate that excess or inappropriate expression of CD154 plays a pivotal role in immune responses in promoting the pathogenesis of SLE.

Several regulatory mechanisms exist in CD154 expression. In posttranslational regulation, cell-surface CD154 is reduced by receptor-mediated endocytosis as a result of binding with CD40[8] or through the release of soluble CD154, which is a proteolytic cleavage product of CD154.[9,10] Ligation of T cell receptor (TCR) initiates rapid augmentation of CD154 gene transcription, and the transient CD154 gene expression gradually decreases in a similar manner to the protein expression.[11,12] The 3′ untranslated region (3′UTR) of CD154 mRNA contains five AU-rich elements, which is a potential sequence motif

affecting mRNA stability as well as that in several transiently expressed genes, including TNF-α, by selective binding of RNA-binding proteins during T cell activation.[13,14] Moreover, other studies have revealed distinct proteins bound to unique sites lacking AU-rich elements in the 3'UTR, e.g., a CU-rich sequence, and increased CD154 mRNA stability.[15,16] These studies suggest the possibility that inadequate posttranscriptional regulation contributes to an increase in the surface expression and mRNA stability of CD154 in lymphocytes from patients with SLE.

To investigate the difference in CD154 regulation during T cell activation between normal and SLE T cells, we examined the surface and mRNA expression of CD154 in CD4+ T cells stimulated with PMA and ionomycin, and analyzed the posttranscriptional regulation of the mRNA stability.

Materials and methods

Patients and controls

Peripheral blood samples were obtained from the 28 Japanese SLE patients enrolled in this study. There were 3 males and 25 females, with an average age of 36.2 years (range 17–45 years, who all fulfilled), the American College of Rheumatology revised criteria for the classification of SLE.[17] We also enrolled 18 age-matched healthy volunteers, 2 males and 16 females, with an average age of 34.4 years (range 22–43 years) as controls. CD4+ T cells from five female controls and the patients with active SLE were used to analyze surface and mRNA expression of CD154. Disease activity was determined using the SLE disease activity index (SLEDAI) scoring system,[18] and the disease of patients with a SLEDAI score ≥ 5 was defined as active. This study was reviewed and approved by the institution's review board, and informed consent was obtained from all subjects enrolled in the study.

Cell preparation and culture conditions

Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll–Hypaque centrifugation (Lymphoprep, AXIS-SHIELD PoC AS, Oslo, Norway), and CD4+ T cells were isolated from total PBMCs by positive selection on CD4-conjugated magnetic beads according to the manufacturer's instructions (Dynal AS, Oslo, Norway). Cells were cultured with RPMI 1640 medium (Sigma Chemical, St. Louis, MO, USA), containing 10% fetal calf serum, 2.05 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen Corp., Carlsbed, CA, USA), in 96 round wells at 37°C and 5% CO_2. Cells were stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin. Since the half-life of CD154 mRNA at 8 and 16 h stimulation with PMA and ionomycin is reported to range from 4.3 to 5.2 h,[11] we conducted some experiments where activated cells were washed with phosphate-buffered solution (PBS) and then incubated with 10 μg/ml actinomycin D (Act D, Sigma Chemical), a transcriptional inhibitor, for 4 h to evaluate the difference in the stability of CD154 mRNA between SLE patients and controls.

Flow cytometric staining

CD4+ T cells at a concentration of 1×10^6 and stimulated with PMA + ionomycin for different lengths of time were incubated with a saturating concentration of FITC-conjugated antihuman CD154 Mab (TRAP1, PharMingen, San Diego, CA, USA) at 4°C for 30 min. After three washes with PBS, cell fluorescence levels were analyzed with a FACScan flow cytometer (BD Bioscience, San Jose, CA, USA).

RNA isolation and Northern blot analysis

Total RNA was isolated from CD4+ T cells at concentrations of 2–8 $\times 10^6$ using an RNeasy kit (QIAGEN, Hilden, Germany). RNA (3.0–5.0 μg) was run in 1.2% agarose/formaldehide gels and transferred to nylon membranes. Membranes were prehybridized at 42°C for 6 h. The CD154 CDNA, isolated from a HeLa cell line transfected with an expression vector encoding the full-length human CD154[10] was used as the probe for Northern blot analysis. Hybridization was carried out with a random primer ^{32}P-labeled CD154 probe (Roche Dignostics, Mannheim, Germany), at 42°C for 40 h, and then washed twice at 42°C for 10 min with 1 × SSPE + 0.1% SDS, followed by two washes at 65°C for 20 min with 0.5 × SSPE + 0.1% SDS, and two more washes at 65°C for 20 min with 0.1 × SSPE + 0.1% SDS. Membranes were exposed and scanned by densitometry using an image analyzer BAStation (Fuji Photo Film Co., Tokyo, Japan), and values were normalized to the signals of the control β_2-microglobulin (β_2M) probe.

Reverse transcriptase–polymerase chain reaction (RT–PCR) and sequence analysis of the 3' untranslated region of CD154 CDNA

PBMCs were washed once with PBS and used immediately for reverse transcriptase – polymerase chain reaction (RT–PCR). RT–PCR was performed as follows: 1 μg total RNA in a total volume of 20 μl was reverse-transcribed into CDNA using oligo-dT primer and M-MuLV reverse transcriptase (Roche Diagnostics), and amplification was performed using 2.5 μl CDNA in a total volume of 50 μl containing 60 mmol/l Tris-HCl (pH 9.5, at 25°C), 25 mmol/l ammonium sulfate, 3.5 mmol/l $MgCl_2$, 0.5 μmol/l of each oligonucleotide primer, 2.5 μmol/l dNTP, and 2.5 U Platinum Taq DNA polymerase (Invitrogen Corp.).

Primer sequences used for CD154 amplification were as follows: sense, 5'-ATGATCGAAACATACAACCAAA-3'; antisense, 5'-TCAGAGTTTGAGTAAGCCAAAG-3'. The size was 846 base pairs.[19] Primer sequences for the control probe β_2M were: sense, 5'-ACCCCCACTGAAAA AGATGA-3'; antisense, 5'-ATCTTCAAACCTCCAT GATG-3'. The PCR products were analyzed electro-

phoretically in 1.2% agarose gels prepared with Tris-borate EDTA buffer, stained with 10 mg/ml ethidium bromide, and photographed.

For direct sequence analysis, PBMCs were stimulated with PMA + ionomycin for 6h before RT–PCR. Three oligonucleotide primers were selected for CD154 3′ UTR, sense-1, 5′-ATACAGCACAGCGGTTAAGC-3′, antisense-1, 5′-AGGCCATAGGAACCCAGAGT-3′, 405 bp, sense-2, 5′-CACCCTCTCGGACAGTTATT-3′, antisense-2, 5′-GTGTTAGAAAGGGGGATTGA-3′, 152 bp, sense-3, 5′-ACACACACACAGAGTCAGGC-3′, and antisense-3, 5′-CAAGTTCACTCTGGAAACAA-3′, 181 bp, to cover the AU-rich elements and CU-rich sequence of the 3′ UTR of CD154 mRNA. Amplifications were performed with 30 cycles of denaturation (93°C, 1 min), annealing (55°C, 2 min), and extension (72°C, 2 min). The PCR products were purified and subjected to direct sequencing using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Fostercity, CA, USA) according to the manufacturer's instructions.

Statistical analysis

The difference between the values for patients with SLE and those for controls were determined by the use of two-way repeated-measures ANOVA and Scheffe's test, or Mann–Whitney's *U*-test with STAT-View software (SAS Institute, NC, USA).

Results

Detection of CD154 mRNA in PBMCs from patients with active SLE

The expression of CD154 mRNA in freshly isolated PBMCs was examined by RT–PCR. CD154 mRNA was detected in the nine patients with active SLE who were examined, but not in the controls (Fig. 1).

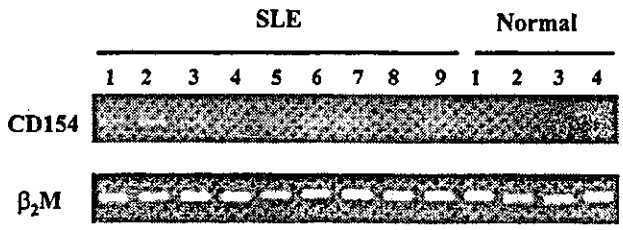

Fig. 1. Detection of CD154 mRNA from peripheral blood mononuclear cells (PBMCs) of active systemic lupus erythematosus (*SLE*) patients. RNA was extracted from freshly isolated PBMCs and used for reverse transcriptase – polymerase chain reaction (RT-PCR) analysis for CD154 and β_2M. The data were obtained from nine patients with active SLE and four control subjects

Surface expression of CD154 on activated CD4+ T cells

To assess surface CD154 expression in response to mitogen stimulation, CD4+ T cells from SLE patients and healthy controls were stimulated with PMA + ionomycin for various time periods and analyzed with flow cytometry. As shown in Fig. 2, the maximum expression was observed at 6h of stimulation in both control and SLE CD4+ T cells, but the percentage (mean ± SD) of CD154+ cells was statistically higher in SLE patients (n = 5, 74.21 ± 8.53%) than in controls (n = 5, 48.45 ± 10.31%) ($P < 0.01$ by two-way repeated-measures ANOVA with Scheff's test). Moreover, prolonged CD154 expression was observed even after 48h stimulation in the SLE T cells, while a sharp decease in expression to the baseline level was recorded for the normal T cells. As previously described,[5,6,11] increased and prolonged expression was observed in T cells from all five SLE patients examined, but not in control T cells. The expression patterns of CD154 in T cells were statistically different between SLE patients and healthy controls ($P < 0.01$ by two-way repeated-measures ANOVA with Scheff's test). Northern blot analysis revealed that the levels of CD154

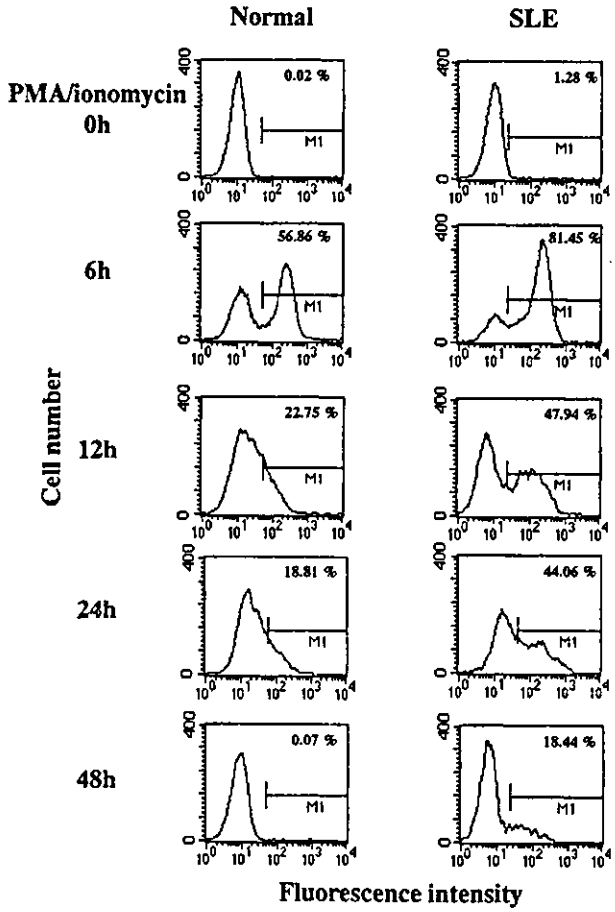

Fig. 2. Kinetics of CD154 expression on activated CD4+ T cells. CD4+ T cells from control subjects (n = 5) and SLE patients (n = 5) were incubated with phorbol myristate acetate (*PMA*) + ionomycin for 0–48h, and stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD154 monoclonal antibodies (Mab). Representative data are shown

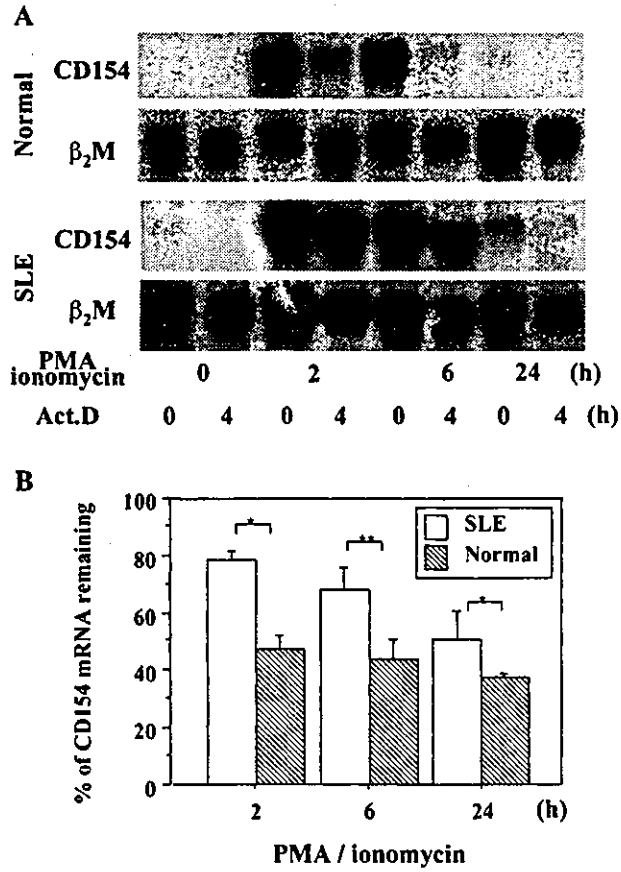

Fig. 3. CD154 mRNA stability of activated CD4+ T cells. CD4+ T cells from control subjects and SLE patients were stimulated with PMA + ionomycin at the time-periods indicated, and then incubated with Act D for 4 h to terminate the transcription before RNA extraction. The amount of mRNA was determined with Northern blot analysis with hybridization of probes for CD154 and β_2M. **A** Representative result of phosphoimaging. **B** The phosphoimage of mRNA was quantified with a densitometer, and then the density of the CD154 mRNA in each lane was standardized with that of β_2M in the corresponding lane. The quantifiable data at 2, 6, and 24 h of stimulation are indicated. The *columns* represent the mean percentage ($n = 5$ for both SLE and control subjects) of the density remaining after Act D treatment, and the *bars* indicate the standard deviation (SD). The percentage of the remaining CD154 mRNA was significantly higher in SLE patients than in the controls at 2, 6, and 24 h after stimulation. $^*P < 0.01$, $^{**}P < 0.05$, by Mann–Whitney's *U*-test

mRNA corresponded to its surface expression (data not shown).

Stability of CD154 mRNA in patients with SLE

The turnover of CD154 mRNA is known to be rapid.[12] To investigate whether mRNA stability is related to the increased and prolonged mRNA expression of CD154 in SLE patients, we examined mRNA stability with Act D treatment for 4 h after PMA + ionomycin stimulation. As shown in Fig. 3A, levels of CD154 mRNA were higher in SLE T cells activated for 2–24 h, and the detection was still confirmed after Act D treatment at each stimulation time period, whereas the levels apparently decreased in the controls. The percentage of CD154 mRNA remaining, representing mRNA stability, was significantly higher in SLE patients than in the controls at 2, 6, and 24 h of stimulation (Fig. 3B, $P < 0.01$, 0.05, and 0.01, respectively, by Mann–Whitney's *U*-test). PMA + ionomycin stimulation has been reported to increase mRNA stability. However, these results revealed that CD154 mRNA degradation was significantly delayed in SLE T cells compared with those of the controls. This indicated that there exists a possibility that the stabilization of CD154 mRNA in SLE T cells contributed to the increased and prolonged expression of CD154 in SLE T cells.

3′ UTR sequence of CD154 CDNA in activated CD4+ T cells

TNF-α gene expression is partially regulated at the post-transcriptional level and, in particular, AU-rich elements in 3′ UTR play an important role in the mRNA turnover.[14] CD154 mRNA, which has considerable sequence homology with TNF-α, is reported to have five AU-rich elements also, and other regions like a CU-rich sequence, which are involved in mRNA stability, in the 3′UTR.[16] We analyzed the 3′ UTR sequence of CD154 CDNA, including those sequences in PMA + ionomycin-activated PBMCs from 28 SLE patients (17 active and 11 inactive) and 18 controls, by direct sequence. However, our results revealed no insertions, defects, or abnormal sequences in five AU-rich elements and a CU-rich sequence of CD154 3′ UTR CDNA from SLE (both active and inactive) patients and controls (data not shown).

Change of CD154 expression and mRNA stability after in vitro culture

It is suggested that continuous activation of T cells in vivo results in various T cell abnormalities in SLE patients.[2,3] To examine whether the kinetics of CD154 expression changes after culture in vitro, we incubated CD4+ T cells for 96 h in the absence of mitogen, and then stimulated the cells with PMA + ionomycin. Culturing for 96 h prior to stimulation resulted in significantly decreased and shortened CD154 expression on SLE T cells ($P < 0.01$ by repeated-measures ANOVA with Scheff's test), which were similar to those of normal T cells (Fig. 4). In normal T cells, no significant change in CD154 expression was demonstrated between those with and without 96 h pre-stimulation culturing. We further evaluated whether the change in CD154 mRNA stability affected the protein expression on SLE T cells in the same condition. Our results revealed that CD154 mRNA in activated SLE T cells was significantly destabilized with preculturing as compared with those without preculturing, and this was similar for the controls (Fig. 5).

Next, we examined the influence of serum factors on CD154 surface expression. SLE and normal CD4+ T cells were precultured in 100% culture medium (described above), or with culture medium consisting of 50% sera from

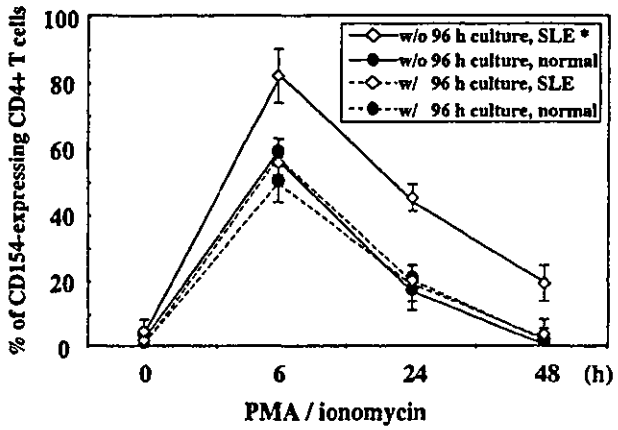

Fig. 4. Kinetics of CD154 surface expression on activated CD4+ T cells after 96 h culture in vitro. CD4+ T cells from SLE patients (n = 5) and control subjects (n = 5) were stimulated with PMA + ionomycin immediately after separation, or cultured in mitogen-free medium for 96 h followed by stimulation with PMA + ionomycin for the durations indicated. The percentage of CD154 + cells was determined with flow cytometry and compared between freshly isolated and 96-h precultured CD4+ T cells. The *symbols* and *bars* indicate the mean percentage of CD154+ cells and the SD of the mean, respectively. Culturing for 96 h prior to stimulation resulted in a significant decrease and shortened CD154 expression on SLE T cells ($P < 0.01$ by repeated-measures ANOVA with Scheff's test). *w/o*, without; *w/*, with; *star*, $P < 0.01$

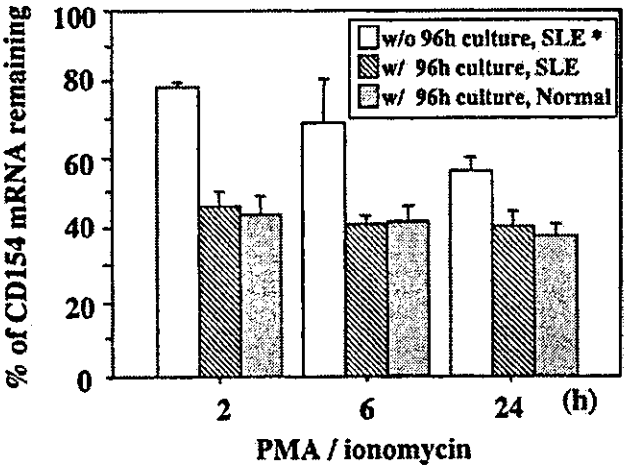

Fig. 5. Decreased CD154 mRNA stability of lupus CD4+ T cells after in vitro culture. CD4+ T cells from SLE and control individuals (n = 3 for both SLE and controls) were cultured in the absence of mitogen for 96 h, and then activated under identical conditions as in Fig. 4. for 2, 6, and 24 h, followed by incubation with Act D for 4 h. The CD154 mRNA was quantified as described in Fig. 3. The percentage of CD154 mRNA remaining after Act D treatment was compared between CD4+ T cells with and without the 96-h pre-stimulation culture. The symbols indicate the mean percentage of CD154 mRNA remaining and the SD of the mean, respectively. Preculture of SLE T cells without stimulation for 96 h decreased CD154 mRNA stability to similar levels to those of control individuals. *w/o*, without; *w/*, with; *star*, $P < 0.01$

active SLE patients and 50% culture medium, for 96 h and then stimulated with PMA + ionomycin. The influence of SLE sera was not demonstrated on CD154 expression in this study (date not shown).

Discussion

The interaction between CD40 and its ligand CD154 is pivotal for the development and maintenance of both humoral and cellular immune systems. The expression of CD154 on CD4+ T cells is transient and tightly regulated to mediate antigen-specific immune responses, while the constitutive presence of CD40 is observed in a wide spectrum of target cells.[3]

As previously described, CD154 mRNA is detected in freshly isolated PBMCs from patients with active SLE, but not from controls (see Fig. 1), and surface expression has also been demonstrated.[5,11] Moreover, a high concentration of soluble CD154 in SLE plasma has been demonstrated, and this may contribute to the production of autoantibodies and consequent inflammation in patients with SLE.[10] These findings indicate that some SLE T cells express CD154 constitutively.

We showed that CD154 mRNA of SLE patients was more stable than that of controls in this study (see Fig. 3). Recent studies have demonstrated that certain nucleotide sequence motifs, e.g., AU-rich elements and CU-rich regions, in the 3′ UTR provide binding sites for proteins to promote the decay of CD154 and several other mRNAs.[15,16,20–22] Although SLE susceptibility is not linked to the x chromosome where CD154 is located,[23] it is still possible that abnormal sequences of mRNA contribute to mRNA stability in a similar way to the TCR ζ chain reported to be deleted in the 3′ UTR by alternative splicing.[24–26] Therefore, we analyzed the nucleotide sequence of 3′ UTR of CD154 mRNA in PMA + ionomycin-activated PBMCs, but no abnormal or unique sequences were recognized in SLE patients when compared with the controls.

In SLE patients, increased expression of HLA-DP, an early activation marker of T cells, has been reported to decrease during 4 days of culture in a mitogen-free medium in vitro.[27] Moreover, impaired production of IL-2 is reported to be recovered by in vitro culture.[27] These findings indicate that some of the T cell abnormalities in SLE patients can be altered when these cells are in the resting condition. On the other hand, it is reported that the hyperexpression of CD154 on SLE T and B cells persisted during culture of SLE PBMCs in vitro.[6] Therefore, we cultured SLE CD4+ T cells alone in vitro for 96 h regardless of continuous T–B interaction and extracelluler stimulation, and then stimulated these cells with PMA + ionomycin. Protein and mRNA expression of CD154 in response to these stimuli was revealed to decrease to normal levels, and furthermore, mRNA stability also decreased to levels similar to those of the controls (see Figs. 4 and 5).

Several cytokines increase CD154 expression, and in particular IL-12 and IL-15 are reported to be increased in SLE patients.[28–30] Furthermore, stromal cell-derived factor-1 and osteopontin are also reported to augment CD154 expression.[31,32] To examine the influence of these serum factors, we determined CD154 expression on activated CD4+ T cells after culturing for 96 h in the presence of 50% sera from active SLE patients prior to PMA + ionomycin

stimulation. There was no difference in CD154 expression between those in the presence or absence of the SLE sera (data not shown), suggesting that the addition of SLE serum only was insufficient to continue CD154 hyperexpression on SLE T cells.

Distinct components of intracellular signaling molecules and transcriptional factors may be involved in the prolonged and increased expression of CD154 in SLE T cells. The TCR signal pathway has three main streams, Ca^{2+}/calcinurin/nuclear factor of activated T cells (NF-AT), Ras/mitogen-activated protein kinase (MAPK), and protein kinase C/nuclear factor-κB pathways.[33–35] Increased calcium influx has been observed after TCR ligation in SLE T cells compared with normal T cells.[36] Translocation of NF-AT from cytosol to nuclear factor, and binding to the CD154 promotor region to induce gene transcription, requires a calcium-dependent calcinurin pathway. Cyclosporin A, an inhibitor of this pathway, failed to inhibit CD154 expression on activated SLE T cell lines with TCR ligation when added at the late phase of activation.[37,38] Furthermore, the Cbl and MAPK, which activate transcriptional factor AP-1, were believed to be candidate molecules regulating the late phase of the activation, including mRNA stability, and to be defected in SLE T cells.[38] However, T cell signaling in vivo may be more complicated, since other accessory molecules are also involved in T–B cell interactions. It was shown that T cell surface molecules CD4, CD28, LFA-3, and ICOS augment CD154 expression.[39–42]

In SLE patients, increased calcium influx after TCR ligation, and abnormalities in the phosphorylation of TCR ζ and Cbl, have been reported.[24–26,38] PMA + ionomycin induce a similar activation pattern of T cells mediated through ligation of both TCR and other co-stimulatory molecules.[37] However, PMA + ionomycin directly stimulates the downstream abnormal signal transduction pathways PKC, Ras, and Ca^{2+} influx.[35,43] We demonstrated that increased expression and mRNA stability of CD154 in SLE CD4+ T cells decreased to normal levels with 96h prestimulation culture. These data indicated that CD154 hyperexpression on SLE T cells could not be due to intrinsic abnormalities in the downstream signal transduction pathways of TCR ligation.

The mechanism of inappropriate CD154 expression in SLE patients in vivo is still ambiguous. The continuous stimulation of T cells with abundant autoantigen-presenting cells may deliver a distinct form of signal transduction cascade, and increase and prolong the expression of CD154 in SLE T cells.

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Treatment of Rheumatoid Arthritis With Humanized Anti–Interleukin-6 Receptor Antibody

A Multicenter, Double-Blind, Placebo-Controlled Trial

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Objective. **Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates the immune response, inflammation, and hematopoiesis. Overproduction of IL-6 plays pathologic roles in rheumatoid arthritis (RA), and the blockade of IL-6 may be therapeutically effective for the disease. This study was undertaken to evaluate the safety and efficacy of a humanized anti–IL-6 receptor antibody, MRA, in patients with RA.**

Methods. **In a multicenter, double-blind, placebo-controlled trial, 164 patients with refractory RA were randomized to receive either MRA (4 mg/kg body weight or 8 mg/kg body weight) or placebo. MRA was administered intravenously every 4 weeks for a total of 3 months. The clinical responses were measured using the American College of Rheumatology (ACR) criteria.**

Results. **Treatment with MRA reduced disease activity in a dose-dependent manner. At 3 months, 78% of patients in the 8-mg group, 57% in the 4-mg group, and 11% in the placebo group achieved at least a 20% improvement in disease activity according to the ACR criteria (an ACR20 response) ($P < 0.001$ for 8-mg group versus placebo). Forty percent of patients in the 8-mg group and 1.9% in the placebo group achieved an ACR50 response ($P < 0.001$). The overall incidences of adverse events were 56%, 59%, and 51% in the placebo, 4-mg, and 8-mg groups, respectively, and the adverse events were not dose dependent. A blood cholesterol increase was observed in 44.0% of the patients. Liver function disorders and decreases in white blood cell counts were also observed, but these were mild and transient. There was no increase in antinuclear antibodies or anti-DNA antibodies. Anti-MRA antibodies were detected in 2 patients.**

Conclusion. **Treatment with MRA was generally well tolerated and significantly reduced the disease activity of RA.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by persistent synovitis and progressive joint damage, and it is often associated with the presence of antiimmunoglobulin autoantibodies, rheumatoid factors (1). Although the causes of RA are not fully understood, proinflammatory cytokines are involved in the development of the disease (2,3). These cytokines and their actions may be potential targets to block for the treatment of RA. Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates the immune response, inflammation, hematopoiesis, and bone metabolism (2). Constitutive overproduction of IL-6 is thought to play pathologic roles in RA. Elevation of IL-6 levels was observed in both serum and synovial fluid in patients with RA (4–6), and serum IL-6 levels correlated with disease activity and radiographic joint damage (7–11).

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Administration of mouse monoclonal anti–IL-6 antibody to 5 patients with RA was shown to ameliorate disease activity, although the effect was transient (12). Therefore, interference with the action of IL-6 may be effective for treating RA.

MRA is a humanized anti–human IL-6 receptor (anti–IL-6R) monoclonal antibody that inhibits the binding of IL-6 to IL-6R (13). The antibody was humanized by grafting the complementarity-determining regions from the murine anti–IL-6R antibody into human IgG1, thereby creating a functioning antigen-binding site in a reshaped human antibody. Safety studies in healthy adult male volunteers revealed that intravenous administrations of MRA were well tolerated. There were trends toward reductions in disease activity in 2 safety and dose-finding studies, 1 of single-dose MRA (14) and 1 of repetitive treatment for 6 months (15), involving small numbers of RA patients in the UK and Japan, respectively. On the basis of these findings, we conducted a multicenter, double-blind, placebo-controlled trial of MRA in patients with established and active RA.

PATIENTS AND METHODS

Patients. The study began in March 2001, and patients were enrolled from April 16, 2001 to November 26, 2001. Eligible patients were age ≥20 years, fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for RA (16), had a history of >6 months of disease activity, and were in functional class I, II, or III according to the Steinbrocker criteria (17). All candidates had been treated unsuccessfully (due to lack of efficacy) with at least 1 disease-modifying antirheumatic drug (DMARD) or immunosuppressant.

No DMARDs, immunosuppressants, parenteral and/or intraarticular use of corticosteroids, plasma exchange therapies, or surgical treatments were allowed during a 4-week washout period before the first dose and throughout the study period. Patients receiving prednisolone (maximum of 10 mg/day) and/or nonsteroidal antiinflammatory drugs (NSAIDs) were eligible if the dosage had not increased during the washout period. Both medications remained stable during the study period. In addition, eligible patients had white blood cell counts of at least 3,500/mm^3 and platelet counts of at least 100,000/mm^3 at enrollment. The necessary degree of disease activity at enrollment was confirmed by a finding of ≥6 swollen joints, ≥6 tender joints, and 1 of the following 2 criteria: a Westergren erythrocyte sedimentation rate (ESR) of at least 30 mm/hour or a C-reactive protein (CRP) level >1.0 mg/dl.

Sexually active premenopausal women were required to use effective contraception during the study period. Women in this category also had to have a negative urine pregnancy test result before enrollment. Patients were excluded if they had a medical history of a serious allergic reaction, significant cardiac, blood, respiratory system, neurologic, endocrine, renal, hepatic, or gastrointestinal diseases, or an active intercurrent infection requiring medication. Patients were not screened for purified protein derivative of tuberculin because most Japanese receive BCG vaccine. Chest radiographs specifically to rule out either active tuberculosis or old granulomatous disease were not required in the study protocol, but were often done to screen for active intercurrent respiratory infections.

Study protocol. The study protocol was approved by the Ministry of Health, Labor and Welfare of Japan and by the ethics committee at each participating center. Before enrollment, patients gave written informed consent, had a complete medical history taken, and underwent a complete examination. The examination consisted of a physical examination, measures of disease activity, and laboratory tests. In addition, serum was obtained for testing blood IL-6, soluble IL-6R (sIL-6R), anti-DNA antibodies, antinuclear antibodies (ANAs), and bone metabolism markers (blood osteocalcin, C-terminal type I procollagen propeptide [PICP]) on day 0 and at week 12 or 4 weeks after the last dose of the study drug. Serum for testing antibodies to MRA (neutralizing antibodies and IgE antibodies) was collected on day 0, every 2 weeks after the administration day, 1 month after the last infusion, and before the extension study when MRA was no longer detectable in the serum.

Baseline clinical assessments included the following: complete count of swollen and tender joints (49 joints evaluated; cervical spine and hips evaluated only for tenderness), physician's and patient's global assessment of disease status on a visual analog scale (VAS) from 0 (asymptomatic) to 100 (severe symptoms), patient's assessment of pain on a VAS from 0 (no pain) to 100 (severe pain), functional disability measured with a modified Health Assessment Questionnaire (18), Westergren ESR, and CRP level (19). Disease activity assessments were repeated on day 0 and every 4 weeks throughout the study. Each patient was assessed by the same rheumatologist at each visit.

Treatment. Patients were randomly assigned to 1 of 3 treatment groups: placebo, 4 mg of MRA per kg of body weight (4-mg group), or 8 mg of MRA per kg of body weight (8-mg group). Patients were administered an allocated study drug 3 times at 4-week intervals for 3 months. MRA was supplied as a sterile liquid formulation with 200 mg of MRA per 10-ml vial. The placebo was a liquid formulation not containing active substance and indistinguishable from the MRA vial. The appropriate amount of MRA was diluted to a total volume of 250 ml in sterile saline and administered over a period of 1 hour by intravenous drip infusion using a 0.2-μm in-line filter under careful monitoring.

Statistical analysis. The primary end point was the incidence of a 20% improvement in disease activity according to the ACR criteria (ACR20) (19) at week 12 with the last observation carried forward (LOCF) method. The closed testing procedure for multiple comparisons was used in the primary dose-response analysis. The first- and second-step comparisons were those for 8 mg/kg MRA versus placebo and 4 mg/kg MRA versus placebo, respectively. Secondary end points included the Disease Activity Score in 28 joints (DAS28) (20), the incidences of 50% and 70% improvement in disease activity according to the ACR criteria (ACR50 and ACR70, respectively), the presence of overall improvement in the ACR criteria from week 0 to week 12, and the different