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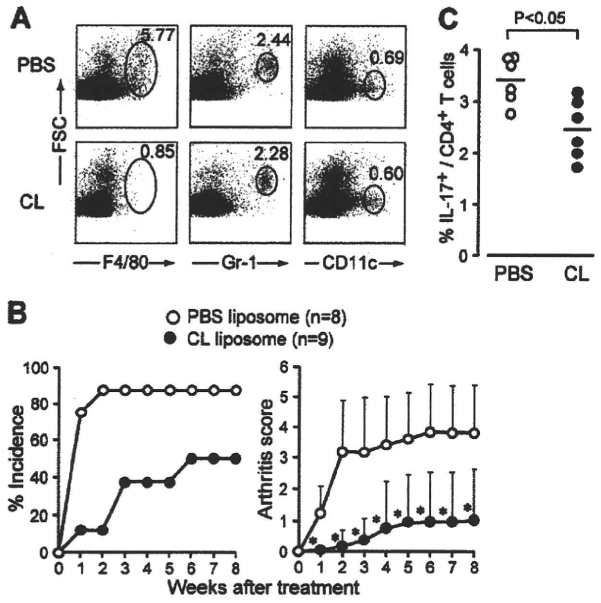

Figure 5. Macrophages are required for in vivo Th17 expansion and induction of arthritis. (A) Specific depletion of macrophages in SKG mice 3 d after i.v. injection of 200 µl CL. One representative out of three independent experiments is shown (numbers indicate percentages). (B) Joint scores of SKG mice i.v. injected with 200 µl PBS or CL 1 d before mannan treatment. *, $P < 0.05$. Error bars are means ± SD. (C) Percentage of IL-17$^+$ CD4$^+$ T cells in the popliteal lymph nodes of SKG mice 2 wk after mannan treatment. Mice were pretreated by an i.v. injection of PBS or CL ($n = 6$). Horizontal bars are the means.

spontaneous differentiation of Th17 cells and triggers autoimmune arthritis (Hirota et al., 2007). Because T cell–APC interaction induces local complement activation (Liu et al., 2008; Strainic et al., 2008), we asked whether such intrinsic complement activation would contribute to Th17 cell differentiation and arthritis induction. In RAG2$^{-/-}$ mice that developed arthritis after transfer of SKG CD4$^+$ T cells, the joint tissue actively transcribed *C3* and *IL-6* mRNA, indicating complement activation and IL-6 production in the affected joint (Fig. 6 A). When CD4$^+$ T cells from C5aR$^{+/+}$ or C5aR$^{-/-}$ SKG mice were transferred to C5aR$^{+/+}$ or C5aR$^{-/-}$ RAG2$^{-/-}$ mice, both CD4$^+$ T cell populations induced arthritis at equivalent incidences and severities in C5aR$^{+/+}$ RAG2$^{-/-}$ mice but were significantly lower in C5aR$^{-/-}$RAG2$^{-/-}$ mice (Fig. 6 B). Thus, C5aR expression by the recipient cells, not donor CD4$^+$ T cells, was required for disease induction. In accordance with the joint scores, the generation of Th17 cells from transferred CD4$^+$ T cells was significantly suppressed in C5aR$^{-/-}$ recipients (Fig. 6, C and D). The generation of IFN-γ–secreting cells was not significantly affected, although there was a tendency toward a higher proportion of IFN-γ$^+$ cells in C5aR$^{-/-}$ recipients. Inhibition of Th17 cell differentiation by C5aR deficiency was not restricted to SKG CD4$^+$ T cells, as Th17 cell differentiation of BALB/c CD4$^+$ T cells during homeostatic proliferation was also suppressed in C5aR-deficient recipients (Fig. 6 E). Collectively, these results indicate that interactions between self-reactive T cells and APCs can preferentially drive the differentiation of the former into Th17 effector cells via complement activation and the resulting C5a action on APCs.

Thus, extrinsic and intrinsic complement activation promotes Th17 cell differentiation and expansion, evoking autoimmune arthritis in SKG mice (Fig. 6 F). It was noted, however, that C5aR deficiency significantly suppressed but did not completely inhibit arthritis development triggered by laminarin, zymosan, or mannan (Fig. 2). This incomplete inhibition could be attributed to the fact that these microbial products not only activate complement but also directly stimulate macrophages and DCs via cell surface–expressed pattern recognition receptors such as TLR and C-type lectin receptors (e.g., Dectin-1, mannose receptor, and DC-SIGN; Yoshitomi et al., 2005; Robinson et al., 2006; Sheng et al., 2006). Indeed, in our co-culture experiments with macrophages, zymosan, laminarin, or mannan at a high dose (1 mg/ml) expanded Th17 cells, presumably via IL-6 production in an MyD88-dependent and -independent manner (Fig. S4). In addition, we observed a synergy between the signals from C5aR and MyD88 for cytokine production by macrophages (Fig. 4 A; Fang et al., 2009). Yet, it is of note that the prototypic TLR agonist LPS or CpG alone failed to elicit arthritis in SKG mice, in contrast to successful arthritis induction by mannan, a prototypic complement activator (Yoshitomi et al., 2005). This indicates that complement activation and C5a production can be a major pathway for driving Th17-dependent autoimmune arthritis in SKG mice.

Because C5a, IL-17, and GM-CSF are commonly capable of enhancing granulopoiesis and neutrophil recruitment (Höpken et al., 1996; Korn et al., 2009), together they would cause robust neutrophil accumulation (Fig. 3 B) and form a positive feedback loop of Th17-mediated inflammation (Fig. 6 H; Sonderegger et al., 2008). In addition, the cartilage surface lacks several complement inhibitors, which might render the joint highly susceptible to complement activation (Matsumoto et al., 2002). It is thus likely that transient synovial inflammation may frequently occur when an individual is exposed to complement-activating microbial products, ICs, autoantibodies, or physical trauma. Such synovial inflammation per se may not be sufficient to trigger chronic arthritis in normal individuals. Yet, if an individual harbors potentially arthritogenic CD4$^+$ T cells (e.g., because of genetic predisposition), such complement-induced synovial inflammation may promote the differentiation/expansion of arthritogenic Th17 cells and instigate chronic arthritis. It is worth noting in this regard that genetic susceptibility to RA is in part determined by the polymorphism of the genes encoding C5 (TRAF1-C5), PTPN22 (which affects TCR proximal signaling, as observed with the SKG ZAP-70 mutation), or STAT4, which might alter Th17 cell function (Vang et al., 2005; Plenge et al., 2007; Remmers et al., 2007). These genetic polymorphisms could promote the production of arthritogenic T cells and their Th17 cell differentiation via

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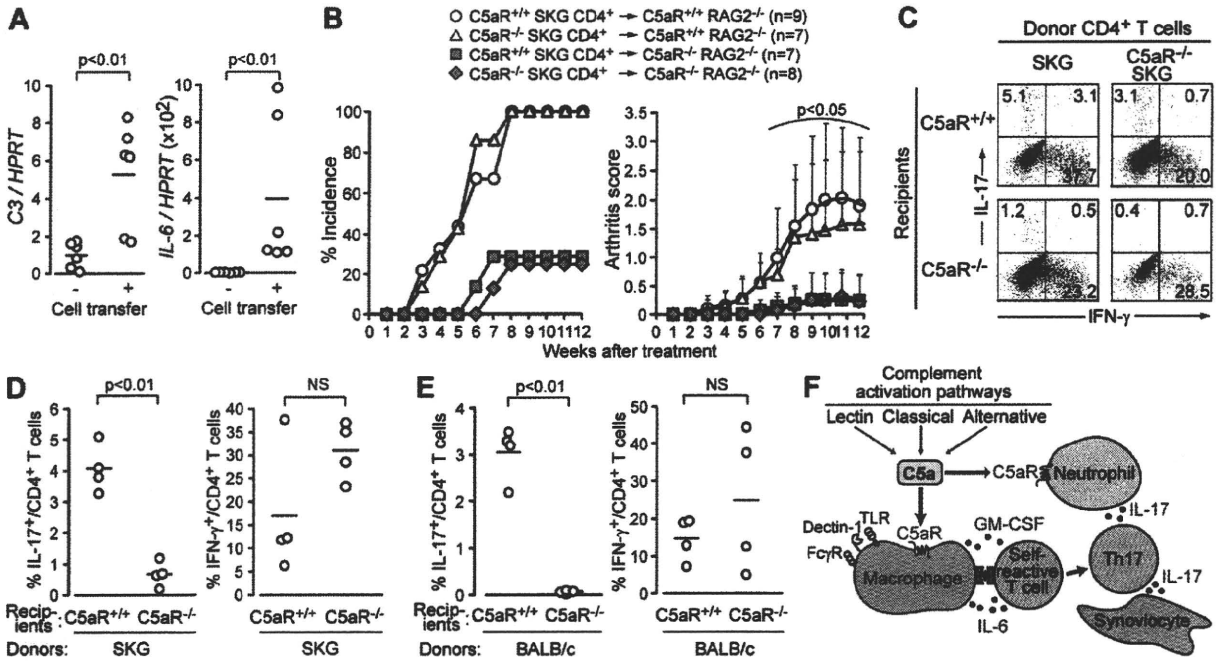

Figure 6. C5a promotes spontaneous differentiation of CD4+ T cells to Th17 cells via homeostatic proliferation. (A) Quantitative RT-PCR for *C3* and *IL-6* mRNA in the joints of RAG2$^{-/-}$ mice 12 wk after transfer of SKG CD4+ T cells ($n = 6$). (B) 10^6 C5aR$^{+/+}$ or C5aR$^{-/-}$ SKG CD4+ T cells were transferred to C5aR$^{+/+}$ or C5aR$^{-/-}$ RAG2$^{-/-}$ mice. Joint scores were assessed every week in two independent experiments. Error bars are means ± SD. (C) 2×10^6 C5aR$^{+/+}$ or C5aR$^{-/-}$ SKG CD4+ T cells were transferred to C5aR$^{+/+}$ or C5aR$^{-/-}$ RAG2$^{-/-}$ mice. Intracellular cytokines in recipient splenic CD4+ T cells were stained on day 7 (numbers indicate percentages). One representative out of four independent experiments is shown. (D and E) Percentages of IL-17+ or IFN-γ+ cells among CD4+ T cells in C5aR$^{+/+}$ or C5aR$^{-/-}$ RAG2$^{-/-}$ mice ($n = 4$ each) after transfer of SKG (D) or BALB/c CD4+ T cells (E). (F) A model for the role of complement activation in Th17-mediated autoimmune arthritis. Horizontal bars in A, D, and E are the means.

complement activation. Although C5aR signaling plays a complex role depending on the cell types (e.g., macrophages or DCs) and additional receptors (e.g., TLRs; Guo and Ward, 2005; Weaver et al., 2010), this report provides evidence that complement activation and C5a production is critically involved in the initiation of certain autoimmune disease, and presumably microbial immunity, by driving Th17 development.

MATERIALS AND METHODS

Mice. C5aR$^{-/-}$, IL-17$^{-/-}$, IL-6$^{-/-}$, RAG2$^{-/-}$, TLR4$^{-/-}$, and MyD88$^{-/-}$ mice (TLR4- or MyD88-deficient mice were provided by S. Akira, Osaka University, Osaka, Japan) were described previously (Hawlisch et al., 2005; Yoshitomi et al., 2005; Hirota et al., 2007). MASP-null mice were generated by crossing MASP1/3$^{-/-}$ and MASP2/sMAP$^{-/-}$ mice (Iwaki et al., 2006; Takahashi et al., 2008). All mice were maintained in a SPF condition in our animal facility in accordance with the guidelines for animal care approved by the Institute for Frontier Medical Sciences, Kyoto University.

Reagents. Recombinant mouse C5a was purchased from R&D Systems. Mannan from *Saccharomyces cerevisiae* was purchased from Sigma-Aldrich and was dissolved in 200 µl PBS before i.p. injection. The endotoxin level in the reagents used in the current study was 0.44 EU/ml for 1 µg/ml C5a and 6.14 EU/ml for 1 mg/ml mannan, 4.01 EU/ml for 1 mg/ml laminarin, and <0.001 EU/ml for plasma-derived C5a, as determined by the LAL test (Wako Chemicals USA, Inc.). Plasma-derived C5a was purified from human EGTA-plasma as previously described (Köhl, 1997). CL was a gift from Roche, and the liposomal preparation was prepared as previously described (Solomon et al., 2005). IgG3-IC was prepared as previously described (Díaz de Ståhl et al., 2003).

Antibodies. For flow-cytometric analysis, anti-CD4 (RM4-5), anti-IL-17 (TC11-18H10.1), anti-IFN-γ (XMG1.2), anti-CD11b (M1/70), anti-CD11c (HL3), anti-Gr-1 (RB6-8C5), and isotype IgG were purchased from eBioscience; anti-GM-CSF (MP1-22E9) was purchased from BD; anti-C5aR (20/70) was purchased from Cedarlane; and anti-F4/80 (A3-1) was purchased from AbD Serotec.

Cell culture. Resident peritoneal macrophages were sorted by MoFlo (Dako) for FSC^{high}, SSC^{high}, CD11b^{high} cells from lavage of the BALB/c peritoneal cavity with 10 ml PBS containing 2% FCS and 2 mM EDTA. Thioglycollate-elicited peritoneal macrophages were prepared as previously described (Zhang et al., 2007). Splenic DCs were sorted for CD11c^{high} cells in BALB/c spleens treated with Librase Blenzyme II (Roche). 2.5×10^4 CD4+ T cells were cultured with 1.25×10^4 macrophages or DCs in RPMI 1640 medium (Sigma-Aldrich) containing 10% FCS, and were stimulated with 0.5 µg/ml anti-CD3 (2C11) with or without 500 ng/ml C5a (R&D Systems), 10 ng/ml TGF-β (PeproTech), or 10 µg/ml anti-IL-6R (MR16-1).

C3 deposition assay. Maxi-Plates (Thermo Fisher Scientific) were coated with 100 µg/ml laminarin, zymosan, or mannan. After blocking, wells were incubated with 2% mouse sera diluted with TBS/Tween/Ca (0.1% BSA, 0.05% Tween 20, 5 mM $CaCl_2$) at 37°C for 30 min. C3c deposited on the well surfaces was detected by anti-human C3c (Dako) followed by horseradish peroxidase–conjugated secondary antibody (Dako), and assessed by ELISA. Supernatants from the C3c deposition assay were assessed for the concentration of C5a by ELISA and expressed as the concentrations in the original sera.

Quantitative RT-PCR. Total RNA of peritoneal macrophages was extracted 24 h after incubation with 500 ng/ml C5a using the RNeasy column

(QIAGEN). Total RNA of joint tissues was extracted by Isogen (Wako Chemicals USA, Inc.) according to the manufacturer's instructions. cDNA was transcribed by reverse transcription (SuperScript III; Invitrogen), and the genes were quantified by the SYBR Green I system using LightCycler (Roche). Gene expression was normalized to expression of the *HPRT* gene. *IL-23*, *C3*, and *IL-6* primer sequences are as follows: *IL-23* forward, 5′-TCCCTACTAGGACTCAGCCAAC-3′; *IL-23* reverse, 5′-TGGGCATCTGTTGGGTCT-3′; *C3* forward, 5′-TTCGTCCTCATCGCACTG-3′; *C3* reverse, 5′-TGTAACTGGCTTCAATATACTCC-3′; *IL-6* forward, 5′-CCACTTCACAAGTCGGAGGCTTA-3′; and *IL-6* reverse, 5′-GCAAGTGCATCATCGTTGTTCATAC-3′.

ELISA. ELISA for C3a, C5a, and cytokines was performed according to the manufacturer's instructions (BD). For measuring in vivo complement activation, futhan (FUT-175; BD) was added to the plasma preparation to prevent ex vivo complement activation.

Clinical assessment of joint scores, intracellular cytokine staining, and preparation of synovial cells. These were performed as described previously (Hirota et al., 2007).

Statistical analysis. The in vivo joint scores were analyzed by the Mann-Whitney *U* test. Unless otherwise mentioned, the Student's *t* test was used for statistical analysis. $P < 0.05$ was considered significant.

Online supplemental material. Fig. S1 shows C3a/C5a production and early Th17 cell expansion after mannan treatment. Fig. S2 depicts the dependency of C5a/TGF-β–induced Th17 cell development on the type of APCs. Fig. S3 shows the effect of co-stimulation and T cell–derived cytokines on IL-6 production by C5a-stimulated macrophages. Fig. S4 depicts TLR-dependent IL-6 production by laminarin, zymosan, or mannan. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092301/DC1.

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Intervention of an Inflammation Amplifier, Triggering Receptor Expressed on Myeloid Cells 1, for Treatment of Autoimmune Arthritis

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Objective. **Triggering receptor expressed on myeloid cells 1 (TREM-1) is inducible on monocyte/macrophages and neutrophils and accelerates tissue destruction by propagating inflammatory responses in disease related to bacterial infections. Its blockade rescues the hosts in murine models of sepsis, to clear the bacteria without impairing the host defense. The aim of this study was to investigate the involvement of TREM-1 in an autoimmune, noninfectious disease.**

Methods. **Synovial tissue specimens from the joints of patients with rheumatoid arthritis (RA) and the joints of mice with collagen-induced arthritis (CIA) were examined for TREM-1 expression, using flow cytometric analysis. Expression of TREM-1 on macrophages was induced by lipopolysaccharide, with or without a cyclooxygenase inhibitor. Rheumatoid synovial cells were stimulated with agonistic anti–TREM-1 antibodies. Recombinant adenovirus encoding the extracellular domain of TREM-1 fused with IgG-Fc (AxCATREM-1 Ig) or synthetic TREM-1 antagonistic peptides were injected to treat CIA, and the clinical manifestations of the antigen-specific T cell and B cell responses were evaluated.**

Results. **TREM-1 was expressed on CD14+ cells in rheumatoid synovial tissue and synovial macrophages from mice with CIA. Unlike murine macrophages, human monocyte/macrophages did not depend on prostaglandin E_2 for up-regulation of TREM-1. Agonistic anti–TREM-1 antibodies promoted tumor necrosis factor α production from rheumatoid synovial cells. Blockade of TREM-1 using AxCATREM-1 Ig and antagonistic peptides ameliorated CIA without affecting the serum levels of anti–type II collagen antibodies or the proliferative responses of splenocytes to type II collagen.**

Conclusion. **TREM-1 ligation contributes to the pathology of autoimmune arthritis. The results of this study implied that blockade of TREM-1 could be a new approach to rheumatic diseases that is safer than the presently available immunosuppressive treatments.**

Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial hyperplasia with massive infiltration of inflammatory cells, which leads to degeneration of cartilage, erosion of bone, and ultimately loss of function in the affected joints. T cells recognizing unknown autoantigens have been proposed to initiate inflammation in the synovial tissue. Presumably, this process is followed by synovial recruitment of macrophages and lymphocytes, which are further activated in the sites of inflammation (1). Activated macrophages contribute to disease progression by producing various proinflammatory cytokines, prostaglandins (PGs), metalloproteinases (MMPs), and nitric oxide (2–4). Although production of these inflammatory molecules is regulated by ligand-triggered activation of cell

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surface receptors, including cytokine receptors, complement receptors, Toll-like receptors (TLRs), and immunoreceptor tyrosine–based activation motif (ITAM)–associated receptors, the roles of the individual receptors in RA pathology are not fully understood.

A triggering receptor expressed on myeloid cells (triggering receptor expressed on myeloid cells 1 [TREM-1]) has been identified as a transmembrane receptor that binds to an ITAM-containing adaptor molecule, DAP12 (5,6). It is expressed on monocyte/macrophages and neutrophils and is up-regulated by various stimuli such as microbial TLR ligands and proinflammatory cytokines (5–7). Treatment with a monoclonal antibody reactive to TREM-1 simulated binding of unknown ligands and stimulated neutrophils and monocytes to produce various proinflammatory cytokines and to express immunostimulatory cell-surface molecules (5). Furthermore, the combination of this antibody and lipopolysaccharide (LPS) induced robust production of proinflammatory cytokines by monocytes, indicating that TREM-1 acts as an amplifier of innate immune responses (5,8,9).

Although natural ligands of TREM-1 remain to be identified, the involvement of TREM-1 has been reported largely in bacterial infections. TREM-1 was up-regulated on myeloid cells in human and murine hosts with sepsis (9,10). Soluble TREM-1 (sTREM-1) molecules, cleaved from membrane-bound TREM-1, were present at high concentrations in sera and bronchoalveolar lavage fluid from patients with bacterial infections (11,12). An increase in the serum level of sTREM-1 has been found to be the most accurate laboratory marker of bacterial infections (11).

TREM-1 activation should be detrimental in the pathology of sepsis, because administration of a TREM-1 extracellular domain fused with the IgG-Fc portion (TREM-1 Ig) or a synthetic peptide containing a putative ligand-binding sequence of TREM-1 protected hosts from lethal LPS challenge and septic bacterial infection (9,13). These treatments decreased tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) production, but residual levels of these cytokines appeared sufficient for clearance of pathologic bacteria (9). TREM-1 blockade by the antagonistic peptides also attenuated experimental inflammatory colitis (14), in which gut commensal bacteria are essential for disease induction (15). Thus, TREM-1 engagement amplifies host immune reactions to bacterial infections and, in some instances, leads to undesired host tissue damage.

For the treatment of RA, new biologic antirheumatic agents have demonstrated superb efficacy. However, they have been associated with a variety of serious infections. TNFα-blocking agents have conferred on patients an increased risk of bacterial as well as fungal infections and reactivation of *Mycobacterium tuberculosis* infection (16,17). The administration of humanized anti–IL-6 receptor monoclonal antibodies exacerbated chronic active Epstein-Barr virus infection (18). Treatment with a chimeric anti-CD20 monoclonal antibody might activate latent JC virus infection, leading to progressive multifocal leukoencephalopathy (19). Because TREM-1 blockade attenuated proinflammatory cytokine production and allowed sufficient control of bacterial infections, it should be a candidate approach to RA treatment that does not impair the immune defense against microbial infections.

We demonstrated recently that LPS-induced up-regulation of TREM-1 on murine macrophages is mediated by PGE_2 (20). Because PGE_2 is released in various inflammatory conditions, cyclooxygenase (COX) inhibitors might act as crucial inhibitors of TREM-1 expression. However, they do not alter the devastating disease course of RA. This implies that TREM-1 engagement is not of importance in the pathology of RA. However, little is known about TREM-1 expression and its regulation in humans.

In the present study, we revealed the differential contribution of PGE_2 to TREM-1 induction in human and murine monocyte/macrophages. We demonstrated TREM-1 expression in synovial tissue from rheumatoid joints and in the joints of mice with collagen-induced arthritis (CIA), an animal model of RA. TREM-1 blockade exerted significant therapeutic effects on CIA. It did not impair T cell and B cell immune responses to the inducing antigen. These results provide evidence that TREM-1 ligation should contribute to the pathology of autoimmune arthritis, and that TREM-1 blockade could be a new therapeutic approach distinct from the presently available treatments for RA.

MATERIALS AND METHODS

Cells. Human synovial tissue specimens were derived from patients with RA undergoing total joint replacement surgery or synovectomy at Shimoshizu National Hospital. Consent forms were completed by the patients before they underwent surgery. RA was diagnosed according to the criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (21). Murine synovial tissues were isolated from the knee joints of mice with CIA. Human and murine synovial cells were prepared as described previously (22,23). Resident peritoneal macrophages from male mice were prepared as described previously (7).

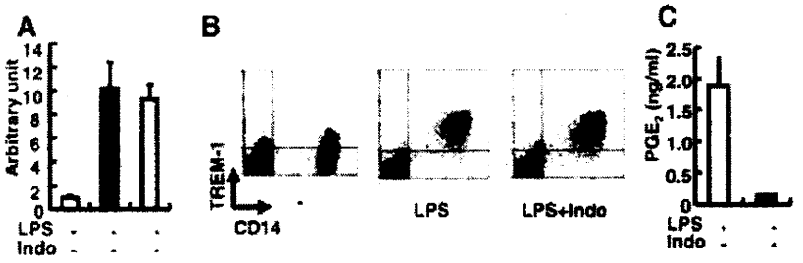

Figure 1. Lipopolysaccharide (LPS)–induced expression of triggering receptor expressed on myeloid cells 1 (TREM-1) on human peripheral blood mononuclear cells (PBMCs) in the presence or absence of a cyclooxygenase inhibitor (indomethacin; Indo). **A,** Human PBMCs were stimulated with LPS in the presence or absence of indomethacin for 24 hours. TREM-1 mRNA in the stimulated PBMCs was quantified with real-time polymerase chain reaction. The amount of each mRNA sample was normalized to that of GAPDH mRNA, and an arbitrary unit was defined (value of untreated cells = 1). **B,** Surface expression of TREM-1 was analyzed by flow cytometry, using anti–TREM-1 and anti-CD14 antibodies. **C,** Prostaglandin E_2 (PGE_2) released in the culture supernatants was quantified with a specific enzyme-linked immunosorbent assay. Values in A and C are the mean and SD.

TREM-1 antagonistic reagents. Replication-defective adenoviruses containing a mouse TREM-1 Ig gene (AxCATREM-1 Ig) and LacZ gene (AxCALacZ) were prepared as described previously (24). Antagonistic TREM-1 peptides, LP17 (LQVTDSGLYRCVIYHPP) and sequence-scrambled control peptides (TDSRCVIGLYHPPLQVY) (13) were synthesized (Invitrogen, Carlsbad, CA).

Quantification of TREM-1 Ig, TNFα, and PGE_2 concentrations. TREM-1 Ig in the sera and the culture supernatants was quantified with an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) to quantify sTREM-1. Specific ELISA kits to quantify TNFα, IL-17, and PGE_2 in the culture supernatants were obtained from R&D Systems and Cayman Chemical (Ann Arbor, MI), respectively.

Detection of TREM-1. Human synovial cells and peripheral blood mononuclear cells (PBMCs) were double-stained with fluorescein isothiocyanate–conjugated anti-human CD14 (Beckman Coulter, Fullerton, CA) and phycoerythrin (PE)–conjugated anti-human TREM-1 monoclonal antibody (R&D Systems). Murine synovial cells were double-stained with allophycocyanin-conjugated anti-mouse CD11b (e-Bioscience, San Diego, CA) and PE-conjugated anti-mouse TREM-1 monoclonal antibody. Isotype controls were used in all experiments. Data were acquired using a FACSCalibur system and were analyzed using CellQuest software (BD Biosciences, San Jose, CA). Quantitative real-time polymerase chain reaction (PCR) was carried out as previously described (4). Carboxymethylcellulose-embedded cryostat sections of frozen synovial samples were incubated with 25 μg/ml of rabbit anti-murine TREM-1 antibody (R&D Systems) or normal rabbit IgG. They were next incubated with biotinylated anti-rat IgG antibody and then incubated with peroxidase-conjugated streptavidin (DakoCytomation, Kyoto, Japan). The samples were treated with diaminobenzidine (DakoCytomation) for immunohistochemical detection and counterstained with hematoxylin.

Activation of TREM-1. Flat-bottomed microtiter plates were precoated with 5 μg/ml of an anti-human TREM-1 monoclonal antibody (R&D Systems) or an isotype-matched control antibody overnight at 4°C. Cells were incubated in these wells for 24 hours for TREM-1 stimulation.

Induction of CIA. Male DBA/1J mice were purchased from Charles River Japan Breeding Laboratories (Tokyo, Japan). All experiments were carried out under the guidelines for animal experiments of Tokyo Medical and Dental University. Induction of CIA, clinical assessment, quantification of joint swelling, and histologic examination were carried out as described previously (25). No LPS was used in the animal experiments. Infiltration of inflammatory cells, transformation of synovial lining, cartilage destruction, and pannus formation were scored in a blinded manner (25). The histologic scores ranged from 0 to 3 (maximum histologic score = 12). IL-17, TNFα, and IL-1β messenger RNA (mRNA) in the joints were quantified as described previously (23,26,27). Type II collagen–specific antibodies in mouse sera and type II collagen–specific T cell responses were quantified as described previously (28). Type II collagen–induced IL-17 and interferon-γ (IFNγ) production by splenocytes was measured with a specific ELISA.

Statistical analysis. Protein concentrations in the supernatants, titers of IgG, ^{3}H-thymidine incorporation, hind paw thickness, and ankle width were compared with Student's paired *t*-test. The arthritis scores and histologic scores were analyzed statistically with the Mann-Whitney U test.

RESULTS

PG-independent TREM-1 up-regulation in human monocyte/macrophages. LPS-induced TREM-1 up-regulation on murine macrophages is mediated by PGE_2 (20). We stimulated human PBMCs with LPS in the

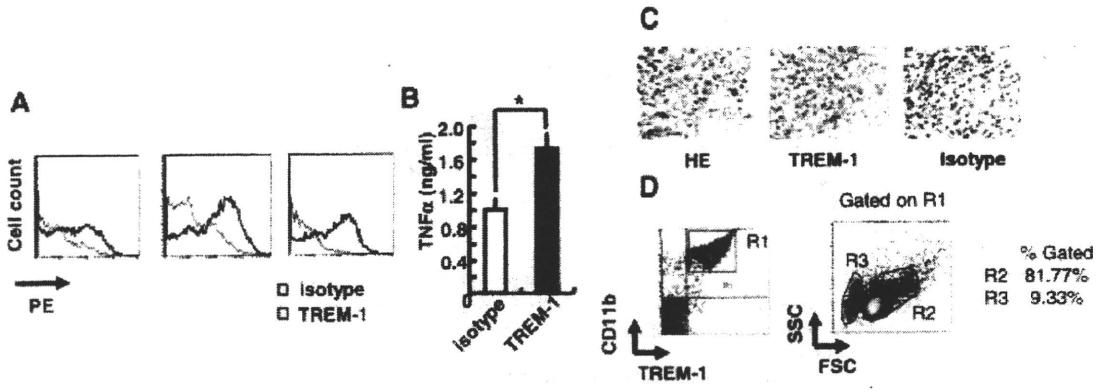

Figure 2. Triggering receptor expressed on myeloid cells 1 (TREM-1) expression in arthritis-affected joints. Synovial cells from rheumatoid synovial tissue of 3 patients with rheumatoid arthritis (RA) were isolated. **A,** Two-color flow cytometry was performed with fluorescein isothiocyanate–labeled anti-CD14 and phycoerythrin (PE)–labeled anti–TREM-1. The panels show TREM-1 expression on the CD14-gated cells from individual patients. **B,** Synovial cells were cultured for 24 hours in the presence of plate-bound agonistic anti–TREM-1 monoclonal antibodies or isotype-matched monoclonal antibodies. Tumor necrosis factor α (TNFα) levels in the supernatants were measured with a specific enzyme-linked immunosorbent assay. Data are representative of 2 experiments and are expressed as the mean and SD of triplicate wells. * = $P < 0.01$. **C,** Frozen sections from the inflamed joints of mice, 7 days after the second immunization with type II collagen, were stained with hematoxylin and eosin (H&E), anti–TREM-1 monoclonal antibody, or an isotype-matched monoclonal antibody. **D,** Isolated cells from the synovial tissues of the collagen-induced arthritis (CIA) joints were analyzed with flow cytometry for TREM-1 and CD11b expression. The cytograms for CD11b+ and TREM-1+ cells (R1) were examined to distinguish between macrophages (R2) and polynuclear cells (R3). SSC = side scatter; FSC = forward scatter.

presence or absence of a COX inhibitor, indomethacin. Quantitative real-time PCR and flow cytometric analyses disclosed that the COX inhibitor did not affect TREM-1 up-regulation on the human cells, at both the mRNA and protein levels (Figures 1A and B), while it abrogated PGE_2 release (Figure 1C). It is known that the cells that express TREM-1 in PBMCs are monocyte/macrophages (5). Actually, almost all TREM-1–positive cells expressed CD14 (Figure 1B). Thus, unlike the situation in murine macrophages, inflammatory stimuli can up-regulate TREM-1 via a PG-independent pathway in human monocyte/macrophages.

Abundant expression of TREM-1 in synovial tissue from rheumatoid joints and CIA joints. To detect TREM-1 expression in synovial tissue from patients with RA, synovial cells were isolated from the rheumatoid joints. Flow cytometric analyses revealed that the CD14+ synovial cells expressed TREM-1 (Figure 2A). Thus, macrophages in rheumatoid synovial tissues expressed TREM-1.

To test the function of the expressed TREM-1, rheumatoid synovial cells were stimulated with immobilized agonistic anti–TREM-1 monoclonal antibodies for 24 hours. Although spontaneous TNFα secretion by the synovial cells was observed, it increased significantly after TREM-1 crosslinking (Figure 2B).

To assess TREM-1 expression in synovial tissue from mice with CIA, a murine model of RA, synovial tissue specimens from affected mice were examined for TREM-1 expression. Immunohistochemical analyses disclosed several TREM-1–positive cells in the synovial tissues (Figure 2C). Flow cytometric analyses revealed that TREM-1 was expressed by isolated CD11b+ synovial cells, most of which were identified as macrophages (Figure 2D) (29). These results demonstrated that TREM-1 is expressed primarily on synovial macrophages in CIA joints.

Adenoviral gene transfer for systemic expression of TREM-1 Ig. It has been shown that in vivo administration of recombinant adenoviruses provokes systemic expression of soluble transgene products (30). To address whether TREM-1 blockade exerts its therapeutic effects on CIA, recombinant adenovirus containing a gene for the extracellular domain of TREM-1 fused with IgG-Fc (TREM-1 Ig) was constructed (AxCATREM-1 Ig) (24). When AxCATREM-1 Ig was injected intravenously into mice, an increase in serum concentrations of TREM-1 Ig persisted for at least 7 days (Figure 3A). Injection of the control adenovirus, AxCALacZ, did not increase serum levels of endogenous sTREM-1, which can be detected with the same assay.

To confirm the bioactivity of the adenoviral

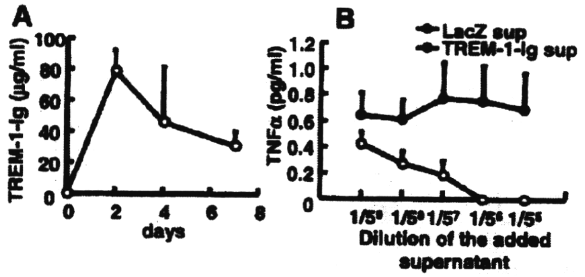

Figure 3. Adenoviral gene transfer of TREM-1 Ig. Mice were treated with intravenous injections of AxCALacZ or AxCATREM-1 Ig (10^9 plaque-forming units/mouse). **A**, Sera were examined at the indicated time points for TREM-1 Ig levels, using enzyme-linked immunosorbent assay (ELISA). Values are the mean and SD results from 5 mice per group. **B**, The culture supernatants of NIH3T3 cells infected with AxCALacZ (LacZ sup) or AxCATREM-1 Ig (TREM-1-Ig sup) were collected at 3 days postinfection. Nine consecutive 5-fold dilutions of the supernatants were added to the culture medium of resident peritoneal macrophages stimulated with plate-bound agonistic anti–TREM-1 monoclonal antibody. After 24 hours, the culture supernatants were examined for TNFα concentrations with a specific ELISA. Data are representative of 2 experiments and are expressed as the mean and SD results from triplicate wells. See Figure 2 for other definitions.

TREM-1 Ig, NIH3T3 cells were infected with AxCATREM-1 Ig or control viruses. The culture medium of AxCATREM-1 Ig–infected cells contained 10 μg/ml of TREM-1 Ig. It was noted that TREM-1 Ig concentrations in sera from AxCATREM-1 Ig–treated mice were higher than 10 μg/ml during the first 7 days after infection. The addition of the supernatant to culture medium of resident peritoneal macrophages inhibited the TNFα production triggered by immobilized anti–TREM-1 monoclonal antibodies in a dose-dependent manner (Figure 3B). These results led us to assume that the serum concentration of TREM-1 Ig in mice treated with in vivo gene transfer should be sufficient to prevent TREM-1 ligation.

Suppression of CIA by TREM-1 Ig. For induction of CIA, mice were immunized twice with type II collagen. After the onset of arthritis (2 days after the second immunization), they were treated with AxCATREM-1 Ig or control AxCALacZ adenoviruses. Evaluation of ankle width, hind paw thickness, and the arthritis score disclosed that CIA was suppressed significantly by the intravenous injection of AxCATREM-1 Ig, in a dose-dependent manner (Figures 4A–C). Control adenoviruses exerted no effects. In histologic examinations, the control joints showed hyperplastic pannus tissues massively infiltrated by inflammatory cells, cartilage destruction, and bone erosion, which are characteristic of the pathology of RA. These features were suppressed in

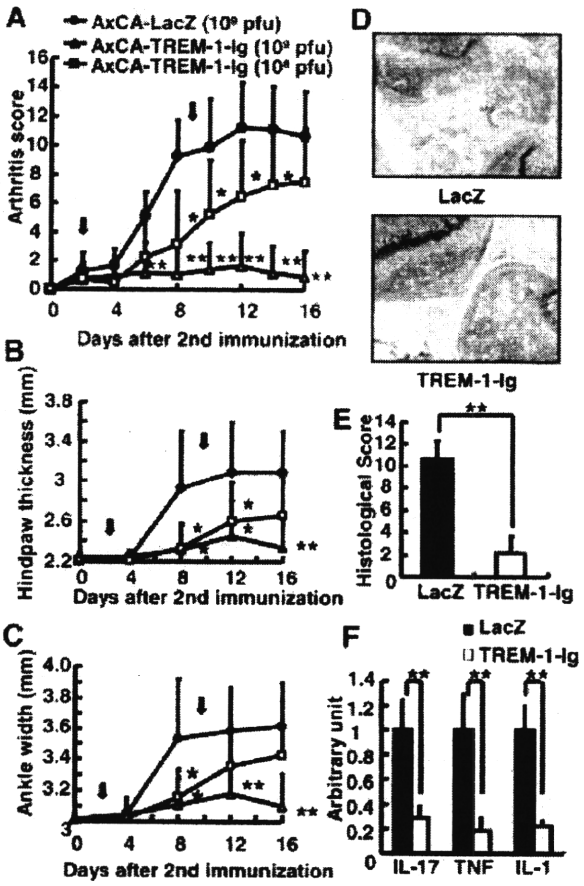

Figure 4. Attenuation of collagen-induced arthritis (CIA) by triggering receptor expressed on myeloid cells 1 (TREM-1) blockade. **A–C**, Mice with CIA were treated with intravenous injections of AxCA-LacZ (10^9 plaque-forming units [PFU]), AxCATREM-1 Ig (10^8 PFU), or AxCATREM-1 Ig (10^9 PFU) adenoviruses in 100 μl phosphate buffered saline at the indicated time points (arrows). The arthritis score (**A**) hind paw thickness (**B**), and ankle width (**C**) were evaluated. **D** and **E**, The joints of AxCALacZ (LacZ)– and AxCATREM-1 Ig (TREM-1 Ig)–treated mice were examined histologically with hematoxyin and eosin staining 16 days after the second immunization. Histologic scores were compared between the 2 groups. Original magnification ×400 in **D**. **F**, The joints of AxCALacZ-treated and AxCATREM-1 Ig–treated mice were collected 14 days after treatment, and RNA was extracted for quantification of interleukin-17 (IL-17), tumor necrosis factor α (TNFα), and IL-1β mRNA, with real-time polymerase chain reaction. Each mRNA level was normalized to that of GAPDH mRNA, and an arbitrary unit was defined (value of LacZ-treated samples = 1). Values are the mean and SD results from 8 mice per group. * = $P < 0.05$; ** = $P < 0.01$, versus the AxCALacZ control group.

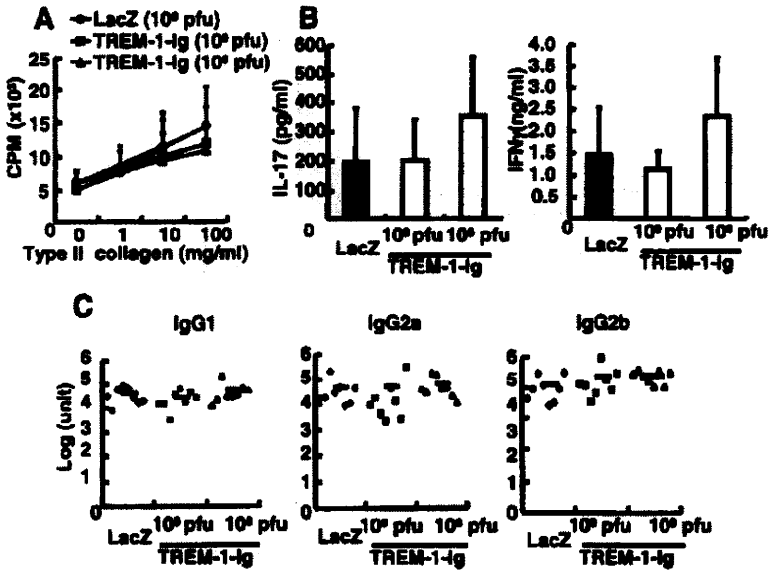

Figure 5. Effects of TREM-1 blockade on antigen-specific T cell and B cell responses. Splenocytes and sera were collected from mice with CIA that were treated with 2 different doses of AxCATREM-1 Ig (10^9 PFU and 10^8 PFU) and those treated with AxCALacZ (10^9 PFU), 16 days after the second immunization. A, Proliferative responses of the splenocytes to different concentrations of type II collagen were assessed with ^{3}H-thymidine incorporation. B, The splenocytes were cultured for 48 hours in the presence of 100 μg type II collagen, and IL-17 and interferon-γ (IFNγ) levels in the supernatants were measured with a specific enzyme-linked immunosorbent assay (ELISA). Values in A and B are the mean and SD results from 4 mice per group. C, Serum concentrations of anti–type II collagen antibodies with IgG1, IgG2a, and IgG2b subclasses were determined with a specific ELISA. Type II collagen–specific antibody units were determined using a reference serum created from pooled sera from arthritic mice. A 1:40 dilution of serum from arthritic mice was assigned a value of 1,000 units/ml. Horizontal lines show the mean for each group. See Figure 4 for other definitions.

synovial tissues from the AxCATREM-1 Ig–treated mice (Figures 4D and E). Expression of IL-17, TNFα, and IL-1β, which is reported to be involved in the pathogenesis of arthritis, was suppressed significantly in the AxCATREM-1 Ig–treated mice (Figure 4F).

Effect of TREM-1 blockade on T cell and B cell responses. TREM-1 ligation triggers differentiation of monocytes into immature dendritic cells (8). Because dendritic cells evoke acquired immunity, we studied the effect of systemic TREM-1 blockade on antigen-specific T cell and B cell responses to type II collagen. When the splenocytes isolated 14 days after treatment from TREM-1 Ig–treated and control mice were cultured with various concentrations of type II collagen, they proliferated equally in response to type II collagen (Figure 5A). No significant differences were observed in IL-17 and IFNγ production by AxCATREM-1 Ig–treated and control mice (Figure 5B). Sera that were derived from

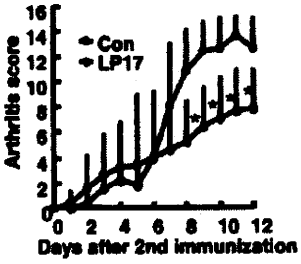

Figure 6. Treatment of CIA with antagonistic TREM-1 peptide. Mice with CIA were treated with intraperitoneal injections of an antagonistic TREM-1 peptide, LP17, or a sequence-scrambled control peptide (Con). Mice received 200-μg injections of these peptides every day, beginning on day 24, when arthritis became evident. Values are the mean and SD results from 4 mice per group. * = $P < 0.05$ versus control. See Figure 4 for other definitions.

TREM-1 Ig–treated and control mice at the same time had comparable levels of IgG1, IgG2a, and IgG2b anti–type II collagen antibodies (Figure 5B). Thus, TREM-1 blockade did not attenuate anti–type II collagen responses by T lymphocytes and B lymphocytes.

Attenuation of CIA by antagonistic TREM-1 peptide. Previous studies demonstrated that the TREM-1 antagonistic peptide LP17 protected mice from LPS-induced lethality (13). To confirm the therapeutic effect of TREM-1 blockade on CIA, TREM-1 engagement was inhibited by daily intraperitoneal injections of LP17. The systemic administration of LP17 suppressed CIA, although the effect was not as complete as that observed following viral gene transfer (Figure 6).

DISCUSSION

We demonstrated here that TREM-1 was expressed on synovial macrophages in joints affected by human and murine autoimmune arthritis. The expressed TREM-1 should be functional, because ligation resulted in enhanced TNFα production by synovial cells. In vivo blockade of TREM-1 ligation ameliorated CIA. Thus, we demonstrated that TREM-1 should be responsible for the pathology of autoimmune diseases that are not related to bacterial infections and that blockade of TREM-1 should be a new therapeutic approach to the treatment of RA.

The therapeutic effects of systemic TREM-1-Ig blockade in CIA were not mediated by disruption of the adaptive immune responses raised against the articular autoantigen. This finding indicated that TREM-1 blockade did not interfere with the antigen presentation promoted by complete Freund's adjuvant. We and other investigators demonstrated that TREM-1 engagement stimulated innate immune cells to produce various proinflammatory cytokines (5,9). Thus, the therapeutic effect should be attributable to attenuation of the inflammatory responses rather than prevention of the adaptive immune responses. Although neutrophil functions can be modified with TREM-1 blockade, it should be noted that TREM-1 ligation stimulated macrophages more effectively than neutrophils to amplify production of proinflammatory cytokines (5,8,9,31).

COX inhibitors ameliorate CIA (32,33) but showed limited efficacy in altering the natural disease course of RA. This discrepancy could be deciphered partly by the fact that COX inhibitors can abrogate TREM-1 up-regulation of murine macrophages but not human monocyte/macrophages. It also indicates that intervention that suppresses TREM-1 expression in the murine system cannot necessarily be applied to the human system. At the moment, direct TREM-1 blockade using TREM-1 Ig or LP17 peptides is the only feasible way to inhibit a common TREM-1 pathway shared by mice and humans.

TREM-1 expression can be up-regulated by various proinflammatory cytokines that are present in the inflamed joints and by ligation of TLRs that are present on rheumatoid synovial macrophages (34,35). Endogenous ligands for TLR-2 and TLR-4 are expressed in RA joints. They include fibrinogen, Hsp60, Hsp70, hyaluronic acid, myeloid-related protein 8/14, and high mobility group box chromosomal protein 1 (36–44). Indeed, TLR inhibition by a dominant negative form of the Toll/IL-1 receptor domain containing adaptor protein molecules suppressed the spontaneous production of proinflammatory cytokines and MMPs from rheumatoid synovial fibroblasts (45). Thus, TREM-1 could act together with many proinflammatory receptors in the inflamed joints.

Adenovirus gene transfer is not currently well tolerated in clinical settings. However, the antagonistic peptide treatment used in this study was not as effective as gene therapy, while the same peptide dose was effective for the treatment of experimental sepsis. This might be partly attributable to the short half-life of the peptides in the body. Identification of natural ligands should promote development of various intervention techniques to treat actual patients. Also, such studies should help to identify what exact ligands activate TREM-1 in arthritic joints.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Kohsaka had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Murakami, Akahoshi, Miyasaka, Kohsaka.

Acquisition of data. Murakami, Toyomoto.

Analysis and interpretation of data. Murakami, Akahoshi.

Construction of the adenovirus vector. Aoki.

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Mortality and cause of death in Japanese patients with rheumatoid arthritis based on a large observational cohort, IORRA

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Objectives: To investigate mortality, cause of death, and risk factors related to mortality in Japanese patients with rheumatoid arthritis (RA).

Methods: The IORRA cohort is a large observational cohort established in 2000 at the Institute of Rheumatology, Tokyo Women's Medical University. Essentially, all RA patients were registered and clinical parameters were assessed biannually. For patients who failed to participate in subsequent surveys, simple queries were mailed to confirm survival. Standardized mortality ratios (SMRs) were calculated and mortality risk factors were analysed using a Cox proportional hazard model.

Results: We analysed 7926 patients (81.9% females; mean age 56.3 ± 13.1 years; mean disease duration 8.5 ± 8.3 years) with RA who enrolled in IORRA from October 2000 to April 2007. During the observational period (35 443.0 person-years), 289 deaths were reported. Major causes of death included malignancies (24.2%), respiratory involvement (24.2%) including pneumonia (12.1%) and interstitial lung disease (ILD) (11.1%), cerebrovascular disease (8.0%), and myocardial infarction (7.6%). As death was not confirmed in all patients, the SMR was deduced to be between 1.46 [95% confidence interval (CI) 1.32–1.60] and 1.90 (95% CI 1.75–2.07) for all patients, between 1.45 (95% CI 1.22–1.70) and 1.70 (95% CI 1.45–1.97) for men, and between 1.46 (95% CI, 1.29–1.65) and 2.02 (95% CI 1.82–2.24) for women. Factors associated with increased mortality included male gender, older age, worse physical disability, positive rheumatoid factor (RF), corticosteroid use, and presence of ILD.

Conclusion: The mortality of Japanese RA patients is comparable to that in previous reports from western countries, even though the causes of death were significantly different.

The mortality of patients with rheumatoid arthritis (RA) has been well established as being worse than that of the general population in the USA and northwestern European countries (1–10). Mortality is usually indicated by the standardized mortality ratio (SMR), which indicates the rate of death of a target cohort compared to a control cohort. SMR is influenced by the characteristics of the target cohort (i.e. whether it is an inception, prevalence-based, community-based, or clinic-based cohort) and the length of the observation period. Except for one or two reports from an RA inception cohort in Sweden (11), the majority of investigators have reported excess mortality in RA patients compared to the general population (1–10, 12). Several reports from the USA, Canada, the UK, and Finland have shown that the SMR for RA patients ranges from 1.27 to 3.0 (1–10, 12). Among these reports, a landmark study from Pincus et al (6) noted that, at 1.60, the SMR of RA was equivalent to that of stage IV Hodgkin disease or three-vessel coronary artery disease. Since this report was published, methotrexate (MTX) has become a pivotal drug in RA treatment, and increased use of MTX has been anticipated to result in a better prognosis (13). The successful introduction of several classes of biological agents at the end of the previous century has greatly improved RA treatment outcomes in western countries. Anti-tumour necrosis factor (TNF) agents have been demonstrated to have potent anti-rheumatic effects with respect to decreasing inflammation and joint destruction. However, it is not yet known whether biological agents have improved the mortality of RA (14, 15) as they have only been used to treat a small percentage of the overall RA population. The mortality associated with RA may not have changed in the past four decades (16, 17).

Large differences exist between different countries and ethnicities with respect to comorbidities and causes of death in RA patients. Ischaemic cardiovascular disease (CVD) followed by malignancy and infection

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are the primary causes of death in RA patients in western countries (7, 8, 10, 18–20). In particular, ischaemic CVD is the biggest concern, because CVD mortality is increased by approximately 50% in RA patients (8, 10, 21). By contrast, interstitial lung disease (ILD), *Pneumocystis* infection, and secondary amyloidosis are rarely observed in western countries (10). The latter conditions are often noted as causes of death or comorbidities in Japanese RA patients (22–28). Precise mortality and causes of death of Asian RA patients has not yet been evaluated except for one report in which Hakoda et al showed that, within the inception cohort of Japanese RA patients among atomic bomb survivors enrolled from 1958 to 1966 and followed-up until 1999, the SMR was 1.60 and the major causes of death were pneumonia, tuberculosis, and liver disease (29). Thus it is crucial to evaluate the mortality and causes of death of Japanese RA patients prior to the biologic era to construct a country-specific treatment strategy for RA. To address this issue, we analysed the mortality of Japanese RA patients using a single institute-based prospective RA cohort, IORRA (30–32).

Patients and methods

Patients

The IORRA cohort was established in October 2000 as a single institute-based large observational cohort of Japanese RA patients conducted at the Institute of Rheumatology, Tokyo Women's Medical University; the study details have been reported previously (30–32). Several publications have described the features of patients in this cohort, including disease activity (33), gastrointestinal involvement (34), fracture (35), arthroplasty (36), depression (31), and incidence of tuberculosis (32). In brief, all patients diagnosed with RA were registered in the IORRA cohort after informed consent was obtained, and they were required to complete and submit a survey biannually. Evaluated parameters included patient assessment of pain and global evaluation by the visual analogue scale (VAS) and disability measured by the Japanese Health Assessment Questionnaire (J-HAQ), which was validated in 2003 (37); physician evaluation of disease activity (swollen joint count, tender joint count, and physician's assessment by VAS); and the following clinical parameters: erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF), total protein (TP), white blood cell (WBC) count, and total cholesterol (T-chol). Patients also self-reported the use of nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs) such as sulfasalazine, bucillamine (38, 39), or MTX, and corticosteroids. Occurrence of adverse events and comorbidities such as interstitial pneumonitis, diabetes mellitus, pneumonia, depression, and hypertension are also reported. Approximately 5000 patients with RA were involved in each phase of the survey, and over 98% of the patients submitted completed questionnaires by prestamped mail.

In this study, we analysed patients with an onset age over 16 years and who participated in the IORRA survey from October 2000 to April 2007.

Follow-up

For patients who failed to participate in subsequent observations, a simple query was mailed to confirm survival biannually. Such queries were sent to approximately 1000 to 1800 patients after each survey, with a total of 15 815 queries sent to patients who failed to submit competed surveys during the observation period.

Death reports

Reports of death were obtained in several ways. First, physicians at affiliated hospitals reported patient deaths directly. Second, bereaved family members reported patient deaths voluntarily or in response to the mailed simple queries described above. Third, police departments reported deaths when they were sudden, unexplainable by disease course, or accidental, or when patients were otherwise found dead.

Causes of death were classified according to the International Classification for Disease (ICD)-10.

Statistical analysis

Mortality rates were adjusted by age and gender using indirect methods, and the rate ratio (SMR) was calculated using the 2000–2006 life tables reported by the Ministry of Health, Labour and Welfare, Japan, (http://www.e-stat.go.jp/SG1/estat/NewList.do?tid=000001028897) using the person-year method. Risk factors for mortality were analysed using the multivariate Cox proportional hazard model and the calculated hazard ratio (HR) for each variable. The following factors were considered in this analysis: gender, age, RA duration, body mass index (BMI), J-HAQ, Disease Activity Score using 28 joint counts (DAS28), RF, ESR, TP, WBC count, T-chol, smoking, comorbidities, and corticosteroid and DMARD use.

Results

Demographics of the IORRA cohort

A total of 7926 patients [35 443.0 person-years; 6493 (81.9%) females] with a mean follow-up period of 4.8 ± 2.1 years were analysed. Mean age at entry to IORRA was 56.3 ± 13.1 years, and mean disease duration at entry was 8.5 ± 8.3 years (Table 1). The average DAS28 score indicating disease activity was 4.0 ± 1.3, and the average J-HAQ score used as an index of physical disability was 0.79 ± 0.74.

Table 1. Demographics and clinical features of patients with rheumatoid arthritis at entry to the IORRA cohort.

n	7926
Women (%)	81.9
RA onset age (years)	47.8 ± 13.1
Age at entry to IORRA	56.5 ± 13.1
Disease duration (years)	8.5 ± 8.3
Mean follow-up period (years)	4.8 ± 2.1
DAS28	4.0 ± 1.3
J-HAQ score	0.79 ± 0.74
Corticosteroids (%)	46.0
Methotrexate (%)	34.2
Sulfasalazine (%)	23.4
Bucillamine (%)	26.0
Biologics (%)	0.3

RA, rheumatoid arthritis; DAS28, Disease Activity Score using 28 joint counts; J-HAQ, Japanese Health Assessment Questionnaire.

The number of patients who were receiving MTX, corticosteroids, and biological agents at entry to IORRA was 2713 (34.2%), 3646 (46.0%), and 24 (0.3%), respectively.

Follow-up

Among a total of 15 815 queries sent to patients who failed to submit subsequent surveys during the observation period, 10 074 responses were obtained. For each mailed follow-up survey, the response rate ranged from 52.3% to 74.1% (average 63.7%). Among the total 7926 patients, 1372 (17.3%) patients were lost to follow-up, regardless of our efforts.

Death reports

In this observation, 289 death reports were received, including those mailed during the follow-up study. The 174 deaths (60.2%) reported by using this follow-up system from family members included the date and cause of death. Thirteen deaths (4.5%) were reported by police departments. Other death reports were obtained from physicians in affiliated hospitals. In the mailed reports, many family members wrote 'interstitial pneumonitis' as the cause of death. However, interstitial pneumonitis has many causes, such as 'RA lung', allergic reaction to MTX, or infection by viruses or *Pneumocystis jiroveci*. When infective pathogens were reported, we classified the cases as 'pneumonia'; otherwise, the reported 'interstitial pneumonitis' cases were classified as ILD.

Causes of death

The causes of death of the RA patients are listed in Table 2. Malignancies (24.2%) and respiratory involvement (24.2%) were the most frequent causes of death. With respect to respiratory involvement, pneumonia (12.1%) and ILD (11.1%) were observed at a similar frequency. Cerebrovascular disease (8.0%) and myocardial infarction (7.6%) also occurred at a similar frequency. Two patients were reported to have secondary amyloidosis and to have died with sepsis or renal failure on haemodialysis. Among 37 patients whose cause of death was not obvious, sudden death was reported in 11 patients.

We compared the frequency of major causes of death of Japanese RA patients to those of the general Japanese population in Table 3 using the 2006 Ministry of Health,

Table 2. Causes of death in Japanese patients with rheumatoid arthritis (35 443.0 person-years).

Cause of death	Total (n = 289)	Men (n = 121)	Women (n = 168)
Malignancy	70 (24.2)	35 (28.9)	35 (20.8)
Respiratory system	70 (24.2)	36 (29.8)	34 (20.2)
Pneumonia	35	19	16
Interstitial lung disease	32	16	16
Other	3	1	2
Cardiovascular system	40 (13.8)	15 (12.4)	25 (14.9)
Myocardial infarction	22 (7.6)	8 (6.6)	14 (8.3)
Heart failure	10	5	5
Aortic dissection	5	1	4
Other	3	1	2
Cerebrovascular	23 (8.0)	9 (7.4)	14 (8.3)
Infection	16 (5.5)	2	14
Septicaemia	9	2	7
Other	7	0	7
GI haemorrhage or perforation	12 (4.1)	4	8
Musculoskeletal	7	1	6
Renal failure	2	0	2
Accident/suicide	4	0	4
Other	8	2	6
Unknown	37	17	20
Sudden death	11	4	11

Values given as n (%).

Table 3. Comparison of causes of death in Japanese patients with rheumatoid arthritis (RA) and the general population.

Cause of death	RA males (%)	General males (%)*	RA females (%)	General females (%)*
Infection	1.7	2.1	8.3	2.3
Malignancy	28.9	34.1	20.8	26.1
Circulatory system	19.8	26.7	23.2	33.5
Ischaemic heart disease	6.6	7.1	8.3	6.8
Cerebrovascular disease	7.4	9.3	8.3	11.1
Respiratory system	29.8	15.5	20.2	14.5
Pneumonia	15.7	9.7	9.5	10.1
Interstitial lung disease	13.2	n.a.	9.5	n.a.
GI bleeding	3.3	0.3	4.8	0.3
Musculoskeletal disease	0.8	0.3	3.6	0.6
Renal disease	0.1	1.7	1.2	2.3
Accident	0.1	8.2	2.4	5.1
Other	22.3	27.2	23.2	35.3

n.a., not available.
*Data from the cause of death of Japanese population using the 2006 Ministry of Health, Labour and Welfare (Japan) report.

Labour and Welfare (Japan) report. Respiratory involvement is a prominent cause of death in both male and female Japanese RA patients. ILD is also a prominent cause of death in RA patients, but is not usually observed as a cause of death in the general Japanese population. Gastrointestinal bleeding also seems to be a frequent cause of death in both male and female RA patients. However, cardiovascular disease does not seem to be a prominent cause of death in RA patients compared to the general Japanese population in this study.

The 70 malignancies included 16 pulmonary cancer cases, nine malignant lymphoma cases, and seven gastric cancer cases. We compared the different causes of malignancy death in male and female RA patients to the general population given in the 2006 Ministry of Health, Labour and Welfare (Japan) report (Table 4). In both female and male RA patients, death by malignant lymphoma was prominent in comparison to the general population, and lung cancer death was prominent in male RA patients.

Mortality

The SMR of the IORRA cohort was calculated based on 2006 Ministry of Health, Labour and Welfare (Japan) data. However, the survival of all patients was not captured in our cohort. Non-response to the mailed queries is a potential source of bias in this type of research survey. Thus, we attempted to analyse mortality in a hypothetical manner. First, hypothesizing that all 1372 patients who were lost to follow-up were alive, the lowest possible SMR was calculated to be 1.03 [95% confidence interval (CI) 0.91–1.15]. Second, hypothesizing that all 1372 patients who were lost to follow-up were dead, the highest possible SMR was determined to be 5.88 (95% CI 5.60–6.17). However, it is unlikely that all patients who were lost to follow-up were either all alive or all dead. Therefore, when we further hypothesized that patients who were lost to follow-up died at the same rate as patients (or family members) who responded, the SMR was determined to be 1.46 (95% CI

Table 4. Comparison of site-specific malignancies as a cause of death in Japanese patients with rheumatoid arthritis (RA) and the general population.

Site of malignancy	RA males (%)	General males (%)*	RA females (%)	General females (%)*
Stomach	15.2	16.5	10.8	15.3
Colon/rectum	3.0	6.9	8.1	8.3
Liver	0.0	11.4	2.7	10.2
Gall bladder	0.0	4.0	8.1	5.1
Lung	45.5	23.2	8.1	19.2
Breast			10.8	3.4
Uterus			5.4	3.3
Ovary			8.1	2.6
Prostate	3.0	4.8		
Malignant lymphoma	12.1	2.5	16.2	2.6
Other	21.2	30.6	21.6	29.9

*Data from the cause of death of Japanese population using the 2006 Ministry of Health, Labour and Welfare (Japan) report.

Table 5. Risk factors for mortality of patients with rheumatoid arthritis by clinical feature.

Factor	HR	95% CI	p-value
Male	3.03	2.04–4.35	< 0.0000001*
Age (/10 years)	2.44	2.06–2.89	< 0.0000001*
Duration (years)			
≤ 1	1.00		
1–5	0.68	0.40–1.17	0.16
5–10	0.75	0.43–1.31	0.31
10–20	0.86	0.51–1.45	0.58
> 20	1.18	0.68–2.06	0.55
BMI			
≤ 18.9	1.00		
18.9–20.4	0.85	0.53–1.35	0.49
20.4–21.9	0.71	0.46–1.10	0.12
21.9–23.8	0.97	0.64–1.48	0.90
> 23.8	0.80	0.52–1.24	0.32
DAS28			
≤ 2.6	1.00		
2.6–3.2	1.49	0.75–2.98	0.26
3.2–5.1	1.35	0.71–2.56	0.36
> 5.1	1.16	0.56–2.42	0.69
J-HAQ score			
0	1.00		
0.0–0.4	1.05	0.60–1.83	0.87
0.4–0.9	0.99	0.57–1.69	0.96
0.9–1.5	1.70	1.02–2.83	0.043*
> 1.5	2.10	1.22–3.59	0.007*
RF			
≤ 14	1.00		
15–42	2.39	1.33–4.28	0.0035*
42–84	2.17	1.21–3.91	0.0095*
84–210	2.32	1.30–4.12	0.0043*
> 210	3.23	1.85–5.63	< 0.000001*
CRP (mg/dL)			
≤ 0.2	1.00		
0.2–0.5	1.33	0.80–2.22	0.27
0.5–1.0	1.26	0.76–2.08	0.37
1.0–2.3	1.00	0.60–1.68	0.99
> 2.3	1.44	0.85–2.44	0.18
T-chol (mg/dL)			
≤ 166	1.00		
166–187	0.83	0.52–1.31	0.43
187–205	0.90	0.57–1.42	0.66
205–227	0.90	0.56–1.43	0.65
> 227	1.12	0.70–1.78	0.64

HR, hazard ratio; CI, confidence interval; BMI, body mass index; DAS28, Disease Activity Score using 28 joint counts; J-HAQ, Japanese Health Assessment Questionnaire; RF, rheumatoid factor; CRP, C-reactive protein; T-chol, total cholesterol.

1.32–1.60) for all patients, 1.45 (95% CI 1.22–1.70) for males, and 1.46 (95% CI 1.29–1.65) for females. As Kauppi et al reported that RA patients who did not respond to mailed queries were 1.65 times more likely to have died over the 2-year follow-up period compared to responders (40), we finally hypothesized that patients who were lost to follow-up would have died at this rate, making the estimated SMR 1.90 (95% CI 1.75–2.07) for all patients, 1.70 (95% CI 1.45–1.97) for males, and 2.02 (95% CI 1.82–2.24) for females.

Risk factors predictive of mortality

Risk factors predictive of overall mortality are listed in Tables 5 and 6. We found that male gender and older age were the major risk factors for mortality. Worse disability was also a strong risk factor. Higher RF positivity was also a stronger and significant risk factor for mortality. Higher disease activity indicated by DAS28 was not a risk factor for mortality in this study. With respect to medication use, patients using corticosteroids had a 1.73–4.18-fold higher risk for mortality compared to those who did not use corticosteroids. Higher hazard risks correlated with higher corticosteroid doses, with patients using corticosteroids > 10 mg/day having a 4.18-fold higher risk for mortality (Table 6). Use of MTX did not influence mortality risk in this study. Pre-existing ILD was a major risk factor for overall death (HR 4.16) (Table 6).

Discussion

This is the first report about mortality rates of RA patients in a large observational cohort in Asia. The results of this analysis demonstrate that the mortality rate in Japanese RA patients is comparable to those of RA patients in western European countries and the USA. It is notable that IORRA is the largest single clinic-based cohort ever evaluated, and essentially represents the pre-biologic era, since infliximab was introduced in 2003 and etanercept in 2005 in Japan; thus, only 0.3% of patients received anti-TNF therapy at

Table 6. Risk factors for mortality of Japanese patients with rheumatoid arthritis by medication and comorbidities.

Factor	HR	95% CI	p-value
Smoking ever	1.83	1.23–2.72	0.0029*
Smoking current	1.23	0.78–1.94	0.38
NSAID	0.70	0.51–0.95	0.022*
PSL (mg/day)			
0	1.00		
1–5	1.73	1.23–2.43	0.0018
6–10	2.39	1.57–3.65	<0.00001*
>10	4.18	1.92–9.11	0.00032*
MTX (mg/week)			
0	1.00		
1–4	1.09	0.76–1.57	0.64
5–8	0.80	0.51–1.25	0.34
≥ 8	0.21	0.03–1.51	0.12
Interstitial lung disease	4.16	1.24–13.95	0.021*
Diabetes mellitus	1.56	0.59–4.09	0.37
Depression	1.97	0.25–15.61	0.52
Hypertension	0.78	0.37–1.65	0.52
Cardiovascular disease	0.97	0.23–4.05	0.96

HR, hazard ratio; CI, confidence interval; NSAID, nonsteroidal anti-inflammatory drug; PSL, prednisolone; MTX, methotrexate.

cohort entry and only 2.1% of patients received anti-TNF therapy during the study observation period.

Investigation of mortality requires a complete follow-up system. In the UK, patients are tracked by the National Health Service Central Register, which maintains computerized records of all patients registered with general practitioners in England and Wales (7, 10). The date and primary cause of death can be extracted from death certificates coded by the Office for National Statistics (ONS) using the ICD-10. Each Scandinavian country (Sweden, Denmark, Finland, Norway, and Iceland) has a national database, which includes a Population Registry, Cancer Registry, Cause of Death Registry, and Hospital Discharge Registry (41). Clinical data about comorbidities and medication use have been readily obtained through a structured review of all clinical records and physician access to death certificates because of the required national registration of all citizens (8, 9). By contrast, in Japan, patients can select and switch between the large numbers of rheumatology physicians without restriction. Physicians are therefore only aware of a patient's death if they participate directly in their final stage of life or are notified by the patient's family members. Japanese physicians do not have access to death certificates because of the unique Japanese public security system. Japan also has no national registry system for cancer or cause of death. Thus, it is nearly impossible to obtain an accurate SMR in Japan.

We actively attempted to contact patients who failed to participate in subsequent observations by mail every 6 months to increase the reliability of this study. Despite this intensive effort, 17.3% of patients were lost to follow-up. To compensate for this shortcoming, we deduced the SMR based on the most probable and reliable hypothesis derived from published papers, because non-response is a potential source of bias in survey research, not only for RA but also for other diseases (40, 42). According to Kauppi et al (40), non-response is associated with increased mortality both in RA patients and in the general population; these researchers found that non-responders were 1.65 times more likely to die than responders over the 2-year follow-up period. While it may not be entirely appropriate to adapt these Finnish results to our Japanese cohort, we believe this method is acceptable for assuming an accurate SMR considering our circumstances. As a result, the deduced SMR of Japanese RA patients in the IORRA cohort ranged from 1.46 to 1.90. Thus, the mortality of Japanese RA patients is worse than that of the general Japanese population but is comparable to that of RA patients in European countries and the USA.

In this study, causes of death in Japanese RA patients were found to be considerably different from those of RA patients in western countries, even when the fact that the causes of death in the general Japanese population are different from those in the general western population, particularly with respect to frequency of infection or pneumonia, was taken into account. Major causes of death in our IORRA cohort were malignancy and respiratory involvement, mainly including pneumonia and ILD. Causes of ILD include RA itself, allergic pulmonary reaction to MTX, or infection by certain viruses or *Pneumocystis jiroveci*. In this study, when the cause of death was reported as 'interstitial pneumonitis' without a known pathogen, the death was classified as ILD. As the incidence of deaths from ILD is very low in the general Japanese population and also in western RA patients (10), death from ILD is a prominent feature of Japanese RA patients compared to both the Japanese general population and western RA patients. Japanese people may be particularly susceptible to interstitial pneumonia or pulmonary fibrosis due to genetic or ethnic differences. Therefore, it is important to investigate in future studies whether biological agents may increase the incidence of ILD-related deaths in Japanese RA patients.

Ischaemic CVD in RA patients is a major concern in terms of both prevalence and mortality in western countries. Many other investigators (8, 10, 19) have reported that the frequency of cardiovascular death in RA patients is high (~50%) and the disease-specific SMR has been reported to be approximately 2. Women with RA are reported to be at higher risk for ischaemic cardiovascular morbidity and mortality than men with RA (20), particularly in patients with high disease activity (43). However, the risk of ischaemic CVD in RA patients has been reported to depend not only on typical cardiovascular risk factors such as age, diabetes mellitus, smoking, or hypertension (19, 43). Endothelial inflammation in RA patients may accelerate atherosclerosis in the context of the RA cytokine network (44), thus vascular endothelial function may be modulated by the introduction of anti-TNF therapy (45).

The incidence of ischaemic cardiovascular death is approximately 7% in the general Japanese population and we observed almost the same frequency in Japanese men and women with RA, respectively. Additional patients whose causes of death were classified as sudden death may also have died from ischaemic CVD because increased unrecognized coronary heart disease and sudden death in RA patients is suggested (46). If these patients are included, the death rate for ischaemic CVD would increase to 1.5-fold that of the general Japanese population. Thus, it appears that ischaemic CVD may also potentially be a major contributor to RA mortality in Japan; however, this must be confirmed in additional studies.

In this study, only two deaths were reported to be caused by secondary amyloidosis. A large difference in the occurrence of secondary amyloidosis exists between individuals of different ethnicities. Amyloidosis is often observed in Japanese, Turkish, and Finnish patients with RA (5, 27, 47, 48), while it occurs at a lower frequency in RA patients in the USA and western Europe. While a larger number of patients were expected to have suffered from amyloidosis, it may not have been reported as the primary cause of death in this analysis.

Japanese RA patients in this study were found to have nearly identical risk factors to those reported from western countries (9, 10): male gender, older age, worse physical disability, positive RF, smoking history, and corticosteroid use were identified as major risk factors. While anticyclic citrullinated peptide (anti-CCP) antibody could also be a potential risk factor that needs to be analysed, anti-CCP data are not available from this observational IORRA study because Japanese health insurance did not begin covering measurement of anti-CCP antibody until April 2007, which was after the observational period of this study. We also identified an additional unique risk factor: interstitial pneumonitis. With respect to the contribution of interstitial pneumonitis to overall mortality, the pre-existence of ILD was revealed as the major risk factor. Therefore, improvement or elimination of pre-existing ILD appears to be crucial to decrease the mortality of Japanese RA patients. The predictive model constructed for this study used only baseline characteristics and was therefore not a time-dependent model, because time-dependent values for each patient from baseline to death were not available in our observational cohort. This may be the limitation of this study.

In conclusion, the prognosis of Japanese RA patients with respect to death is equivalent to that of RA patients in western countries; however, the causes of death are substantially different. The mortality of Japanese RA patients described here represents the pre-biologic era. It is important to clarify whether new therapeutic strategies, with either anti-TNF agents or other biological agents, can be used to improve not only RA disease activity but also mortality in Japanese RA patients.

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