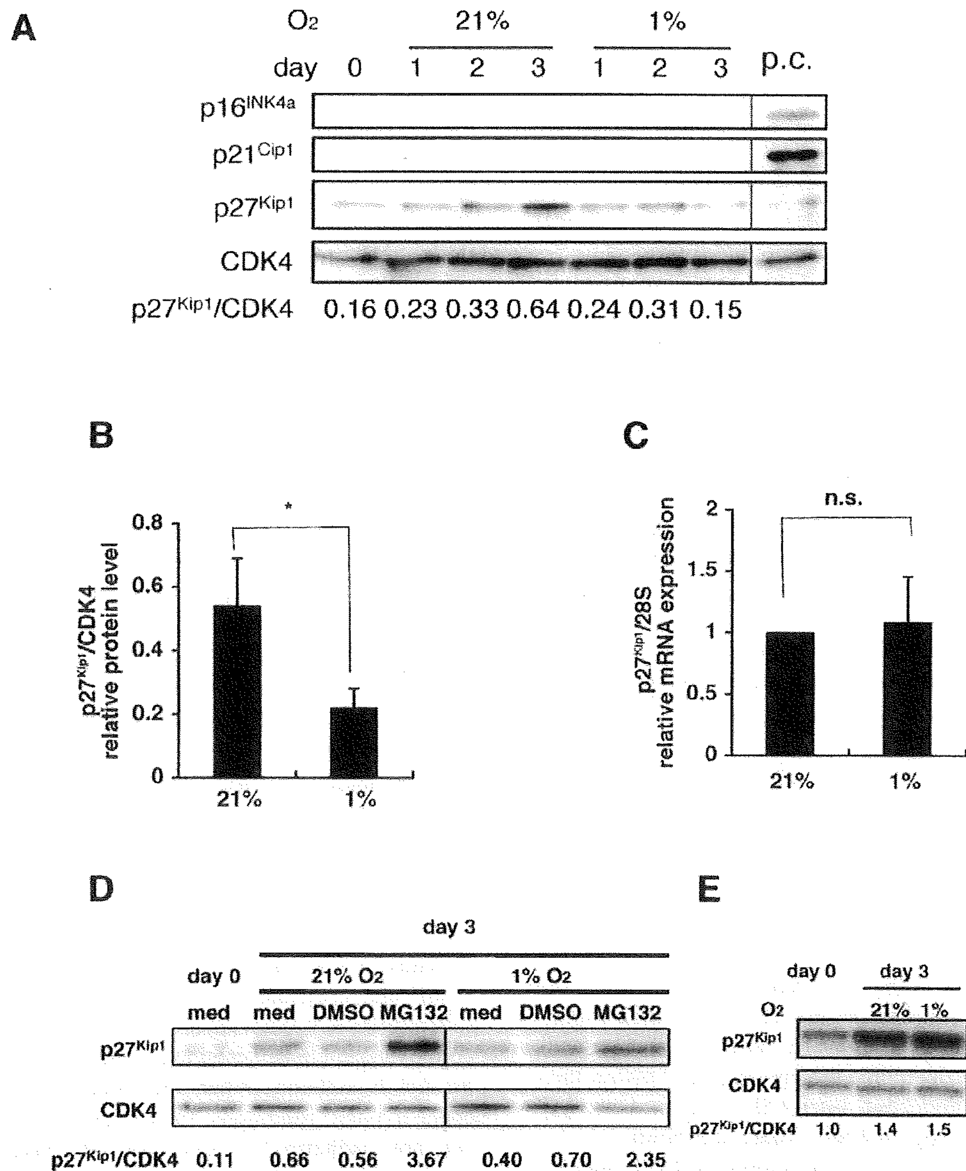

Figure 2. Downregulation of p27[Kip1] expression by hypoxia. A. RASF were cultured at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions, and whole-cell lysates were collected after indicated number of days. Expression of CDKI p16[INK4a], p21[Cip1], and p27[Kip1] by RASF was immunodetected by Western blot. CDK4 was stained as loading control. Expression levels of p27[Kip1] protein standardized with those of the CDK4 protein in the same blot are shown at the bottom. pc: positive control. B. p27[Kip1] levels cultured under normoxic (21% O_2) and hypoxic (1% O_2) conditions for 3 days were standardized with levels of CDK4. Columns and bars represent mean and SD of 3 samples. *$p < 0.05$. C. RASF were cultured for 3 days at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions before extraction of total RNA. p27[Kip1] mRNA expression was quantified by real-time PCR using 28S ribosomal RNA as an endogenous control. Columns and bars represent mean and SD of 3 samples. ns: not significant. D. RASF were cultured at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions, and whole-cell lysates were collected after 3 days. RASF were treated with culture medium alone (med), 0.1% DMSO, or 2.5 μM MG132 during the last 24 hours of culture. p27[Kip1] expression by RASF was immunodetected by Western blot. Representative results of 2 samples are shown. Expression levels of p27[Kip1] protein standardized with those of the CDK4 protein in the same blot are shown at the bottom. E. Human dermal fibroblasts were cultured for 3 days at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions before extraction of whole-cell lysates. Expression of p27[Kip1] and CDK4 was assessed as in A. Representative blots of 2 independent experiments are shown.

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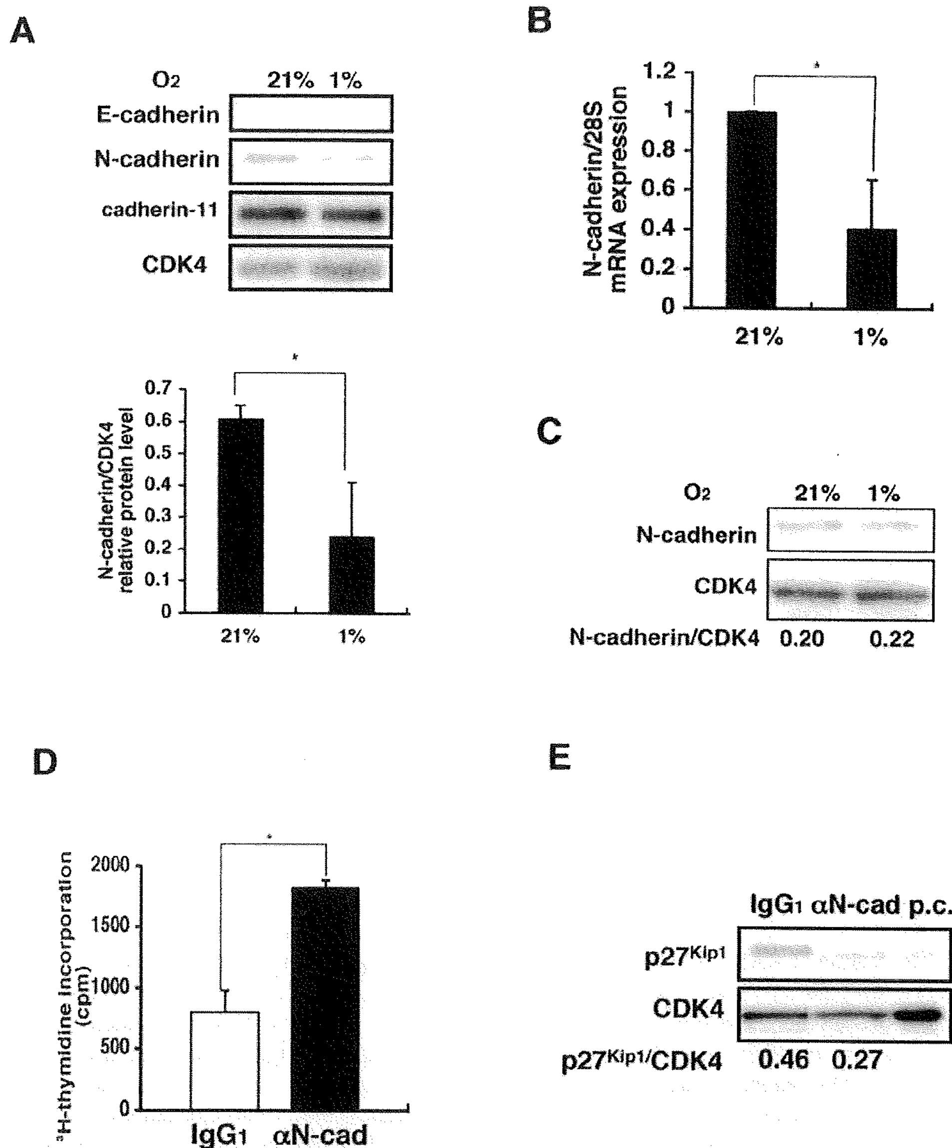

Figure 3. Downregulation of N-cadherin expression by hypoxia. A. RASF were cultured at high density under normoxic (21% O_2) or hypoxic (1% O_2) conditions for 3 days before collection of whole-cell lysates. N-cadherin protein expression was immunodetected by Western blot. Representative blots of 3 samples are shown in the upper panel. Expression levels of N-cadherin protein were standardized with those of CDK4 protein (lower panel). Columns and bars represent mean and SD of the 3 samples. *$p < 0.05$. B. RASF were cultured as in A before extraction of total RNA. N-cadherin mRNA expression was quantified by real-time PCR using 28S as an endogenous control. Columns and bars represent mean and SD of the 3 samples. *$p < 0.05$. C. Expression of N-cadherin and CDK4 in human dermal fibroblasts cultured under normoxic (21% O_2) or hypoxic (1% O_2) conditions was assessed as in A. Representative blots of 2 independent experiments are shown. D. RASF were cultured at high density, and treated with anti-N-cadherin-blocking mAb (αN-cad) or isotype IgG1 control mAb. After 3 days, effect of N-cadherin blockade on RASF proliferation was examined by ^{3}H-thymidine incorporation. Representative results of 3 samples are shown. Columns and bars represent mean and SD of 5 wells. *$p < 0.05$. E. Expression of $p27^{Kip1}$ and CDK4 by RASF treated with αN-cad or IgG1 for 3 days were immunodetected by Western blot. Representative results of 2 independent experiments are shown. pc: positive control

their contact inhibition. The accelerated proliferation was mediated by suppressed expression of N-cadherin, which should otherwise upregulate $p27^{Kip1}$ expression on their homophilic interaction. It has been reported that hypoxia stimulates RASF to produce several inflammatory mediators and proteinases that are involved in the pathology of RA[2,5]. We observed that hypoxia may contribute to RA pathology by promoting proliferation of RASF as well (Figure 4).

Hypoxia did not affect logarithmic-phase proliferation of RASF, but abrogated their contact inhibition by modulating N-cadherin and subsequent $p27^{Kip1}$ expression. The same

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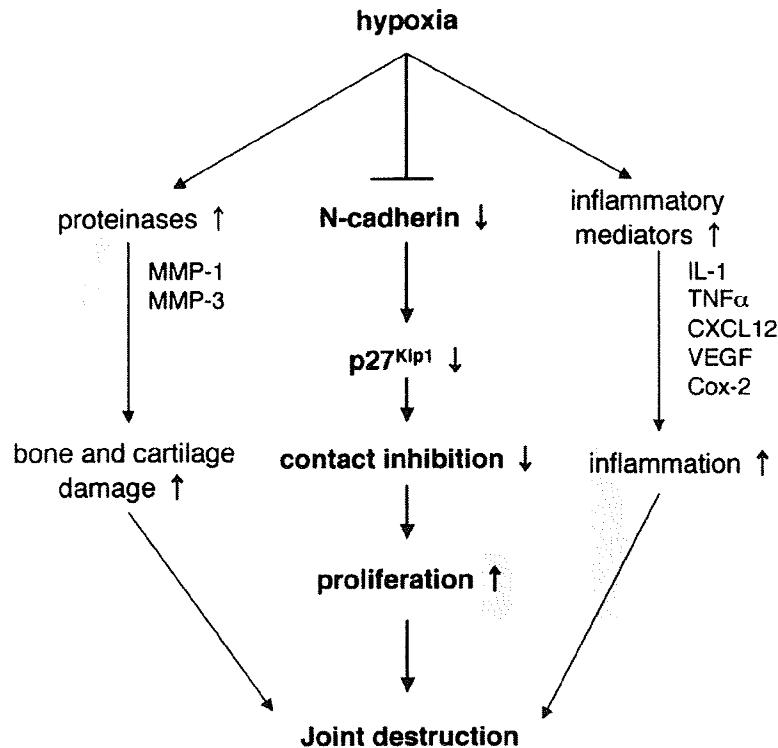

Figure 4. Abrogation of contact-dependent proliferative inhibition of RASF by hypoxia. Hypoxia downregulates N-cadherin and subsequently $p27^{Kip1}$ expression to augment RASF proliferation. It also upregulates inflammatory mediators and proteinases involved in RA. These results suggest that hypoxia in rheumatoid synovial tissues should contribute to joint destruction through multiple pathways. MMP: matrix metalloproteinase; VEGF: vascular endothelial growth factor.

changes were not observed in HDF. Thus, hypoxia-induced augmentation of proliferation via N-cadherin and $p27^{Kip1}$ might be one specific feature of RASF. RASF were found to be prone to express a few types of CDKI in longterm *in vitro* cultures[14]. Although the short-term hypoxic culture in our experiments disclosed only its effect on $p27^{Kip1}$, we assume that expression of other CDKI might be affected by hypoxia.

Our results showed that RASF proliferation was augmented in the presence of 1% O_2, but not in the presence of 3% O_2. Treuhaft, *et al* reported that the knees of 30% of patients with RA exhibited profound hypoxia, with pO_2 less than 10 mm Hg, and the lowest pO_2 in a rheumatoid joint was 8 mm Hg[4]. Thus, 1% O_2 corresponds to the lowest levels in rheumatoid joints. Further, it has been reported that movement of inflamed joints can reduce the blood flow in synovial capillaries, and thus further aggravate synovial hypoxia[30]. Hypoxia may augment RASF proliferation in such severely hypoxic rheumatoid joints, especially during exercise.

The time-course experiments revealed that the relative ^{3}H-thymidine incorporation by RASF was increased gradually with time, and peaked after 72 hours of culture. Further, hypoxia downregulated the expression of $p27^{Kip1}$ and N-cadherin to a statistically significant level. However, the absolute level of ^{3}H-thymidine incorporation by RASF peaked after 48 hours, and decreased thereafter, i.e., at 72 hours, even under hypoxic conditions. These data suggest that hypoxia-induced augmentation of RASF proliferation does not completely overcome contact inhibition. After culture for 48 hours, hypoxia augmented RASF proliferation at a statistically significant level, and downregulated the protein expression of $p27^{Kip1}$, but the latter effect was not significant. Thus, some mechanism other than the attenuation of $p27^{Kip1}$ protein expression may be involved in the hypoxia-induced augmentation of RASF proliferation.

It is known that quite a few cellular responses to hypoxia are mediated by hypoxia inducible factor-1 (HIF-1)[31]. Its expression was upregulated in the rheumatoid synovial tissues, reflecting the hypoxic environment of the affected joints[2]. In a separate experiment, HIF-1 in cultured RASF was upregulated artificially with $CoCl_2$ [2]. However, this treatment did not affect the RASF proliferation or the protein level of $p27^{Kip1}$ (data not shown), suggesting that an HIF-1-independent pathway should operate in the hypoxia-triggered augmentation of RASF proliferation.

Cadherins maintain the integrity of multicellular structures. One of the intracellular outcomes of cadherin activation is induction of CDKI, including $p21^{Cip1}$ and $p27^{Kip1}$, that inhibit cell-cycle progression. E-cadherin interaction mediates contact-dependent proliferative inhibition by

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increasing the level of p27^{Kip1} expression in thyroid cancer cells[32], while N-cadherin interaction induces contact inhibition through upregulation of p27^{Kip1} in CHO cells[26]. These data point to a link between cadherin signaling and CDKI protein expression. In addition, hypoxia suppresses E-cadherin expression on cancer cell lines[33] and N-cadherin expression in cortex proximal renal tubules[34]. Thus, there is another link between hypoxia and cadherins.

RASF expressed N-cadherins, but not E-cadherins. Recently, it was reported that cadherin-11 is expressed by RASF and contributes to organization of the lining-like structure of synovial tissues[28,29]. A cadherin-11–Fc fusion protein and an anti-cadherin–11-blocking mAb prevented and reduced arthritis in mouse models[35]. Thus, cadherins, which should control proliferative reactions to hypoxia, appear to be crucial molecules for formation and maintenance of the rheumatoid synovial structure.

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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).

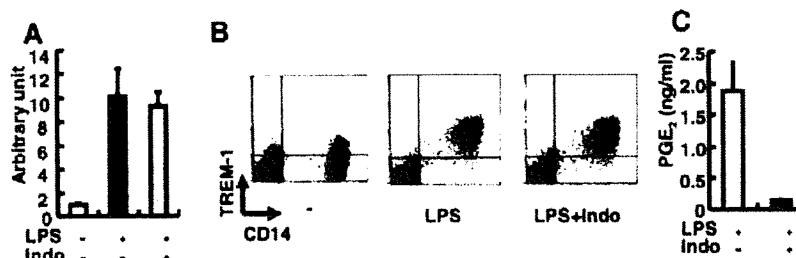

Figure 1. Lipopolysaccharide (LPS)–induced expression of triggering receptor expressed on myeloid cells 1 (TREM-1) on human peripheral blood mononuclear cells (PBMCs) in the presence or absence of a cyclooxygenase inhibitor (indomethacin; Indo). **A,** Human PBMCs were stimulated with LPS in the presence 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

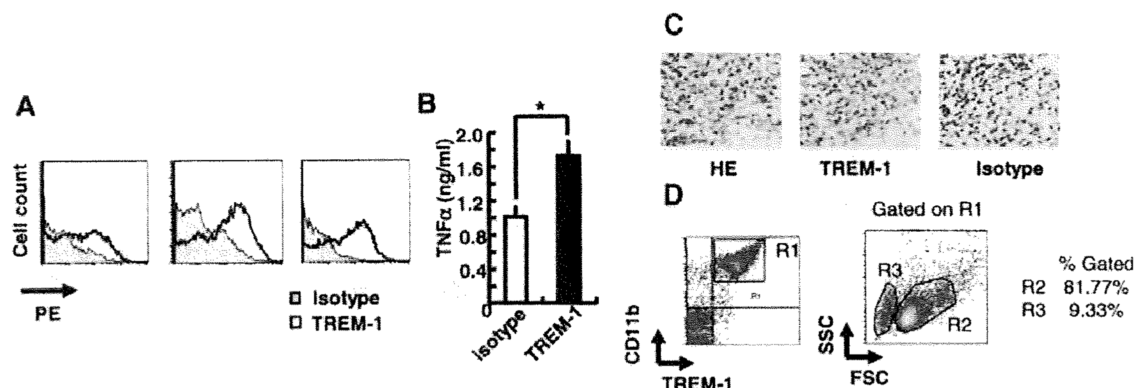

Figure 2. Triggering receptor expressed on myeloid cells 1 (TREM-1) expression in arthritis-affected joints. Synovial cells from rheumatoid synovial tissue of 3 patients with rheumatoid arthritis (RA) were isolated. **A,** Two-color flow cytometry was performed with fluorescein isothiocyanate–labeled 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

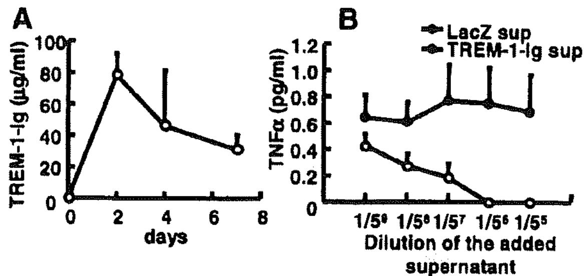

Figure 3. Adenoviral gene transfer of TREM-1 Ig. Mice were treated with intravenous injections of AxCALacZ or AxCATREM-1 Ig (10^9 plaque-forming units/mouse). **A**, Sera were examined at the indicated time points for TREM-1 Ig levels, using enzyme-linked immunosorbent assay (ELISA). Values are the mean 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 destruc-

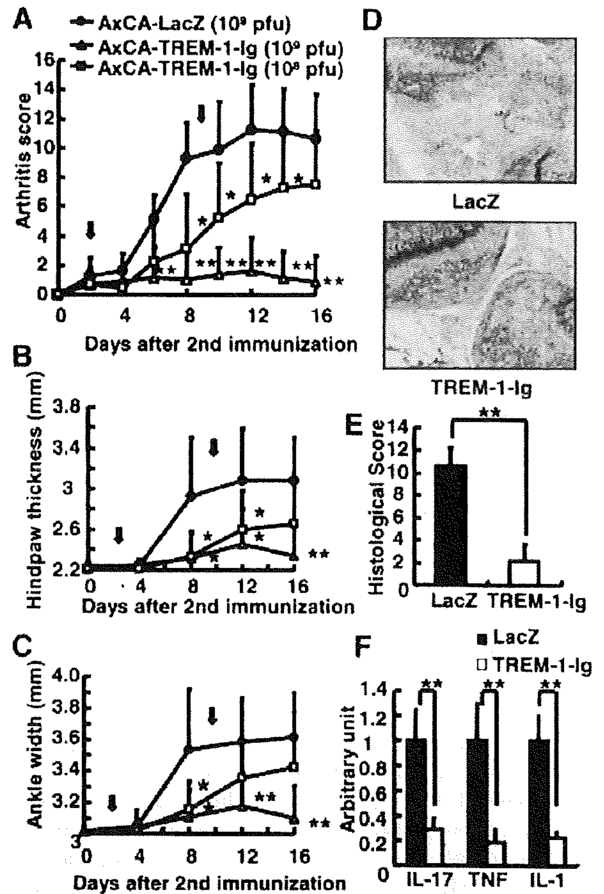

Figure 4. Attenuation of collagen-induced arthritis (CIA) by triggering receptor expressed on myeloid cells 1 (TREM-1) blockade. **A–C**, Mice with CIA were treated with intravenous injections of AxCA-LacZ (10^9 plaque-forming units [PFU]), AxCATREM-1 Ig (10^8 PFU), or AxCATREM-1 Ig (10^9 PFU) adenoviruses 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.

tion, and bone erosion, which are characteristic of the pathology of RA. These features were suppressed in

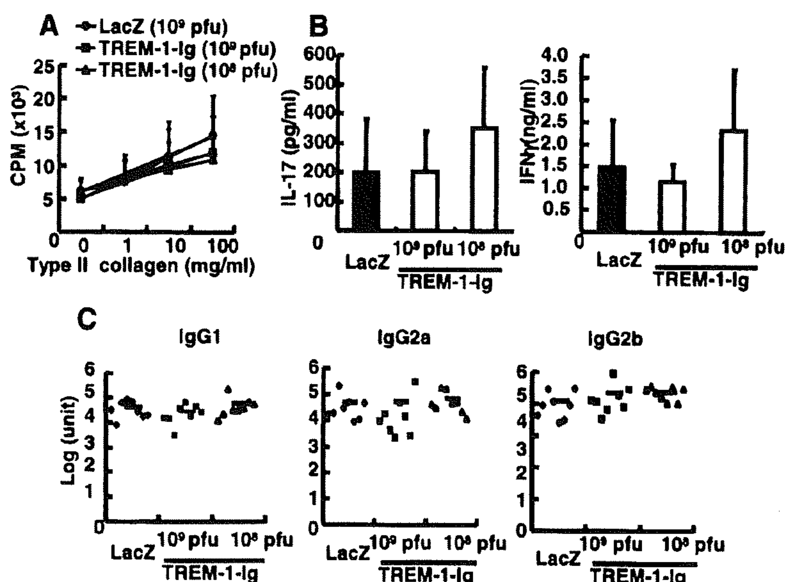

Figure 5. Effects of TREM-1 blockade on antigen-specific T cell and B cell responses. Splenocytes and sera were collected from mice with CIA that were treated with 2 different doses of AxCATREM-1 Ig (10^9 PFU and 10^8 PFU) and those treated with AxCALacZ (10^9 PFU), 16 days after the second immunization. **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

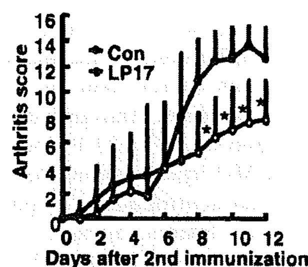

Figure 6. Treatment of CIA with antagonistic TREM-1 peptide. Mice with CIA were treated with intraperitoneal injections of an antagonistic TREM-1 peptide, LP17, or a sequence-scrambled control peptide (Con). Mice received 200-μg injections of these peptides every day, beginning on day 24, when arthritis 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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Complement activation in patients with primary antiphospholipid syndrome

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ABSTRACT

Objective: To investigate the significance of complement activation in patients with primary antiphospholipid syndrome (APS).

Methods: Thirty-six patients with primary APS, 42 control patients with non-systemic lupus erythematosus (SLE) connective tissue diseases, and 36 healthy volunteers were analysed retrospectively. Serum complement levels (C3, C4, CH_{50}) and anaphylatoxins (C3a, C4a, C5a) were examined in all subjects, and serum complement regulatory factors (factor H and factor I) were measured in patients with primary APS. Plasma anticoagulant activity was determined in a mixing test using the activated partial thromboplastin time.

Results: Serum complement levels were significantly lower in patients with primary APS than in patients with non-SLE connective tissue diseases (mean (SD) C3: 81.07 (17.86) vs 109.80 (22.76) mg/dl, $p<0.001$; C4: 13.04 (8.49) vs 21.70 (6.96) mg/dl, $p<0.001$; CH_{50}: 31.32 (8.76) vs 41.40 (7.70) U/ml, $p<0.001$) or healthy volunteers. Only two healthy subjects with low serum C4 levels showed hypocomplementaemia, whereas most patients with primary APS showed raised serum C3a and C4a. No subjects showed raised C5a. Patients with primary APS with low serum C3 or C4 had significantly higher levels of C3a or C4a than healthy controls. No patients had low serum complement regulatory factors. Among patients with primary APS, hypocomplementaemia was significantly more common in those with high anticoagulant activity than in those with low or normal activity.

Conclusion: Hypocomplementaemia is common in patients with primary APS, reflecting complement activation and consumption, and was correlated with anticoagulant activity, suggesting that antiphospholipid antibodies may activate monocytes and macrophages via anaphylatoxins produced in complement activation.

Antiphospholipid syndrome (APS) is a clinical condition characterised by recurrent venous/arterial thrombosis and pregnancy morbidity in the presence of antiphospholipid antibodies (aPL).[1] Despite the strong association between aPL and clinical manifestations, the pathogenic role of aPL has not been fully elucidated[2]; however, this role is possibly multifactorial in nature. The engagement of aPL on cell surfaces promotes intracellular signalling[3 4]; in endothelial cells, this leads to expression of procoagulant activity and adhesion molecules[5] that in turn increases leukocyte adherence to endothelial cells.[6] In monocytes, it leads to the upregulation of tissue factor[7 8] that can enhance thrombin-induced platelet activation/aggregation.[9] Thrombosis is also favoured by aPL-induced depression of fibrinolysis[10] and by aPL interference with natural anticoagulant activities.[11–13]

Complement is part of the innate immune system and represents one of the effector arms of antibody-mediated immunity.[14] The complement system is commonly activated in systemic lupus erythematosus (SLE) and is strongly associated with the physiopathology of inflammation, as suggested by the low serum complement concentration with increased deposition at sites of tissue damage. Complement-derived inflammatory mediators (anaphylatoxins) such as C3a, C4a and C5a increase vascular permeability, activate platelets[15] and neutrophils,[16] and promote release of cytokines such as tumour necrosis factor (TNF) α from monocytes,[17] with simultaneous induction of systemic inflammation and coagulation.

A number of studies on murine models have highlighted how complement activation is essential for aPL-induced pregnancy morbidity.[18 19] C5a, the most powerful inflammatory anaphylatoxin, seems to be crucial in clinical manifestation in these models.[18] These findings have provided a new insight, suggesting that tissue injury in APS may be caused by a complement-mediated inflammatory process, rather than by thrombosis alone.[20] Since complement activation in aPL-related thrombosis has not been examined clearly, this study was performed to evaluate the prevalence and relevance of hypocomplementaemia in patients with primary APS.

PATIENTS AND METHODS

Patients

This study was performed with a retrospective and cross-sectional design and included 36 patients with primary APS treated at Hokkaido University Hospital from 1996 to 2006. Primary APS was diagnosed according to the classification criteria for APS,[1 21] with exclusion of patients who fulfilled criteria for SLE.[22] Thirty-six age and gender-matched healthy volunteers and 42 non-SLE patients with connective tissue disease (15 systemic sclerosis, 9 Sjögren's syndrome, 11 polymyositis/dermatomyositis, 5 mixed connective tissue disease, 1 overlap syndrome and 1 allergic granulomatous angiitis) were enrolled as controls. The non-SLE connective tissue disease control group comprised consecutive patients attending our autoimmune disease and rheumatology clinic who matched as controls and agreed to join this study. None of the participants had complications associated with infection, malignancy, impaired circulation or tissue ischaemia at the time of blood collection and all were negative for C-reactive protein.

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Table 1 Characteristics of the patients

Characteristics	Primary APS (n = 36)	Control* (non-SLE) (n = 42)
Gender (F:M)	27:9	37:5
Age (years), mean (range)	46 (18–75)	52 (13–77)
Number of SLE criteria fulfilled†, mean (SD)	2.08 (0.78)	2.00 (0.83)
Manifestation		
Venous thrombosis (n)	16	2
Arterial thrombosis (n)	19	4
Pregnancy morbidity (n)	3	
Time since last thrombotic event (years), mean (SD)	4.2 (3.6)	

*Control group includes cases of systemic sclerosis (n = 15), Sjögren's syndrome (n = 9), polymyositis/dermatomyositis (n = 11), mixed connective tissue disease (n = 5), overlap syndrome (n = 1) and allergic granulomatous angiitis (n = 1); †revised American College of Rheumatology SLE Classification Criteria (1997). APS, antiphospholipid syndrome; SLE, systemic lupus erythematosus.

Clinical records were reviewed retrospectively or patients were interviewed at the time of blood sample collection, or both. Arterial events such as stroke, myocardial infarction and iliac artery occlusion were confirmed by CT scan, magnetic resonance imaging (MRI) or angiography, as required. Deep vein thrombosis and pulmonary thrombosis were defined as venous thrombosis and confirmed by angiography or scintigraphy. Clinical manifestations of APS were diagnosed by rheumatologists with reference to imaging tests and clotting assays.

Plasma and serum sample collection

Blood was drawn by venepuncture and collected into different tubes. Blood samples for clotting assays were collected in plastic tubes containing 0.105 M citrate, immediately centrifuged at 3000 rpm for 15 min at 4°C, filtered through a 0.22 μM pore size membrane to obtain platelet-free plasma, and stored at −80°C until use. In the patients with primary APS, the plasma levels of D-dimer and fibrin degradation product (FDP) were determined using LPIA ace D-dimer II (Mitsubishi Kagaku Iatron, Tokyo, Japan) and Nanopia P-FDP (Daiichi Pure Chemical Co, Tokyo, Japan) kits, respectively. Blood samples for anaphylatoxin determination were collected in EDTA tubes containing nafamostate mesilate and centrifuged immediately to avoid cold in vitro complement activation.[23] Blood samples for measurement of serum complement were drawn in plain tubes, allowed to clot and then centrifuged before measurement.

The study was done in accordance with the Declaration of Helsinki and the Principles of Good Clinical Practice. Approval was obtained from the local ethics committee, and informed consent was obtained from each subject before enrolment.

aPL determination

The levels of IgG/M anticardiolipin (aCL) and IgG/M anti-β_2-glycoprotein I (β_2GPI) antibodies were measured using a standard aCL ELISA[24] and an in-house ELISA assay,[25] respectively. The positive cut-off values of the assays were set at the 99th centile for 134 healthy controls, according to laboratory criteria for APS.[1] IgG/M phosphatidylserine-dependent antiprothrombin (aPS/PT) antibodies were assayed as previously described.[26] Lupus anticoagulant (LA) was determined by two clotting assays using an opto-mechanical coagulation analyser (Start4, Diagnostica Stago, Asnières, France) based on the guidelines of the Subcommittee on Lupus Anticoagulant/Phospholipid-Dependent Antibody.[27] For the activated partial thromboplastin time (aPTT), a sensitive reagent with a low phospholipid concentration (PTT-LA, Diagnostica Stago) was used for screening, with confirmation using a Staclot LA kit (Diagnostica Stago). The dilute Russell's viper venom time was determined using a Gradipore LA test (Gradipore, Frenchs Forest, NSW, Australia). LA was considered positive when at least one of these tests was positive for LA, and was arbitrarily classified into either strong or weak LA according to the anticoagulant potential, as follows. One volume of sample plasma was mixed with four volumes of normal pooled platelet-free plasma, and the clotting time of the mixture was measured using PTT-LA. LA was defined as strong if the aPTT ratio (1:4 mixed plasma/normal plasma) was >1.28 and weak if this ratio was <1.28.

Serum complement and anaphylatoxin determination

Complement components C3, C4 and C5 were determined by a nephelometric method that gives normal ranges of 86–160, 17–45 and 9–13 mg/dl, respectively. CH_{50} activity was determined by the Mayer method, with a normal range of 30–45 U/ml. Serum anaphylatoxin levels were determined by radioimmunoassay (complement C3a des-Arg [^{125}I], complement C4a des-Arg [^{125}I], complement C5a des-Arg [^{125}I], Human Assay, GE Healthcare Bioscience, London, UK), with normal ranges of

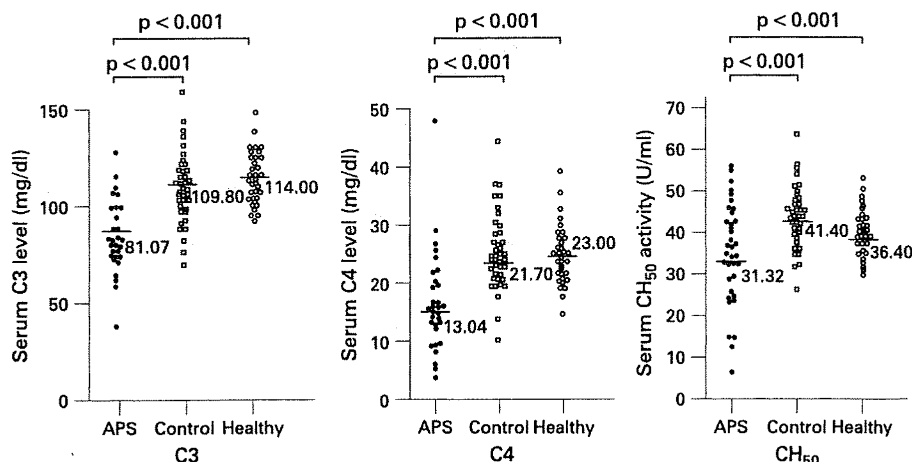

Figure 1 Serum complement levels. C3, C4 and CH_{50} levels in patients with primary antiphospholipid syndrome (APS), patients with non-systemic lupus erythematosus (SLE) connective tissue diseases and healthy volunteers. Control, patients with non-SLE connective tissue diseases; healthy, healthy volunteers. Statistical analysis by Student *t* test.

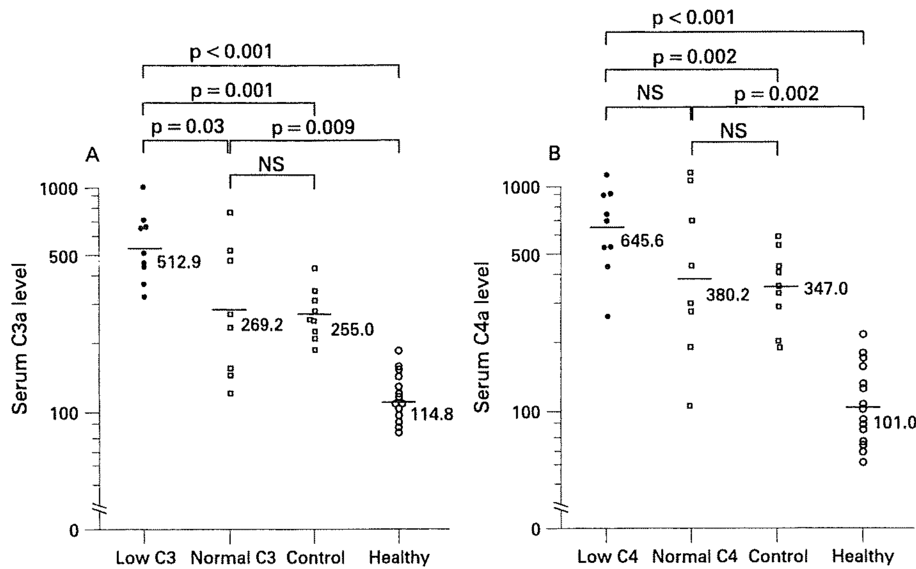

Figure 2 Serum anaphylatoxin levels. Serum C3a and C4a levels were measured in 17 patients with primary antiphospholipid syndrome (APS), 9 patients with non-systemic lupus erythematosus (SLE) connective tissue diseases and 15 healthy volunteers. The bars and figures in the graphs represent the mean levels of anaphylatoxins in each groups. (A) Comparison of C3a levels between patients with primary APS with low or normal C3, control patients and healthy volunteers. (B) Comparison of C4a levels between patients with primary APS with low or normal C4, control patients and healthy volunteers. Control, patients with non-SLE connective tissue diseases; healthy, healthy volunteers. Statistical analysis by Student *t* test.

50–200 ng/ml for C3a, 50–250 ng/ml for C4a and <10 ng/ml for C5a. C3, C4 and CH_{50} were measured in all participants, C5 was determined in 10 patients with primary APS, and anaphylatoxins were examined in 17 patients with primary APS, 9 control non-SLE patients and 17 healthy controls.

Plasma TNFα level determination

Plasma TNFα levels were examined in 22 patients with APS using sandwich ELISA (Endogen Human TNFα ELISA kit: Pierce Biotechnology, Rockford, Illinois, USA).

Measurement of serum complement regulatory factor

Serum levels of complement regulatory factor H (C3b-related C5 activation inhibitor) and factor I (C4b-related C3 activation inhibitor) were measured in 16 and 13 patients with APS, respectively, by radioimmunoassay (Monoclonal Antibody to Human Factor H, Monoclonal Antibody to Human Factor I, Quidel Corporation, San Diego, California, USA). The normal ranges are 22.8–41.7 mg/dl for factor H and 3.3–14.4 mg/dl for factor I, according to the manufacturer's instructions.

Measurement of serum immune complex

Serum levels of immune complex were measured in 33 patients with primary APS and 22 patients with non-SLE connective tissue disease, by enzyme immunoassay (Immunocomplex mRF Nissui, Nissui Pharmaceutical, Tokyo, Japan). The normal range is <4.2 μg/ml, according to the manufacturer's instruction.

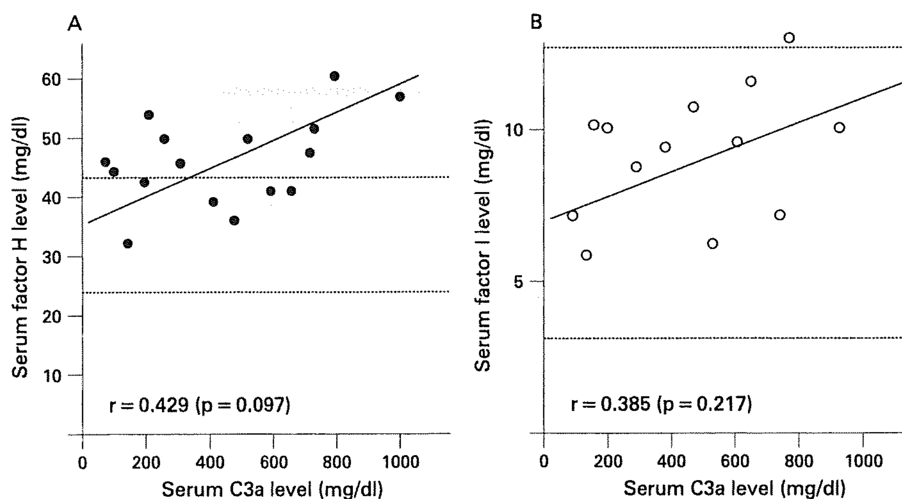

Figure 3 Complement regulatory factor levels in patients with primary antiphospholipid syndrome (APS). Serum factor H (A) and factor I (B) were measured in 16 and 13 patients with primary APS, respectively. Dotted lines represent the upper and lower limits of the normal ranges. Statistical analysis by Pearson correlation coefficient.

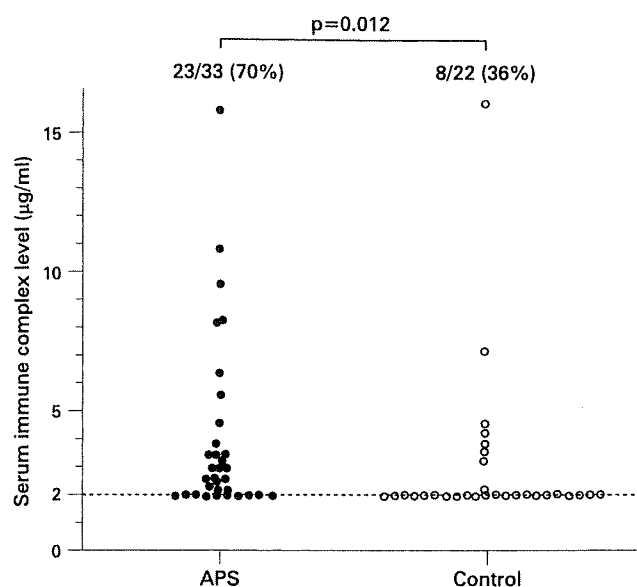

Figure 4 Serum immune complex levels. Serum immune complex levels were examined in patients with primary antiphospholipid syndrome (APS) and non-systemic lupus erythematosus (SLE) connective tissue disease controls. Figures at the top of the scatter diagram represent the ratio of the patients with detectable serum immune complex level. The dotted line represents the detection limit (2.0 µg/ml) of serum immune complex level. Control, patients with non-SLE connective tissue diseases. Statistical analysis by Fisher's exact test.

Statistical analysis

A Student *t* test, Mann–Whitney non-parametric test, Pearson correlation coefficient or Fisher's exact test was used as appropriate. In categorical analysis, the relative risks were expressed as odds ratios with 95% confidence intervals (95% CI). SPSS II for Windows was used for all calculations.

RESULTS

Serum complement levels and prevalence of hypocomplementaemia in primary APS

Criteria for SLE were investigated in the subjects by chart review or interview of the patients. The number of lupus criteria met by the primary APS group did not differ from that for patients with non-SLE connective tissue diseases (table 1). Signs of acute thrombosis were not detected in any patients with primary APS at the time of blood collection, and no significant increase of plasma D-dimer or FDP was found in these patients. The mean (SD) period after the last manifestation in patients with primary APS was 4.2 (3.6) years (range 0.3–10) (table 1). The patients with primary APS showed a higher prevalence of reduced levels of C3, C4 and CH_{50} than patients with non-SLE connective tissue diseases (C3: 69.4% vs 9.52%, OR = 21.59, 95% CI 6.18 to 75.42; C4: 61.1% vs 7.1%, OR = 15.32, 95% CI 4.48 to 52.31; CH_{50}: 47.2% vs 2.4%, OR = 36.68, 95% CI 4.54 to 296.26). No healthy volunteers had a reduced complement level, but two had a low serum C4 level. The mean levels of C3, C4 and CH_{50} were lower in patients with primary APS than in patients with non-SLE connective tissue disease or healthy volunteers (fig 1). Additionally, serum complement levels of patients with non-SLE connective tissue disease with a past history of thrombosis (C3: 112 (13.5); C4: 20.0 (6.32); CH_{50}: 40.4 (4.54)) did not show significant differences with the patients without a past history of thrombosis.

Serum anaphylatoxin levels

Most patients with primary APS showed raised serum C3a and C4a levels (C3a: 13/17, 76%; C4a: 14/17, 82%), but none showed raised C5a (0/17). Patients with primary APS with a low serum C3 concentration had significantly higher levels of C3a than those with normal C3, but there was no significant difference in C4a levels between patients with low and normal C4 (fig 2). The non-SLE patients had significantly lower levels of C3a and C4a than patients with primary APS with low serum C3 and C4 concentrations. However, there were no significant differences in C3a and C4a levels between control patients and patients with primary APS with normal C3 and C4 levels. C3a and C4a levels of control patients with past history of thrombosis (n = 4; C3a: 2 68 (73.1); C4a: 362 (62.1)) did not show significant differences from patients without a past history of thrombosis. No healthy volunteers showed raised serum anaphylatoxin levels.

Plasma TNFα levels in patients with primary APS

Raised plasma TNFα was found in 7/22 (32%) of patients with primary APS. The prevalence of raised TNFα was greater in patients with hypocomplementaemia than in those with the normal serum CH_{50} activity (63% vs 14%, OR = 10, 95% CI 1.26 to 79.34).

Serum complement regulatory protein levels

Serum complement regulatory factor H and factor I were measured in 16 and 13 patients with primary APS, respectively. These patients did not have reduced levels of factor H or factor I (fig 3). Serum factor H levels tended to be raised in patients with high C3a serum levels, but the increase was not statistically significant (fig 3).

Serum immune complex levels

The positive ratio of serum immune complex in patients with primary APS was 23/33 (70%), which was significantly increased compared with ratio of patients with non-SLE connective tissue disease (8/22 (36%), OR = 4.03, 95% CI 1.28 to 12.6) (fig 4).

Correlation between clinical manifestations and hypocomplementaemia

Clinical manifestations of APS occurred as venous thrombosis, arterial thrombosis and pregnancy morbidity in 16/36 (44.4%), 19/36 (52.8%) and 3/27 (11.1%) cases, respectively. No particular manifestation was correlated with hypocomplementaemia. The relative risks for having those manifestation in patients with low CH_{50} were 1.28 (95% CI 0.34 to 4.82), 1.55 (0.41 to 5.78), 1.88 (0.16 to 22.8), respectively. All patients received warfarin and/or antiplatelet agents, but none received heparin or its derivatives.

Six patients with non-SLE connective tissue diseases had past histories of thrombosis (one venous thrombosis and three arterial thrombosis) and no patients showed hypocomplementaemia.

Correlation between aPL levels and hypocomplementaemia

The prevalences of IgG-aCL, IgM-aCL, IgG-anti-β_2GPI, IgM-anti-β_2GPI, IgG-aPS/PT, IgM-aPS/PT and LA were 26/36 (72%), 9/36 (25%), 21/36 (58%), 8/36 (22%), 21/36 (58%), 18/36 (50%)

Table 2 Correlation between aPL and hypocomplementaemia (low CH_{50})

aPL	n	Normal CH_{50}	Low CH_{50}	OR (95% CI)
aCL IgG*				
Positive	26	12	14	0.86 (0.20 to 3.69)
Negative	10	5	5	
aCL IgM*				
Positive	9	4	5	0.86 (0.19 to 3.92)
Negative	27	13	14	
Anti-β_2GPI* IgG				
Positive	21	11	10	1.65 (0.43 to 6.31)
Negative	15	6	9	
Anti-β_2GPI* IgM				
Positive	8	3	5	0.6 (0.12 to 3.01)
Negative	28	14	14	
aPS/PT* IgG				
Positive	21	14	7	8.0 (1.69 to 38.0)
Negative	15	3	12	
aPS/PT* IgM				
Positive	18	11	7	3.14 (0.80 to 12.3)
Negative	18	6	12	
LA				
High	14	3	11	6.42 (1.37 to 30.1)
Low/normal	22	14	8	

*IgG and IgM were tested in all 36 samples.
aCL, anticardiolipin antibody; aPL, antiphospholipid antibodies; aPS/PT, phosphatidylserine-dependent antiprothrombin antibody; anti-β_2GPI, anti-β_2-glycoprotein I antibody; CI, confidence interval; LA, lupus anticoagulant; OR, odds ratio.

and 30/36 (83%), respectively, in patients with primary APS. Neither IgG/M-aCL nor IgG/M-anti-β_2GPI was correlated with hypocomplementaemia (low CH_{50}), but the presence of IgG/M-aPS/PT positively correlated with hypocomplementaemia (table 2), which occurred significantly more often in patients with primary APS with high LA than in those with low or negative LA (11/14 (79%) vs 8/22 (36%), OR = 6.42, 95% CI 1.37 to 30.1; table 2).

DISCUSSION

Our results show that hypocomplementaemia is frequently found in patients with primary APS. The high serum C3a and C4a levels and the correlation between serum C3a concentrations and low C3 suggests that hypocomplementaemia in these patients is due to complement activation rather than complement deficiency. None of the patients with primary APS had reduced factor H or factor I levels, indicating that complement activation is not caused by deficiency of these factors but presumably by enhanced immune complex formation. In primary APS, immune complex formation might have a bearing on the anticoagulant potential of aPL, given the positive correlation between strong LA and hypocomplementaemia. This relationship is also related to plasma TNFα released by procoagulant and proinflammatory cells, further supporting a role for complement activation in some manifestations in patients with APS.

Concurrent reduction of C3, C4 and CH_{50} was the most common profile in our patients, reflecting activation of the classical complement pathway. The proinflammatory effect of C5a is relevant to the pathogenesis of miscarriages in the APS animal model, but none of the patients had raised serum C5a. The finding of lower serum C5a compared with C3a and C4a is consistent with reports in other diseases, including SLE.[28–30] C5a is a strong inflammatory mediator and regulatory factors such as factor H or factor I inhibit C3b-dependent activation of C5. In this study, patients with primary APS with high serum C3a levels tended to have raised factor H, and persistent C3 activation may lead to upregulated production of regulatory factors; however, none of the patients were in the acute phase of thrombosis at the time of blood collection. It is likely that the behaviour of C5a in primary APS is similar to that in SLE flares,[30] but complement activation is common during pregnancy[31] and C5 activation may occur in APS pregnancy. A recent report provides evidence to show that serum of patients with APS can induce tissue factor production on neutrophils and this effect was shown to be due to C5a activation.[32] Anaphylatoxins, especially C5a, are extremely labile and are quickly degraded by serum protease, and thus blood was examined immediately; however, it is possible that C5a was degraded in some cases.

Infection, injury or other biological stresses can activate the complement system.[33] Accumulating evidence indicates that tissue ischaemia or platelet aggregation can induce complement activation,[34 35] therefore, thrombosis itself is one of the incidents which might induce complement activation. However, none of the participants in this study had complications associated with infection, malignancy, impaired circulation, tissue ischaemia, or thrombosis at the time of blood collection and all were negative for C-reactive protein. The hypothesis is further suggested by the data that non-SLE controls who had a history of thrombosis did not show hypocomplementaemia. No patients were receiving heparin or its derivatives, which are known to modify complement activation. Additionally, most of the patients with APS were receiving one or more antiplatelet agents and blood of all patients with primary APS was not examined at the acute stage of thrombosis. In SLE or other immune complex-mediated diseases, immune complex formation can promote activation of the classical pathway. We showed in this study that raised immune complex levels were frequently found in patients with primary APS, suggesting the circulating immune complex potentiates the triggering of complement activation, ultimately leading to thrombotic events. Those immune complexes may include an antigen–antiphospholipid antibody complex in patients with primary APS, as previously described.[36] We found that LA activity was correlated with hypocomplementaemia, and LA from APS plasma may reflect the sum of multiple aPL involved in the pathophysiology of APS. Therefore, our results partially support the hypothesis that an aPL–autoantigen complex drives complement activation.

The prevalence of aPS/PT in patients with hypocomplementaemia was higher than that in patients with a normal CH_{50} level. IgG1 has been proposed as a dominant subclass in aCL in patients with thrombotic events,[37] but we[7] and others[38] have found that IgG2 is the dominant IgG subclass of aCL or anti-β_2GPI in APS. Since IgG2 is less effective in activating complement than other subclasses, aPS/PT might be more potent in activating complement than aCL or anti-β_2GPI. Additional mechanisms may contribute to complement activation in patients with primary APS. In SLE, reduced complement receptor type 1 levels on erythrocytes and impaired CD55 and CD59 expression (downregulators of the complement system) have been proposed as additional mechanisms of complement activation.[39] Equivalent data are unavailable in primary APS, but since many patients with primary APS evolve towards SLE, there may be common mechanisms of complement activation in SLE and APS.

We have previously reported that raised plasma TNFα levels in APS are not associated with HLA haplotypes including class

III.[40] In this study, some patients with hypocomplementaemia showed raised TNFα. Although the relationship of hypocomplementaemia and plasma TNF levels shown in this study was borderline, as TNFα is produced by activated monocytes, complement activation might accelerate prothrombotic reactions in APS, further supporting a role for complement activation in some manifestations in patients with APS. In the animal APS pregnancy model, TNFα has been proposed as a candidate therapeutic target,[41] and our data in patients with APS partly support this idea. Recognition of cross-talk between complement activation and prothrombotic status highlights an important role for the complement system in the physiopathology of primary APS, since complement activation may participate in coagulation processes and contribute to tissue damage. Taken together, these results suggest that the complement system could be a potential therapeutic target in patients with APS.

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Competing interests: None.

Ethics approval: Approval obtained from the local ethics committee.

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STAT4 single nucleotide polymorphism, rs7574865 G/T, as a risk for antiphospholipid syndrome

Genetic factors are hypothesised to play a role in the susceptibility to antiphospholipid syndrome (APS) based on several family studies in patients with anti-phospholipid antibodies (aPL) and/or clinical manifestations of APS. APS can occur alone (primary APS) or in conjunction with systemic lupus erythematosus (SLE). Therefore, APS and SLE may, in part, share a common mechanism for disease onset or progression. Likewise SLE, human leukocyte antigen (HLA) associations have been reported in APS.[1] In addition, polymorphisms of target antigens for aPL and thrombotic genetic risk factors have been described.[1] Despite the numerous studies on the immunogenetic predisposition to APS, only a few genes such as HLA region have been identified.

Signal transducer and activator of transcription 4 (STAT4) is a transcription factor expressed on myeloid cells, T cells, dendritic cells, monocytes and so forth. Since the STAT4 pathway plays crucial roles in the Th1 and Th17 lineages,[2 3] alterations in the STAT4 pathway may lead to autoimmune and/or chronic inflammatory disorders. The STAT4 gene is located in the chromosome 2q that has been considered to be associated with SLE and RA.[4 5] Recently, Remmers *et al*[6] demonstrated that the STAT4 haplotype tagged by rs7574865 G/T single nucleotide polymorphism (SNP) was strongly associated with rheumatoid arthritis (RA) and SLE in the North American population. Moreover, recent genome wide association study confirmed not only HLA but also STAT4 associations in Caucasian patients with SLE.[7] STAT4 association was also found in Korean patients with RA [8] and Caucasian patients with primary Sjögren syndrome (SS).[9] In this study, we investigated rs7574865, the most strongly associated SNP of STAT4, in Japanese patients with APS and compared them with healthy controls.

We enrolled 74 patients with APS (37 with primary APS and 37 with secondary APS complicated with SLE) and 414 ethnically matched healthy controls. Written informed consent was obtained from each participant. All patients with APS met the criteria for the classification of APS.[10] In total, 70 patients met thrombotic criterion and 19 patients met obstetrical criterion. Genomic DNA samples were extracted from peripheral blood. Genotyping of rs7574865 was performed using TaqMan Genotyping Assay kit. Genotype and allele frequencies in each group were compared using the χ^2 test and the related risk for having APS was approximated by the odds ratios.

Genotypes of rs7574865 did not show any significant deviation from Hardy–Weinberg equilibrium in healthy control and APS groups. The T allele frequency of rs7574865 in APS (42.6%) was significantly elevated compared with that in healthy controls (31.6%). When analysed only in patients with primary APS, the T allele frequency of rs7574865 (48.6%) was higher still (table 1). This association was still observed after stratification by clinical manifestations of APS.

Our results show, for the first time, the positive correlation between the T allele of STAT4 rs7574865 and APS. Moreover, the correlation was even enhanced when focusing on primary APS, indicating that this SNP is also associated with APS itself. Our data suggest that the STAT4 SNP plays a crucial role, independent of ethnicity, in the pathogenesis of autoimmune disorders, including RA, SLE, SS and APS.

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Table 1 The allele frequencies of STAT4 SNP, rs7574865 G/T, in controls and patients with APS and primary APS

	T allele	G allele	p Value	OR (95% CI)
Controls (n = 414)	262 (31.6%)	566 (68.4%)	–	–
APS (n = 74)	63 (42.6%)	85 (57.4%)	<0.001	1.60 (1.12 to 2.29)
Primary APS (n = 37)	36 (48.6%)	38 (51.4%)	0.003	2.05 (1.27 to 3.30)
Thrombosis (n = 70)	58 (41.4%)	82 (58.6%)	0.023	1.53 (1.06 to 2.21)
Obstetrical complication (n = 19)	19 (50.0%)	19 (50.0%)	0.018	2.16 (1.12 to 4.15)

The p value and OR (95% CI) for each group were determined in comparison with controls.
APS, antiphospholipid syndrome; OR, odds ratio; SNP, single nucleotide polymorphism; STAT4, signal transducer and activator of transcription 4 (STAT4).