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Expression of CD45 isoforms correlates with differential proliferative responses of peripheral CD4+ and CD8+ T cells

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ABSTRACT

CD4+ T cells express IL-2 receptor complexes to the same level as CD8+ T cells when the two T cell populations were stimulated simultaneously. However, the activation of downstream signaling molecules, such as Jaks, was increased in CD8+ T cells. Although equivalent amounts of CD45, which acts as a Jak phosphatase, was expressed on the two T cell populations, those on the CD8+ T cells have less protein tyrosine phosphatase activity than those on the CD4+ T cells. Furthermore, we find that different CD45 isoforms dominate in the two populations; CD45RO on proliferating CD4+ T cells and CD45RBC on proliferating CD8+ T cells. In addition, NIH3T3 cells expressing the CD45RBC transgene had more phosphorylated Jak1 and grew faster than those with the CD45RO transgene. Thus, the expression of specific CD45 isoforms on T cells correlates with their proliferative response to IL-2, suggesting that controlling cells expressing specific CD45 isoforms could correct excessive or insufficient immune responses.

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1. Introduction

CD8+ T cells, unlike CD4+ T cells, are known to undergo extensive clonal expansion in response to viral infection [1–3]. The expanded cells are prone to persist, and thus clonal expansions of CD8+ T cells are frequently found in the peripheral blood from healthy individuals [4,5]. This is more frequent in the elderly than in the young where they can occupy as much as 40% of the total peripheral T cell repertoire [4–6]. This age-related increase in clonal expansion may be responsible for impaired immunocompetence in the elderly. Also, we found that expansion of pathogenic CD8+ T cell clones persist in the peripheral blood of patients with polymyositis, a chronic cytotoxic CD8+ T cell-driven autoimmune disease [7]. The clonal expansions remained even after treatment of the disease and might lead to relapse. The expansions of CD8+ T cells must be partly attributable to the fact that they can be easily activated by promiscuous TCR stimuli [8]. However, the molecular basis for their massive expansion in response to IL-2 in comparison with CD4+ T cells has not yet been addressed. Investigating the specific mechanisms of CD8+ T cell proliferation could help us to develop new therapeutic strategies to reverse immunological senescence and to suppress CD8+ T cell-mediated autoimmunity.

Because T cell expansion subsequent to triggering of antigen receptors is regulated largely by cytokines that stimulate common γ chain cytokine receptors (γc) [9–12], pharmacological inhibition of the γc signaling pathways represents a promising approach for the treatment of autoimmune diseases [13,14]. However, suppression of these cytokines can inhibit proliferation of T cells engaged in both normal and abnormal immune responses, and might induce generalized immune suppression. To minimize the risk of immune suppression, treatment for autoimmune disease should target specific molecules other than γc and/or specific cells associated with the pathogenic responses.

To determine the possibility of treating CD8+ T cell-mediated autoimmune diseases through a disease specific molecular targeting approach, we investigated the γc signaling pathway in CD4+ and CD8+ T cell subsets and aimed to find regulatory molecules which are specific to CD8+ T cell proliferation using an *in vitro* study system. In this study system, CD8+ T cells expand more vigorously and frequently than CD4+ T cells, as was reported in an *in vivo* system [1] and we found that CD45, one of the regulatory molecules of γc signaling pathways [15], was differentially expressed and active in each T cell subset.

CD45 is a transmembrane glycoprotein that has two tandemly duplicated protein tyrosine phosphatase (PTPase) homology

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domains in its long cytoplasmic tail [16,17]. It regulates signaling of two types of receptors on T cells; it dephosphorylates the Src family protein tyrosine kinases, Lck and Fyn, to potentiate antigen receptor signaling [18,19] and also functions as a Jak PTPase to attenuate cytokine receptor signaling [15]. A number of mechanisms are hypothesized for the regulation of CD45 PTPase activity: (1) CD45 homo-dimerization, which is modulated by sialylation and O-glycosylation of the extracellular domain [20], (2) CD45-associated protein, which inhibits CD45 homo-dimerization [21], and (3) casein kinase 2, which phosphorylate CD45 [22]. However, the precise mechanisms are still unclear.

CD45 molecules are expressed in several configurations on the cell surface. This is because of alternative splicing of three exons (A, B, and C) that encode its extracellular domains. Although alternative splicing can theoretically recombine the three exons to generate eight different CD45 isoforms, only five isoforms (CD45RO, RB, RAB, RBC, and RABC) are expressed as proteins at significant levels in human or murine lymphocytes [23,24]. Differential function of the distinct CD45 isoforms has been suggested, although the data is inconclusive. Bottomly and her coworkers reported that a $CD4^+$ T cell line expressing the CD45RO isoform preferentially activates the Ras-MAP kinase signaling pathway compared with those expressing the CD45RABC isoform when stimulated with antigen [25]. They also showed that CD45RO was more effective in triggering antigen receptor signaling than the CD45RABC and BC isoforms in T cell lines [26] while a second report disclosed contradictory results [27].

For this study we assumed that CD45 isoform regulation could dictate the differential proliferation of $CD4^+$ and $CD8^+$ T cells, and might be a target for therapeutic intervention in human diseases. We first report that the differential IL-2-induced proliferative response between $CD4^+$ and $CD8^+$ T cells is because of the difference in their IL-2-sensitivity. We also show that the CD45 isoforms on activated $CD8^+$ T cells in the periphery are distinct from those on $CD4^+$ T cells. Because the major CD45 isoform on the $CD8^+$ T cells was not detected on $CD4^+$ T cells, targeting that CD45 isoform could become a new therapeutic strategy for $CD8^+$ T cell-mediated immune abnormalities.

2. Materials and methods

2.1. Mice

C57BL/6J mice were purchased from the Charles River Laboratories Japan Inc. (Kanagawa, Japan). All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals approved by Tokyo Medical and Dental University.

2.2. Cells

Splenic T cells were prepared from 7- to 10-week-old mice using a pan T cell isolation kit (Miltenyi Biotec, Auburn, CA). T cells were stained with anti-CD62L (MEL-14; BD Pharmingen, San Diego, CA) and anti-CD44 mAbs (IM7; BD Pharmingen) to collect naive $CD62L^+CD44^{low}$ T cells using an EPICS ELITE cell sorter (Beckman Coulter, Hialeah, FL). In some experiments, $CD4^+$ and $CD8^+$ T cells were positively selected with anti-CD4 and CD8 conjugated magnetic microbeads (Miltenyi Biotec). Cultures were maintained in RPMI 1640 (Sigma–Aldrich, St. Louis, MO) containing 10% FBS (Hyclone, Logan, UT), 50 μM 2-ME, penicillin G, and streptomycin (Invitrogen, Carlsbad, CA). A murine NIH3T3 fibroblast cell line was purchased from the American Type Culture Collection (Manassas, VA) and maintained in DMEM (Sigma–Aldrich) containing 10% FBS, penicillin G, and streptomycin. Retroviral NIH3T3 transfectants were maintained under the same conditions except for the addition of 4 μg/ml puromycin (Calbiochem, La Jolla, CA).

2.3. Transgene construction and generation of retroviral NIH3T3 transfectants

Full length cDNA of mouse CD45 was isolated by PCR from the pARV-BC and pARV-CD45null plasmid [28], which contained mouse CD45RBC and CD45RO cDNAs, respectively. They were subcloned into the pMXs-IRES-puro retroviral vector (pMXs-IP) [29] using the In Fusion kit (Takara Bio Inc., Shiga, Japan). The recombinant pMXs-IP and control pMXs-IRES-EGFP vectors [29] were transfected into PLAT-E packaging cells [29] using Fugene 6 (Roche Applied Science, Indianapolis, IN). NIH3T3 cells were infected with the recombinant viruses in the presence of 8 μg/ml polybrene(R) (Sigma–Aldrich).

2.4. Proliferation assays

T cells from C57BL/6J mice were stimulated with immobilized anti-CD3ε mAbs (145-2C11, BD Pharmingen) in the presence or absence of 5 μg/ml anti-CD28 mAbs (37.51, BD Pharmingen) for 3 days and then cultured with 100 U/ml recombinant human IL-2 (R&D systems, Minneapolis, MN). Some T cells were treated with a synthetic CD45 inhibitor, dephostatin (Sigma–Aldrich), 30 min before supplementation of IL-2. Proliferation of T cells was assessed by cell counting and flow cytometry. Division of T cells was assessed with CFSE labeling using the CFDA SE cell tracer kit (Invitrogen) and apoptotic cell death of T cells was determined by 7-Amino Actinomycin D (7-AAD) and annexin V staining (Annexin V-FITC/7-AAD kit, Beckman Coulter). NIH3T3 transfectants were plated at 5×10^3 cells per micro-titer well and pulsed with [^{3}H]thymidine for 12 or 24 h to assess their thymidine incorporation.

2.5. Flow cytometry

Anti-CD4, CD25, CD122, and CD132 (H129.19, PC-61, TM-β1, and 4G3, BD Pharmingen), anti-CD8 (KT3, Beckman Coulter), and anti-CD45 (104-2, Southern Biotechnology Associates, Birmingham, AL) mAbs were used for cell staining.

2.6. Immunoblotting and immunoprecipitation

Cells were lysed with ice-cold RIPA lysis buffer (Upstate Biotechnology) with protease inhibitor cocktails (Roche diagnostics, Basel, Switzerland). Whole cell lysates were immunoprecipitated with anti-Jak3 mAbs (B32-32, Sigma–Aldrich) using the Protein G immunoprecipitation kit (Sigma–Aldrich). SDS-PAGE fractionated whole cell lysates or immunoprecipitates were probed with Abs reactive to phosho-Jak1 (pJak1), phospho-STAT5 (pSTAT5), phosho-ERK (pERK), Akt, phospho-Akt (pAkt) (Cell Signaling Technology, Beverly, MA), Jak1, Jak3 (Sigma–Aldrich), ERK, phosphotyrosine, casein kinase 2 (Upstate Biotechnology, Waltham, MA), or CD45-associated protein [30]. CD45 molecules were stained with mAbs reactive to a common epitope shared by all CD45 isoforms (anti-pan-CD45 Abs, Santa Cruz Biotechnology, Santa Cruz, CA), exon A, exon B, or exon C-dependent epitopes of CD45 protein (14.8, 16.A, or DNL-1.9, BD Pharmingen). The bound Abs were visualized with ECL (Amersham Biosciences) using HRP-conjugated anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, UK), or HRP-conjugated anti-mouse IgG (Southern Biotechnology Associates).

2.7. Measurement of CD45 activity

Activity of CD45 protein tyrosine phosphatase was assessed as described elsewhere [31]. Naive ($CD62^{+}CD44^{low}$) $CD4^{+}$ and $CD8^{+}$ T cells cell were stimulated with immobilized anti-CD3ε mAbs for 3 days and then cultured with IL-2 for 5 days. Proteins immunoprecipitated from each T cell lysate with anti-CD45 mAbs (104-2) were incubated with phosphotyrosine peptide (Biomol, Plymouth Meeting, PA). Dephosphorylation reactions were stopped by the addition of Malachite Green reagent (Biomol) and the OD at 650 nm was measured. The amount of total CD45 in the lysates was evaluated by sandwich ELISA using mouse anti-CD45 mAbs (104-2), rat anti-CD45 mAbs (YW62.3, Oxford Biotechnology, UK), and HRP-conjugated anti-rat IgG (Southern Biotechnology Associates). Bound antibodies were quantified with TMB (Zymed Laboratories, South San Francisco, CA) as a substrate.

2.8. Statistical analysis

All values were expressed as means ± SE, and statistically examined with Student's *t*-test using Exsas software (version 7.12; Arm, Osaka, Japan).

3. Results

3.1. $CD8^{+}$ T cells are more susceptible to in vitro proliferative stimuli than $CD4^{+}$ T cells

Splenic $CD4^{+}$ and $CD8^{+}$ T cells from C57BL/6J mice were stimulated *in vitro* with immobilized anti-CD3ε mAbs, and then with IL-2. In response to this stimulation, $CD8^{+}$ T cells expanded at a substantially higher rate than $CD4^{+}$ T cells during the entire culture period (Fig. 1A). In 5 days, the $CD8^{+}$ T cells that initially had consisted of a minor population compared with $CD4^{+}$ T cells grew to be the dominant population (Fig. 1B). CFSE labeling of the cells showed that $CD8^{+}$ T cells divided at a faster rate than $CD4^{+}$ T cells in response to this stimulation (Fig. 1C) with little difference in the percentage of apoptotic cells (Fig. 1D). Similar proliferative differences were also observed when anti-CD28 mAb was included (Fig. 1E). It has been shown that memory T cells respond more rapidly than naive T cells to antigens [32,33]. Staining of the splenic $CD4^{+}$ and $CD8^{+}$ T cells from C57BL/6J mice with CD62L and CD44 mAbs revealed that the $CD8^{+}$ T cells contained more cells with a $CD62L^{+}CD44^{high}$ memory phenotype than $CD4^{+}$ T cells (Fig. 1F). This bias in the naive/memory T cell ratio might be responsible for the proliferative

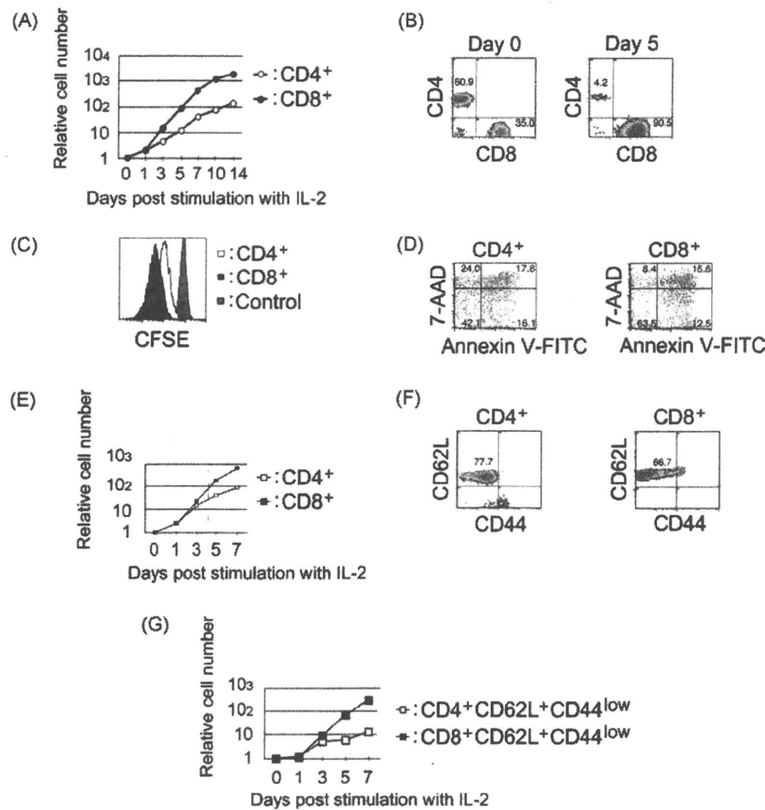

Fig. 1. $CD8^{+}$ T cells expand more rapidly than $CD4^{+}$ T cells following *in vitro* stimulation. Splenic T cells from C57BL/6J mice were stimulated with immobilized anti-CD3ε mAbs for 3 days and then cultured with IL-2 for 2 weeks. (A) $CD4^{+}$ (○) and $CD8^{+}$ (●) T cell numbers relative to their respective cell numbers at initiation of IL-2 stimulation are shown. Data is representative of three independent experiments. (B) T cells before (day 0: the *left panel*) and 5 days (the *right panel*) after stimulation with IL-2 were stained with anti-CD4 and CD8 mAbs. The percentages of $CD4^{+}$ and $CD8^{+}$ T cells in the culture are shown. Data is representative of three independent experiments. (C) Splenic T cells from C57BL/6J mice were stimulated with immobilized anti-CD3ε mAbs for 3 days and then cultured with IL-2 following CFSE labeling. T cells at 3 days post-stimulation with IL-2 were stained with anti-CD4 or CD8 mAbs and the CFSE intensity of $CD4^{+}$ (□) and $CD8^{+}$ (■) T cells was analyzed. The CFSE intensity of T cells before the IL-2 stimulation is shown as control staining. Data is representative of two independent experiments. (D) Splenic T cells from C57BL/6J mice were stimulated in the same way, and stained with FITC-conjugated annexin V and 7-AAD instead of CFSE. The percentages of annexin V and/or 7-AAD positive T cells in the culture are shown. Data is representative of two independent experiments. (E) Splenic T cells from C57BL/6J mice were stimulated with immobilized anti-CD3ε mAbs together with soluble anti-CD28 mAbs for 3 days and then cultured with IL-2 for 1 week. $CD4^{+}$ (□) and $CD8^{+}$ (■) T cell numbers relative to their respective cell number at initiation of IL-2 stimulation are shown. Representative data of two independent experiments are shown. (F) Splenic T cells from C57BL/6J mice stained with anti-CD62L and CD44 mAbs were analyzed. Flow cytometry data is representative of two independent experiments. (G) Proliferation of naive ($CD62L^{+}CD44^{low}$) T cells cultured for 2 weeks. Naive T cells from C57BL/6J mice were stimulated with immobilized anti-CD3ε mAbs together with soluble anti-CD28 mAbs for 3 days and then cultured with IL-2. Naive $CD4^{+}$ (□) and $CD8^{+}$ (■) T cell numbers relative to their respective cell numbers at initiation of IL-2 stimulation are shown. Data is representative of two independent experiments.

difference between CD4⁺ and CD8⁺ T cell preparations. Thus, we purified CD62L⁺CD44^{low} naive T cell populations to stimulate them with anti-CD3ε mAbs and IL-2. Dominant CD8⁺ T cell proliferation was observed even among the naive T cells (Fig. 1G).

3.2. IL-2-induces Jak phosphorylation more intensively in CD8⁺ T cells than in CD4⁺ T cells

The differential proliferative response of CD4⁺ and CD8⁺ T cells has been attributed to the differential upregulation of IL-2 receptor complexes on anti-CD3ε mAb stimulation and/or differential signal transduction through the IL-2 receptor. To test these possibilities, T cells stimulated with anti-CD3ε mAbs for 72 h were examined for their expression of IL-2 receptor α, β, and γ components (CD25, CD122, and CD132). CD25 is equally expressed by the two populations while CD122 and CD132 were increased in CD4⁺ T cells (Fig. 2A). Thus, the IL-2 receptor expression cannot account for the differential response to IL-2.

Immunoblot analyses of cell lysates from the IL-2 stimulated T cells showed that phosphorylation of Jak1 and Jak3, which are phosphorylated following IL-2 stimulation, was increased in CD8⁺ T cells (Fig. 2B and C). Reflecting this hyperphosphorylation of Jaks in CD8⁺ T cells, phosphorylation of STAT5, Akt, and ERK was also increased in CD8⁺ T cells (Fig. 2D–F). Since expression of the IL-2 receptor complex was not increased in CD8⁺ T cells, these results showed that CD8⁺ T cells express IL-2 receptor complexes more susceptible to IL-2 stimulation than CD4⁺ T cells.

3.3. CD45 protein tyrosine phosphatase activity is significantly higher in CD8⁺ T cells than in CD4⁺ T cells

It is known that CD45 PTPase activity potentiates signaling through T cell antigen receptors. At the same time, CD45 attenuates cytokine receptor signaling through Jak, since cytokine receptor signaling depends on Jak dephosphorylation by CD45 [18,19]. Hyperphosphorylation of Jaks and their substrates might be because of lower CD45 PTPase activity in CD8⁺ T cells. Immunoprecipitated CD45 from CD8⁺ T cell lysates dephosphorylated tyrosine phosphorylated peptides less efficiently than CD45 from CD4⁺ T cell lysates (Fig. 3A) while the amount of CD45 in the lysates was equal (Fig. 3B). This demonstrated that the two types of cells had different PTPase activity.

To determine if this difference contributes to the dominant proliferation of CD8⁺ T cells over CD4⁺ T cells, dephostatin, a synthetic CD45 PTPase inhibitor, was added to the culture medium in the T cell proliferation assay. Pre-treatment with dephostatin enhanced T cell Jak1 and STAT5 phosphorylation in a concentration-dependent manner (Fig. 3C and D). Dephostatin also promoted proliferation of CD4⁺ more than that of CD8⁺ T cells and the ratio of CD8⁺/4⁺ T cell number decreased in a concentration-dependent manner (Fig. 3E). We also examined the levels of CD45-associated protein and casein kinase 2 in each T cell subset, both reported to regulate CD45 PTPase activity [21,22], and found no differences between the CD4⁺ and CD8⁺ T cell populations (Fig. 3F and G). These results showed that pharmacological attenuation of CD45 PTPase activity, resulting in enhanced activation of IL-2 receptor signaling, might bring CD4⁺ T cells close to CD8⁺ T cells in terms of their proliferative response.

3.4. CD4⁺ and CD8⁺ T cells express distinct CD45 isoforms

As was stated earlier, one of the factors dictating CD45 PTPase activity is their isoform. To identify CD45 isoforms expressed by the activated CD4⁺ and CD8⁺ T cells, they were fractionated and examined with immunoblot analyses using Ab that recognize all CD45 isoforms. This method revealed that CD4⁺ and CD8⁺ T cells expressed CD45 molecules with distinct molecular weights: 180 kDa by CD4⁺ T cells and 210 kDa by CD8⁺ T cells (Fig. 4A). CD4⁺ T cells expressed another CD45 molecule to a lesser extent with a molecular weight of 190 kDa. Considering the contribution of each exon product in the total molecular weight of CD45, the major CD45 isoform expressed by CD4⁺ T cells is CD45RO, without any alternatively spliced exons, and the minor molecule has an additional exon. CD8⁺ T cells should express CD45 molecules with two additional exons. On analysis using exon product-specific mAbs, CD45 on proliferating CD4⁺ T cells contained products of exon B while those on proliferating CD8⁺ T cells contained products of exons B and C (Fig. 4B). Thus, proliferating CD4⁺ T cells express CD45RO and CD45RB while proliferating CD8⁺ T cells express CD45RBC.

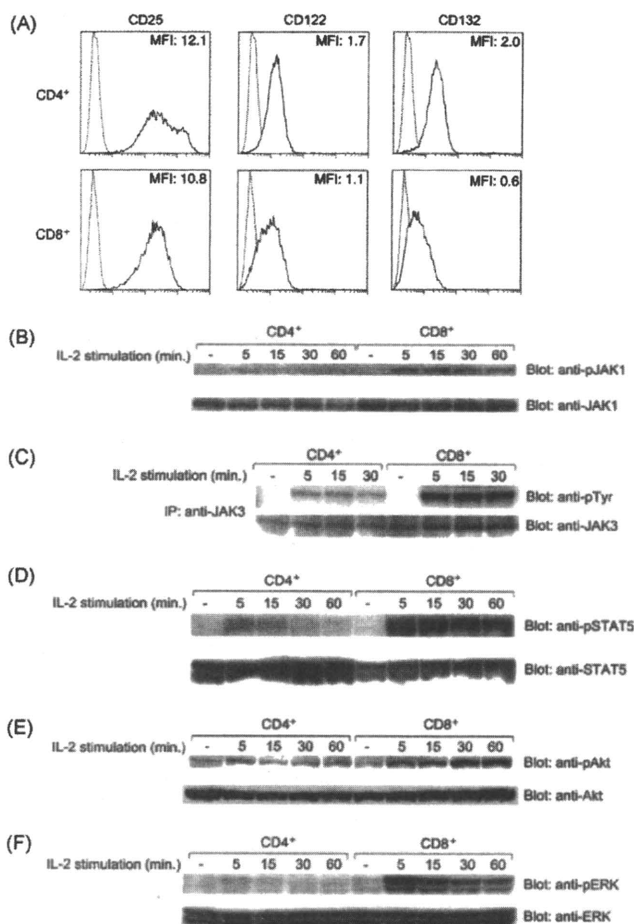

Fig. 2. IL-2 receptor complex expression is reduced but IL-2-triggered signaling is transduced more efficiently in CD8⁺ T cells than in CD4⁺ T cells. Splenic CD4⁺ and CD8⁺ T cells from C57BL/6J mice were stimulated with immobilized anti-CD3ε mAbs for 3 days and then cultured with IL-2 for 5 days. (A) Surface CD25, CD122, and CD132 molecules on CD4⁺ and CD8⁺ T cells were stained with specific mAbs (solid lines) or isotype control Abs (dotted lines), and analyzed by flow cytometry. The mean fluorescence intensity (MFI) of each sample is shown. (B–F) CD4⁺ and CD8⁺ T cells were stimulated and cultured as in (A). They were then stimulated with IL-2 and lysed at the indicated time points after initiation of IL-2 stimulation. pJak1 (B, the *upper panel*) and Jak1 (B, the *lower panel*) in the same lysates were immunodetected with specific Abs. Jak3 proteins were immunoprecipitated from the cell lysates with specific Ab and immunoblotted with anti-phosphotyrosine mAbs (C, the *upper panel*) and with Abs against Jak3 (C, the *lower panel*). pJak1, pSTAT5, pAkt, and pERK (D–F, the *upper panels*, respectively) and Jak1, STAT5, Akt, and ERK (D–F, the *lower panels*, respectively) were detected as in B. Immunoblots and flow cytometric analyses are representative of three independent experiments.

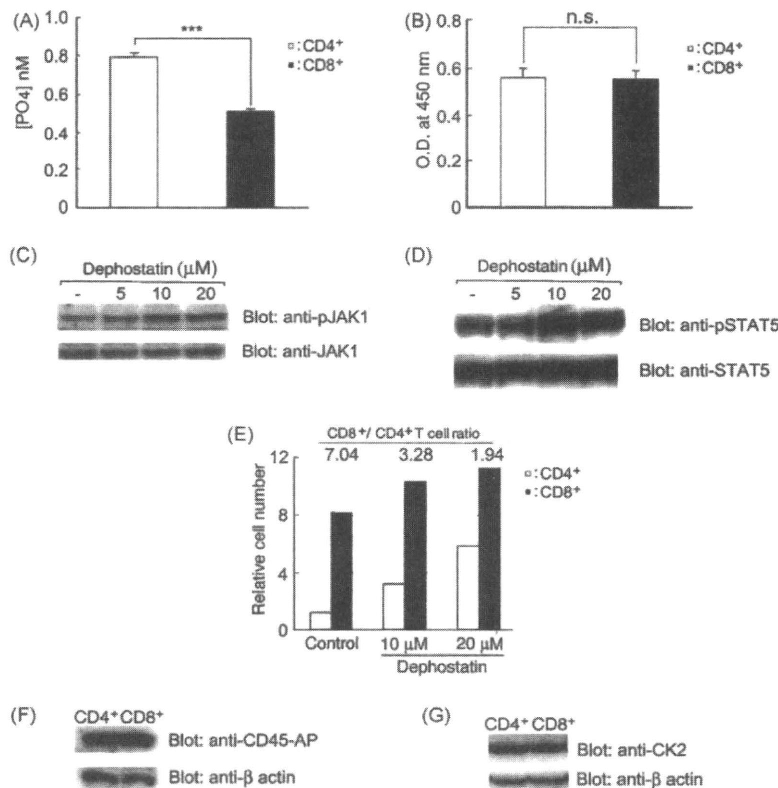

Fig. 3. CD8+ T cells express CD45 with lower tyrosine phosphatase activity than CD4+ T cells. Naive (CD62+CD44^{low}) CD4+ and CD8+ T cells were stimulated with immobilized anti-CD3ε mAbs for 3 days and then cultured with IL-2 for 5 days. (A) The same number of CD4+ (open column) and CD8+ (solid column) T cells were lysed and the CD45 PTPase activity derived from the two populations was quantified with phosphotyrosine peptides as CD45 PTPase substrates. The columns and bars represent the mean and SE of three samples. Data is representative of two independent experiments. ***$P<0.001$. (B) The amount of total CD45 protein in the lysates from (A) was determined. Data is representative of two independent experiments. (C and D) Splenic T cells from C57BL/6J mice were stimulated and cultured as in Fig. 2A. They were then treated with dephostatin at the indicated concentration followed by supplementation of IL-2. Cells were lysed 5 min after IL-2 stimulation. pJak1 (C, the *upper panel*), Jak1 (C, the *lower panel*), pSTAT5 (D, the *upper panels*) and STAT5 (D, the *lower panels*) in the same lysates were immunodetected with specific Abs. Immunoblot analyses are representative of two independent experiments. (E) Splenic T cells from C57BL/6J mice were activated with immobilized anti-CD3ε mAbs for 3 days and then cultured with IL-2 for 4 days with or without dephostatin. CD4+ (open column) and CD8+ T cell (solid column) numbers are relative to the number of CD4+ T cells without dephostatin treatment in the culture and CD8+/CD4+ T cell ratios are shown. The data is representative of two independent experiments. (F and G) Splenic T cells from C57BL/6J mice were stimulated and cultured as in Fig. 2A. CD4+ and CD8+ T cells were positively selected with anti-CD4 and CD8 conjugated magnetic microbeads for preparing cell lysates. CD45-associated protein (CD45-AP; F, the *upper panel*), casein kinase 2 (CK2; G, the *upper panel*), and β actin (F and G, the *lower panel*) were detected with specific Abs. The data are representative of two independent experiments.

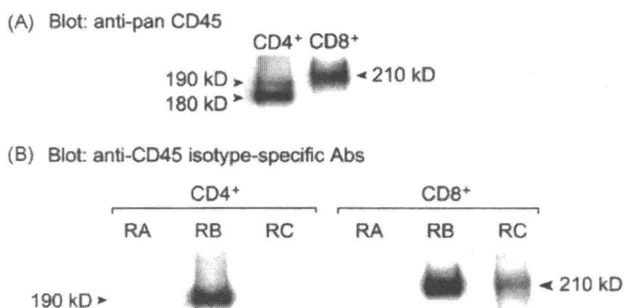

Fig. 4. Activated CD4+ and CD8+ T cells express distinct CD45 isoforms. (A) Naive CD62L+CD44^{low}CD4+ and CD8+ T cells were stimulated with immobilized anti-CD3ε mAbs for 3 days and then cultured with IL-2 for 5 days. CD45 molecules in SDS-fractionated cell lysates were detected with mAbs against pan-CD45. Molecular weights predicted by molecular mass markers (not shown) are indicated on the sides. (B) The same lysates were blotted for CD45RA, CD45RB, and CD45RC. Molecular weights predicted by molecular mass markers (not shown) are indicated on the sides. The data is representative of two independent experiments.

3.5. CD45 isoforms preferentially expressed by CD4+ and CD8+ T cells contribute to differential cell proliferation

To demonstrate that the dominance of different CD45 isoforms on activated CD4+ or CD8+ T cell populations leads to different Jak PTPase activity and cellular proliferation, the CD45RO and CD45RBC genes were introduced to cells that do not express intrinsic CD45. We therefore retrovirally transfected NIH3T3 fibroblastoid cells with CD45RO or CD45RBC. We used this method because (1) T cells do not develop normally in CD45 null mice [34,35], (2) it is hard to introduce genes into normal T cells or the CD45 negative BW5147(T200$^-$) T cell line, (3) a large amount of CD45 molecules are express on primary T cells, making it unreasonable to asses the effects of the CD45 transgenes, (4) NIH3T3 cells do not express CD45 (data not shown), and (5) their Jak1 molecules were phosphorylated constitutively in FBS-supplemented conventional culture medium (Fig. 5A). Although flow cytometric analysis with CD45 Ab showed that CD45RO and CD45RBC-transduced NIH3T3 cells expressed CD45 molecules at comparable levels (Fig. 5B), phosphorylation of Jak1 was especially reduced in the CD45RO-transduced cells compared with control retrovirus-infected cells (Fig. 5A). In concordance with the differential phosphorylation levels of Jak1,

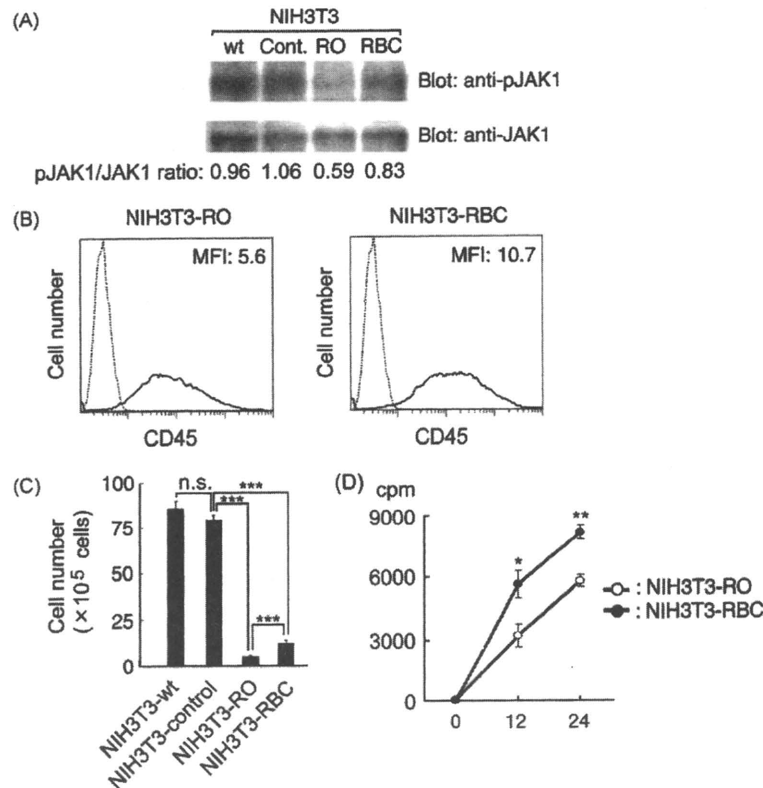

Fig. 5. CD45 has isoform-specific Jak phosphatase activity and inhibits NIH3T3 cell proliferation in a PTPase activity dependent manner. (A) pJak1 (the *upper panel*) and Jak1 (the *lower panel*) in cell lysates from CD45RO and CD45RBC-transduced cells (RO and RBC, respectively), control retrovirus-infected cells (cont.) and unmanipulated NIH3T3 cells (wt) were detected with specific Abs. The levels of pJak1 and Jak1 were quantified using NIH image (version 1.61) and the pJak1/Jak1 ratio was calculated. The data is representative of two independent experiments. (B) CD45 on the transfectants was stained with anti-CD45 mAbs (solid lines) or isotype control Abs (dotted lines), and analyzed by flow cytometry. The mean fluorescence intensity (MFI) of each sample is shown. The data are representative of two independent experiments. (C) Proliferation of unmanipulated NIH3T3 cells (NIH3T3-wt), control retrovirus-infected cells (NIH3T3-control), CD45RO and CD45RBC-transduced cells (NIH3T3-RO and NIH3T3-RBC, respectively) was assessed by cell counting. NIH3T3-wt, NIH3T3-control, NIH3T3-RO, and NIH3T3-RBC cells were plated at 5×10^5 cells per T-25 flask and cultured with 10% FBS containing DMEM for 5 days. The total cell numbers of each cell line at day 5 (5 days after culture) are shown. The columns and bars represent the mean and SE of six samples. Data is representative of two independent experiments. $^{***}P < 0.001$. (D) Proliferation of CD45RO and CD45RBC-transduced cells (NIH3T3-RO and NIH3T3-RBC, respectively) was assessed by a [^{3}H]thymidine incorporation assay. Assays were performed in triplicate and the data is shown as the mean and SE of three samples. Data is representative of two independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ (NIH3T3-RO vs NIH3T3-RBC).

NIH3T3 transfectants expressing CD45RO grew significantly slower than those expressing CD45RBC (Fig. 5C and D). These results show that the difference in CD45 isotype can dictate cellular proliferation through Jak dephosphorylation.

4. Discussion

$CD8^+$ T cells expanded more rapidly than $CD4^+$ T cells in response to IL-2 following anti-CD3ε mAb stimulation *in vitro*. During proliferation, Jak1, Jak3, and other molecules downstream of the IL-2 receptor were more activated in $CD8^+$ T cells than in $CD4^+$ T cells. Activated $CD4^+$ and $CD8^+$ T cells expressed distinct CD45 isoforms that differentially phosphorylated Jaks, leading to differential proliferative responses. Susceptibility of $CD4^+$ and $CD8^+$ T cells to proliferative stimuli appears to be at least partly regulated by CD45 isoform expression.

In contrast to $CD4^+$ T cells, $CD8^+$ T cells undergo extensive expansion following host infection with *Listeria* [1]. There are also many reports of the clonal expansion of $CD8^+$ T cells, not of $CD4^+$ T cells, in normal elderly humans [4,5]. To discern what controls the intrinsic difference in the proliferative responses of $CD4^+$ and $CD8^+$ T cells, we employed an *in vitro* system that mimicked the $CD8^+$ T cell-dominating *in vivo* T cell proliferation. Although it is yet to be determined to what extent the CD45 isoforms regulate IL-2 responses *in vivo*, this study provides insight into the dominant proliferative response of $CD8^+$ T cells.

It has been known that proliferation of $CD8^+$ T cells is preferentially driven by IL-7 and IL-15 [10,11]. However, these cytokines were not detected in the culture supernatants (data not shown). CIS, SOCS1, and SOCS3, negative regulators of cytokine signaling in $CD4^+$ and $CD8^+$ T cells [36–38], were equally expressed by both types of T cells expanded with anti-CD3ε mAbs and IL-2 (data not shown). Thus, the dominant proliferative response of $CD8^+$ T cells appears to be attributable solely to the lower Jak PTPase activity of their CD45.

CD45 molecules on human $CD4^+$ T cells have been used as a marker of naive and memory T cells since CD45 isoform expression shifts from CD45RA to CD45RO on stimulation with specific antigens *in vivo* [32]. Although several methods were employed in this study, immunoblot analysis with a panel of CD45 isotype-specific antibodies was the only tool to comprehensively examine cells for their entire CD45 isoform repertoire. This technique was able to detect CD45 isoforms expressed on T cells, although not enough protein could be extracted from cells before activation. We tried PCR to amplify CD45 cDNA derived from $CD4^+$ and $CD8^+$ T cells T cells using primers specific to the exons flanking the alternatively spliced exons. However, possibly because of mispriming of the primers, the amplified products failed to

discriminate any differences in the cDNA sequences (data not shown).

It has been shown that CD45 PTPase activity depends on its isoform [20]. Indeed, Bottomly et al. reported that the CD45RO isoform has higher PTPase activity than the CD45RABC and BC isoforms in T cells [26]. On the other hand, Xu and Weiss proposed that CD45 PTPase activity is regulated by homo-dimerization of CD45 molecules [20]. According to this, CD45RO, which is the least modified isoform, should have lower PTPase activity than CD45RBC. However, the dimer hypothesis is controversial and they did not measure CD45 PTPase activity generally or on Jak kinases. Thus, several factors may account for regulation of CD45 PTPase activity in some experimental settings. One of these molecules is CD45-associated protein [21], which was reported to up-regulate the CD45 PTPase activity. However, no difference in the protein level of CD45-associated protein was observed between $CD4^+$ and $CD8^+$ T cells (data not shown). Other studies suggested that the CD45 PTPase activity is augmented by phosphorylation of specific serine residues by casein kinase 2 [22]. We examined the activated $CD4^+$ and $CD8^+$ T cells for the protein level of casein kinase 2, and failed to see any difference.

We demonstrated here that CD45 on the $CD8^+$ T cells has less protein tyrosine PTPase activity than CD45 expressed on $CD4^+$ T cells, and also different CD45 isoforms dominate in the two populations. In addition, pharmacological inhibition of CD45 activity drove the proliferation of $CD4^+$ T cells close to that of $CD8^+$ T cells. While it was shown that dephostatin did not inhibit serine/threonine phosphatases and protein kinases [39], no inhibitory activity of dephostatin on PTPases other than CD45 has been reported. The involvement of other PTPases in IL-2R signaling of T cells has not been reported. Furthermore, we observed that SHP-1, which can dephosphorylate STAT [40], was equally expressed by $CD4^+$ and $CD8^+$ T cells (data not shown). These facts led us to believe that the events observed in Fig. 3E were because of the inhibitory effect of dephostatin on CD45 activity. However, since the possibility that dephostatin also suppresses other PTPases cannot be ruled out, the use of this pharmacological inhibitor alone is not enough to conclude that differential CD45 PTPase activity is responsible for the proliferative responses of $CD4^+$ and $CD8^+$ T cells. Therefore, we further tested this hypothesis using gene transfer techniques.

Because of the reasons described in Section 3, we introduced the CD45RO and CD45RBC genes into NIH3T3 cells and demonstrated the effects of CD45RO and CD45RBC on Jak1 phosphorylation and proliferation. Although we tried to detect PTPase activity of the NIH3T3 transfectants directly, it was not feasible because the transfectants did not grow rapidly enough to be examined by the dephosphorylation assay. We also tried to check the effects of FBS starvation on JAK1 phosphorylation in NIH3T3 cell to mimic the cytokine-inducible Jak1 activation. However, we could not see a down modulation in Jak1 phosphorylation without introducing cell death. Therefore, we could not clearly show the effects of the CD45 transgenes on cytokine-induced Jak1 phosphorylation and proliferation of the transfectants. However, these data strongly support our hypothesis that specific CD45 isoforms expressed on T cells correlates with their proliferative response to IL-2.

Since the Jak-STAT pathway plays a primary role in lymphocyte proliferation triggered by cytokine receptor signaling [41], pharmacological inhibition of Jaks, STATs, and other related molecules, including cytokines, should be effective in the treatment of autoimmune diseases [42]. In fact, some chemical compounds have been synthesized for this purpose [42,43]. However, these compounds can suppress both normal and abnormal immune responses and thus may induce excessive immune suppression. In contrast, since CD45 positively regulates antigen receptor signaling [17,18], modulating CD45 to up-regulate its PTPase activity should not suppress the initial T cell activation triggered by antigens but instead inhibit inappropriate expansion. Thus, it may offer a new tool to correct abnormal T cell homeostasis in autoimmune diseases.

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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).

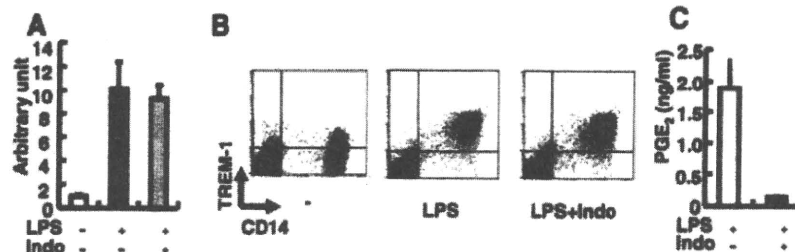

Figure 1. Lipopolysaccharide (LPS)–induced expression of triggering receptor expressed on myeloid cells 1 (TREM-1) on human peripheral blood mononuclear cells (PBMCs) in the presence or absence of a cyclooxygenase inhibitor (indomethacin; Indo). **A,** Human PBMCs were stimulated with LPS in the presence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

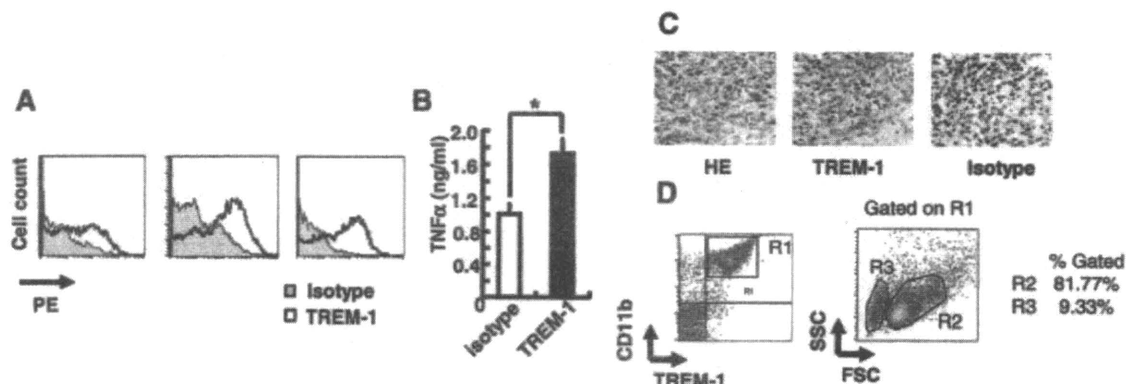

Figure 2. Triggering receptor expressed on myeloid cells 1 (TREM-1) expression in arthritis-affected joints. Synovial cells from rheumatoid synovial tissue of 3 patients with rheumatoid arthritis (RA) were isolated. **A,** Two-color flow cytometry was performed with fluorescein isothiocyanate–labeled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

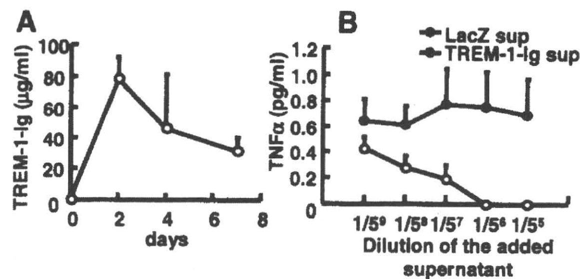

Figure 3. Adenoviral gene transfer of TREM-1 Ig. Mice were treated with intravenous injections of AxCALacZ or AxCATREM-1 Ig (10^9 plaque-forming units/mouse). **A,** Sera were examined at the indicated time points for TREM-1 Ig levels, using enzyme-linked immunosorbent assay (ELISA). Values are the mean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

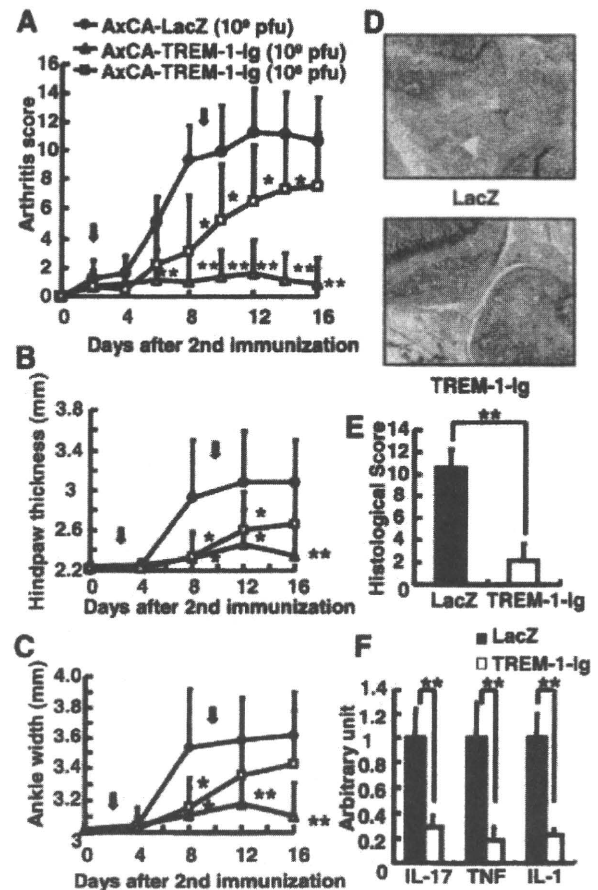

Figure 4. Attenuation of collagen-induced arthritis (CIA) by triggering receptor expressed on myeloid cells 1 (TREM-1) blockade. **A–C,** Mice with CIA were treated with intravenous injections of AxCALacZ (10^9 plaque-forming units [PFU]), AxCATREM-1 Ig (10^8 PFU), or AxCATREM-1 Ig (10^9 PFU) adenoviruses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

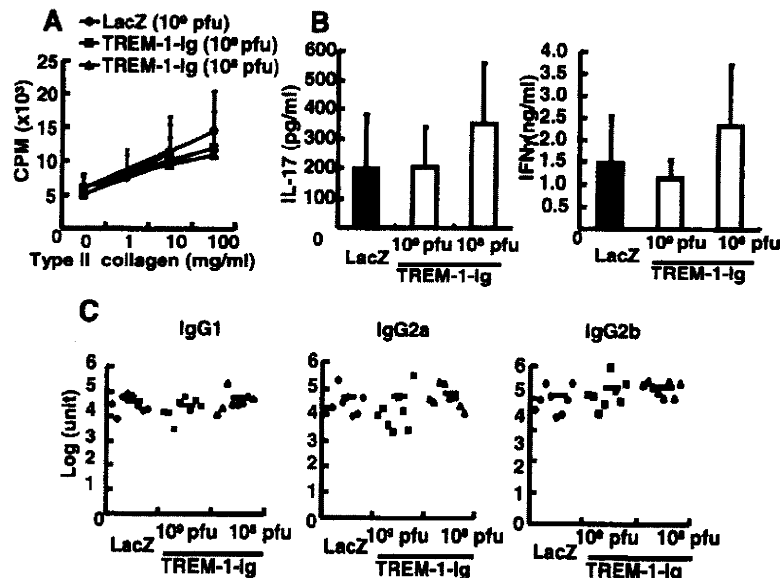

Figure 5. Effects of TREM-1 blockade on antigen-specific T cell and B cell responses. Splenocytes and sera were collected from mice with CIA that were treated with 2 different doses of AxCATREM-1 Ig (10^9 PFU and 10^8 PFU) and those treated with AxCALacZ (10^9 PFU), 16 days after the second immunization.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

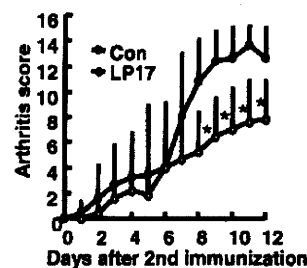

Figure 6. Treatment of CIA with antagonistic TREM-1 peptide. Mice with CIA were treated with intraperitoneal injections of an antagonistic TREM-1 peptide, LP17, or a sequence-scrambled control peptide (Con). Mice received 200-μg injections of these peptides every day, beginning on day 24, when arthritis 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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The Association of a Nonsynonymous Single-Nucleotide Polymorphism in *TNFAIP3* With Systemic Lupus Erythematosus and Rheumatoid Arthritis in the Japanese Population

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***Objective.* Genome-wide association (GWA) studies in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) in Caucasian populations have independently identified risk variants in and near the tumor necrosis factor α (TNFα)–induced protein 3 gene (*TNFAIP3*), which is crucial for the regulation of TNF-mediated signaling and Toll-like receptor signaling. The aim of this study was to assess the role of *TNFAIP3* in the development of SLE and RA in Japanese subjects.**

***Methods.* We selected 2 single-nucleotide polymorphisms (SNPs) from previous GWA studies. Rs2230926 is a nonsynonymous SNP in *TNFAIP3* and is associated with SLE, while rs10499194 is an intergenic SNP associated with RA. We then performed 2 independent sets of SLE case–control comparisons (717 patients and 1,362 control subjects) and 3 sets of RA case–control comparisons (3,446 patients and 2,344 control subjects) using Japanese subjects. We genotyped SNPs using TaqMan assays.**

***Results.* We observed a significant association between rs2230926 and an increased risk of SLE and RA in the Japanese population (for SLE, odds ratio [OR] 1.92, 95% confidence interval [95% CI] 1.53–2.41, $P = 1.9 \times 10^{-8}$; for RA, OR 1.35, 95% CI 1.18–1.56, $P = 2.6 \times 10^{-5}$). The intergenic SNP rs10499194 was also associated with SLE and RA, while the risk allele for RA in Caucasians was protective against the diseases in our population.**

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***Conclusion.* We demonstrated a significant association between the nonsynonymous variant in *TNFAIP3* and the risk for SLE and RA in the Japanese population. *TNFAIP3*, similar to *STAT4* and *IRF5*, may be a common genetic risk factor for SLE and RA that is shared between the Caucasian and Japanese populations.**

Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) represent multigenic diseases and are considered to be caused by interactions between susceptibility genes and environmental factors that result in an abnormal immune response. In fact, familial and linkage studies have provided strong evidence for the role of multiple genetic factors in the development of SLE and RA (1). In addition, association-based approaches in candidate loci using single-nucleotide polymorphisms (SNPs) have also identified several genes that contribute to these diseases. More recently, genome-wide association (GWA) studies in SLE and RA have revealed many susceptibility genes and pathways that contribute to disease development (2).

Familial and linkage studies have also shown familial aggregation of RA, SLE, and other immune-mediated diseases (1). In fact, several gene polymorphisms, including *PTPN22*, *STAT4*, and *IRF5* variants, have been shown to predispose to SLE and RA. Recent GWA studies in Caucasian populations have also identified the tumor necrosis factor α (TNFα)–induced protein 3 gene (*TNFAIP3*) as another common genetic risk factor for SLE and RA (3–6). *TNFAIP3*, also known as the A20 protein, is a negative regulator of the NF-κB signaling pathway that is essential in the pathogenesis of both SLE and RA (7). The association of *TNFAIP3* with diseases has been independently reported in SLE and RA, and it is of great interest that the peaks in association in the GWA studies are different between SLE and RA. In Caucasian populations, the significantly associated SNP markers for SLE, including the nonsynonymous SNP termed rs2230926, are located in the *TNFAIP3* region, while those for RA are located in the intergenic region between *TNFAIP3* and the oligodendrocyte transcription factor 3 gene (*OLIG3*). In addition to the difference in the diseases themselves, the association between *TNFAIP3* polymorphisms and these diseases in the Asian populations remains unclear (8).

In order to elucidate a genetic role for *TNFAIP3* in the development of SLE and RA in the Japanese population, we investigated 2 independent case–control cohorts of patients with SLE and 3 independent cohorts of patients with RA.

PATIENTS AND METHODS

Subjects. The subjects in the SLE study group comprised 2 cohorts of Japanese patients with SLE and unrelated control subjects. An SLE case–control cohort from the RIKEN (SLE cohort 1) consisted of 376 patients (mean age 43.2 years, 90.3% women) and 934 unrelated control subjects (mean age 52.6 years, 25.0% women). An SLE case–control cohort at Hokkaido University (SLE cohort 2) consisted of 341 patients (mean age 46.2 years, 88.3% women) and 428 unrelated control subjects (mean age 47.7 years, 28.7% female). All patients with SLE fulfilled the 1997 American College of Rheumatology (ACR) revised criteria for SLE (9).

The subjects in the RA component of the study comprised 3 cohorts of Japanese patients with RA and unrelated control subjects. The first cohort of patients with RA from BioBank Japan (RA cohort 1) consisted of 1,112 patients (mean age 60.5 years, 89.7% female, 69.7% positive for rheumatoid factor [RF]), and 934 unrelated control subjects. The second cohort from RIKEN (RA cohort 2) consisted of 830 patients (mean age 64.3 years, 83.7% women, 75.0% RF positive), and 658 unrelated control subjects (mean age 48.6 years, 57.4% women). The 934 unrelated control subjects in the first cohort of RA patients were the same as those used in SLE cohort 1. An RA case–control cohort from the Institute of Rheumatology Rheumatoid Arthritis (IORRA) cohort (RA cohort 3), which is a prospective observational cohort of patients with RA studied at Tokyo Women's Medical University, comprised 1,504 patients (mean age 59.3 years, 84% women, 88% RF positive), and 752 control subjects (mean age 38.4 years, 50% women). All patients with RA met the 1987 ACR (formerly, the American Rheumatism Association) revised criteria for a diagnosis of RA (10).

All subjects entered into this study were self-identified as Japanese and were recruited through several medical institutions located in Japan. DNA samples from the patients in the first cohort of RA patients in BioBank Japan were provided by the Leading Project for Personalized Medicine from the Ministry of Education, Culture, Sports, Science and Technology, Japan (11). All subjects provided informed consent prior to their participation in this study, and the study was preapproved by the ethics committee of each institution.

SNPs. For the selection of SNPs required to genotype in and near *TNFAIP3*, we reviewed previous GWA studies of SLE and RA (3–6). We then selected 2 SNPs, rs2230926 and rs10499194. SNP rs2230926 is a nonsynonymous variant in exon 3 of *TNFAIP3* and was strongly associated with SLE in the GWA study by Musone et al (5). Although the GWA study of SLE by Graham et al indicated that rs5029939, located in intron 2 of the gene, is most significantly associated with a predisposition to SLE (6), there is strong linkage disequilibrium (LD) (r^2 = 0.86) between these SNPs according to HapMap phase II data for Japanese and evidence that rs5029939 may be substituted by rs2230926 (Figure 1). Two previous GWA studies in RA revealed that rs10499194 and rs6920220, which are located between *TNFAIP3* and *OLIG3*, were significantly associated risk variants for RA (3,4). The HapMap data for Japanese individuals indicate that the minor allele frequency (MAF) of rs6920220 is 0.011, and that the MAF for control subjects in RA cohort 3 (IORRA) was <0.01. Results of a recent study in Korean populations also indicated