Successful Treatment of Animal Models of Rheumatoid Arthritis with Small-Molecule Cyclin-Dependent Kinase Inhibitors[1]

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Intraarticular gene transfer of cyclin-dependent kinase (CDK) inhibitors to suppress synovial cell cycling has shown efficacy in treating animal models of rheumatoid arthritis. Endogenous CDK inhibitors also modulate immune function via a CDK-independent pathway. Accordingly, systemic administration of small molecules that inhibit CDK may or may not ameliorate arthritis. To address this issue, alvocidib (flavopiridol), known to be tolerated clinically for treating cancers, and a newly synthesized CDK4/6-selective inhibitor were tested for antiarthritic effects. In vitro, they inhibited proliferation of human and mouse synovial fibroblasts without inducing apoptosis. In vivo, treatment of collagen-induced arthritis mice with alvocidib suppressed synovial hyperplasia and joint destruction, whereas serum concentrations of anti-collagen type II (CII) Abs and proliferative responses to CII were maintained. Treatment was effective even when therapeutically administered. Treated mice developed arthritis after termination of treatment. Thus, immune responses to CII were unimpaired. The same treatment ameliorated arthritis induced by K/BxN serum transfer to lymphocyte-deficient mice. Similarly, the CDK4/6-selective inhibitor suppressed collagen-induced arthritis. Both small-molecule CDK inhibitors were effective in treating animal models of rheumatoid arthritis not by suppressing lymphocyte function. Thus, the two small-molecule CDK inhibitors ameliorated arthritis models in a distinctive way, compared with other immunosuppressive drugs. *The Journal of Immunology*, **2008, 180: 1954–1961.**

Rheumatoid arthritis (RA)[3] is a chronic inflammatory disease characterized by synovial hyperplasia with massive infiltration of inflammatory cells in the affected joints. This hyperplasia leads to degeneration of cartilage, erosion of bone, and ultimately functional loss of joints. Although the etiology of RA remains elusive, T cells recognizing unknown autoantigens have been proposed to initiate inflammation in synovial tissues. This mechanism is followed by local recruitment of leukocytes, which are further activated in the inflamed sites (1). Immune complexes, compliment activation, and activation of lymphocytes, macrophages, neutrophils, mast cells, and synovial fibroblasts are part of the aberrant humoral and cellular network (2). Cytokines derived from these cells induce intense proliferation in synovial fibroblasts. Activated fibroblasts become another source of inflammatory cytokines and mediators including tissue-degrading proteinases and prostaglandins. In this manner, the hyperplastic synovial tissue, called a pannus, serves as a nidus for further spread of destructive inflammation, producing cartilage-degrading enzymes and invading the bone matrix of rheumatoid joints (3).

Therapeutic agents of a biologic nature, such as mAbs against TNF-α, soluble TNFR, anti-IL-6R Ab, and IL-1R antagonist, were recently brought into clinical use with the aim of interrupting inflammatory cytokine circuits in joints affected by RA. Although these agents often are more effective than conventional antirheumatic drugs, quite a few patients fail to obtain expected clinical benefit from these medications. Other new drugs such as abatacept, tacrolimus, and rituximab are given to suppress upstream targets such as T and B lymphocytes. These anti-inflammatory agents share similar limitations in clinical response (4). More seriously, these medications often render patients immunocompromised, making them susceptible to serious infections (4).

As mentioned earlier, a salient characteristic in rheumatoid pathology is overgrowth of synovial fibroblasts to form a hyperplastic pannus. This feature has led us to explore cell cycle regulation as a new therapeutic approach to treatment of RA. This antiproliferative treatment focuses on cyclin-dependent kinase (CDK) inhibitors (CDKI) as primary cell cycle regulators. These endogenous nucleoproteins inhibit CDKs that bind to cyclins to initiate catalytic activation (5). CDKs phosphorylate a retinoblastoma (RB) gene product, which results in release of E2F transcription factors for cell cycle progression (5). Based on structural and functional characteristics, CDKIs have been grouped into two distinct families, INK4 and Cip/Kip (5, 6). The INK4 proteins, consisting of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$, form binary complexes with CDK4 or CDK6, which otherwise would promote

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Received for publication November 19, 2007. Accepted for publication November 27, 2007.

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[1] This work is supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; smCDK, small-molecule CDK; CIA, collagen-induced arthritis; CII, type II collagen; FLS, fibroblast-like synoviocyte; MFLS, mouse fibroblast-like synoviocyte; RB, retinoblastoma; LN, lymph node.

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G_1/S phase transition in the cell cycle. In contrast, the Cip/Kip proteins, consisting of $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, bind to all cyclin-CDK complexes.

We have reported that $p16^{INK4a}$ and $p21^{Cip1}$ were induced readily in vitro in cultured RA fibroblast-like synoviocytes (FLSs). Intra-articular gene transfer of $p16^{INK4a}$ or $p21^{Cip1}$ ameliorated disease in animal models of RA, including collagen-induced arthritis (CIA) in mice and adjuvant arthritis in rats (7–9). These studies have indicated that induction of $p16^{INK4a}$ and $p21^{Cip1}$ expression in synovial tissue should hold promise as a new therapeutic strategy for RA treatment. Notably, because cell cycle regulation therapy suppresses a phase of rheumatoid pathology distinct from that suppressed by anti-inflammatory drugs, combination with another antirheumatic drug might act synergistically.

To apply gene transfer to treatment of patients with RA, vectors and protocols need be optimized to avoid unexpected tragic complications (10). An alternative to gene therapy as a way to inhibit CDK activity is the use of small-molecule (sm) compounds that inhibit CDK. Because uncontrolled CDK activity drives neoplastic cell cycling in some tumor cells, CDK activity represents an attractive target in development of anti-cancer therapeutics (11). In response to these smCDK inhibitors, some tumor cells stop proliferation and then also undergo apoptotic cell death (12–14). Thus, many smCDK inhibitors have been developed as candidate oncostatic drugs with a prototype being alvocidib (formerly called flavopiridol) (11). This synthetic flavone, the first CDKI to undergo clinical trials (11), inhibits the kinase activity of multiple CDKs such as CDK1, 2, 4, 6, and 7 (15). Structure-activity studies have shown that alvocidib interferes with binding of ATP to the adenine-binding pocket of CDK2 (16). However, although alvocidib generally proved safe in clinical trials, its efficacy was limited except for a subtype of malignant cells (11, 17). Other smCDK inhibitors, such as staurosporine, R-roscovitine, and BMS-387032, also have been studied in clinical trials, showing good tolerability (11).

The primary known role for CDKI is suppression of CDKs required for cell cycle progression. However, our group as well as other investigators have shown that $p16^{INK4a}$ and $p21^{Cip1}$ also have immunomodulatory effects (7, 18, 19). Notably, overexpression of $p21^{Cip1}$ in RA FLSs reduced production of inflammatory cytokines and tissue-degrading enzymes involved in rheumatoid pathology. This is at least partly a result of inhibition of JNK by $p21^{Cip1}$ (18). Thus, immunomodulation by CDKI, which potentially contribute to antiarthritic effects of CDKI gene transfer, would not necessarily depend on inhibition of CDK activity, raising a question as to whether inhibition of CDK per se can control arthritis. Of special interest here is inhibition of CDK4/6, which are shared targets of $p16^{INK4a}$ and $p21^{Cip1}$.

In the present study we evaluated how systemic administration of two smCDK inhibitors affects animal models of RA. One was alvocidib, while the other was a newly synthesized CDK4/6-selective inhibitor. Systemic administration of either compound inhibited arthritis without obvious immunosuppression or side effects. The results argue that alvocidib exerted its effects primarily by CDK4/6 inhibition.

Materials and Methods

Reagents

Alvocidib was provided by Aventis Pharmaceuticals. A stock solution of alvocidib prepared in DMSO was diluted to working concentrations before each experiment. In addition, a CDK4/6-selective inhibitor compound A (*N*-{5-[2-(cyclohexyloxy)-6-methylpyrimidin-4-yl]-1,3-thiazol-2-yl}-5-[(4-methylpiperazin-1-yl)methyl]pyrazin-2-amine), which was characterized as compound 4 in the previous report (20), was synthesized at Merck-Banyu. It was dissolved in DMSO for in vitro analyses and in 5% glucose containing 10 mM citrate buffer (pH 4) for treatment of mice. Human and mouse IL-1β and TNF-α were purchased from WAKO.

Cells

FLSs were prepared from synovial tissues of patients with RA who underwent total joint replacement surgery or synovectomy at Tokyo Medical and Dental University Hospital (Tokyo, Japan) or National Shimoshizu Hospital (Chiba, Japan). RA was diagnosed according to the criteria of the American College of Rheumatology (21). Written consent forms concerning experimental use of resected tissues were completed by patients before surgery. All procedures in the present studies were approved by the ethics committees of RIKEN and Tokyo Medical and Dental University. Mouse fibroblast-like synoviocytes (MFLSs) were prepared from synovial tissues from knee joints of CIA mice as previously described (22). Although FLSs were used from 7 to 11 passages, MFLSs were used from 6 to 11 passages. Both were cultured in DMEM (Sigma-Aldrich) supplemented with L-glutamine, penicillin, streptomycin, and 10% FBS (Sigma-Aldrich). Purity of the fibroblasts was assessed with flow cytometry (23). CD14 or HLA class II were not expressed by FLSs, suggesting that macrophages and dendritic cells were not present in FLSs.

Proliferation assays

From 2000 to 5000 cells/well were grown overnight in 96-well plates and stimulated with 10 ng/ml IL-1β and 10 ng/ml TNF-α. These cytokine concentrations were determined to be optimal in preliminary experiments. Alvocidib (1–300 nM), compound A (0.01–10 μM), or DMSO alone was placed in wells in the presence of 10% FBS. After 24–48 h, BrdU was added and culture was continued for 20 h. Then incorporation of BrdU was quantified by ELISA with a BrdU cell proliferation ELISA kit (Exalpha Biologicals).

Cell cycle analysis

Cells were stimulated for 24 h in the presence or absence of smCDK inhibitors, washed with PBS, and resuspended in 0.15% Triton X-100/PBS before staining with propidium iodide (50 μg/ml) and examination for DNA content with a FACSCaliber flow cytometer (BD Biosciences).

Animal models of arthritis

CIA was induced in 7-wk-old male DBA/1 mice purchased from Japan Charles River Breeding Laboratories. Each mouse was immunized with 200 μg of bovine type II collagen (CII; Collagen Research Center) emulsified with CFA (Difco) by injection at the tail base. Immunization was repeated 21 days after primary immunization.

Serum-transfer arthritis was induced by transfer of serum from arthritic K/BxN mice, which spontaneously develop arthritis resembling RA. These mice were provided by Drs. C. Benoist and D. Mathis (Joslin Diabetes Center, Boston, MA) (24). Preliminary titration experiments showed that injection of 300 μl of serum into the peritoneal cavity transferred arthritis consistently in RAG2-null mutant ($RAG2^{-/-}$) and C57BL/6 wild-type mice. All procedures met institutional regulations for animal experiments.

Assessment of arthritis

Arthritis in each limb of arthritic mice was assessed clinically by visual scoring from 0 to 4: 0, no swelling; 1, detectable swelling in one joint; 2, non-severe swelling in two or more joints; 3, severe swelling in two or more joints; and 4, severe swelling in two or more joints including digital swelling. Maximal score for an individual animal was 16. Joint swelling was quantified by measuring hindpaw thickness and the ankle width within a micrometer (Ozaki Manufacturing). Arthritis scores were analyzed statistically with unpaired Student's *t* test.

In histological examination, hindpaws were obtained and fixed in 10% buffered formalin, decalcified in 10% EDTA, and embedded in paraffin. Sections (4 μm) were stained with H&E for histologic examination. Infiltration of inflammatory cells, transformation of synovial lining, cartilage destruction, and pannus formation were independently scored in a blind manner from 0 to 3 as previously described (25). Maximum histological score was 12. Histological scores were analyzed statistically with unpaired Student's *t* test. Radiographs of formalin-fixed hindpaws were obtained with a cabinet soft x-ray apparatus (CMB-2; Softex).

Measurement of serum levels of anti-CII Abs

Serum samples were collected at indicated time points and examined for anti-CII Ab concentrations by ELISA. Briefly, wells of 96-well plates were

coated overnight at 4°C with bovine CII in PBS, washed with PBS-0.05% Tween 20, and blocked with 2% BSA in PBS. Samples were incubated for 2 h at room temperature. After washing, HRP-conjugated rabbit anti-mouse IgG1, IgG2a, or IgG2b Ab (Zymed Laboratories) was added. Reaction product formed with 3,3',5,5'-tetramethylbenzidine was measured as OD at 450 nm. A standard mixture serum from arthritic mice was placed on each plate in serial dilution. Arbitrary units for anti-CII IgG1, IgG2a, and IgG2b Abs were determined with this standard curve.

Western blot analysis

Whole-cell lysates (15 μg of protein) were separated by SDS-PAGE on 7.5% polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Anti-phospho RB (Ser^{807}/Ser^{811}) Ab (catalog no. 9308; Cell Signaling Technology) at 1/1000 dilution and anti-RB mAb (clone G3-245; BD Pharmingen) at 1 μg/ml were used as primary Abs. Bound Abs were visualized with peroxidase-conjugated secondary Ab (Amersham Biosciences) at 1/3000 dilution and ECL system (ECL; Amersham Biosciences).

Immunohistochemistry

Paraffin-fixed tissue sections were deparaffinized and incubated with anti-phospho RB (Ser^{807}/Ser^{811}) Ab. They were then incubated with biotinylated anti-rabbit IgG Ab and with ABC reagent (VectaStain Elite ABC kit; Vector Laboratories). Color was developed with diaminobenzidine (Kirkegaard & Perry Laboratories), whereas the sections were counterstained with hematoxylin. Diaminobenzidine-positive cells and stained nuclei were counted by observers in a blind manner at three independent sites per slide to calculate percentage of nuclei with phosphorylated RB gene product. The data were analyzed statistically with unpaired Student's *t* test.

Lymph node (LN) cell responses to CII

Single-cell suspensions derived from inguinal LNs (4×10^5 cells per well in a 96-well plate) were stimulated with denatured CII (0–100 μg/ml) in RPMI 1640 medium (Sigma-Aldrich) supplemented with L-glutamine, penicillin, streptomycin, 2-ME, and 10% FBS (Sigma-Aldrich). After 72 h, BrdU was added and culture was continued for 17 h to quantify incorporated BrdU.

Results

Alvocidib suppresses proliferation of synovial fibroblasts

FLSs from the inflamed joints of rheumatoid patients were isolated and cultured in the presence of alvocidib, a pan-CDKI. MFLSs from arthritic joints of CIA mice were isolated and treated in the same way. When alvocidib was present in culture medium, growth of FLS and MFLS was inhibited in a concentration-dependent manner (Fig. 1*A*). No cell death was visually apparent at concentrations below 300 nM in FLSs and 150 nM in MFLSs. We next analyzed the cell cycle in these fibroblasts after treatment with alvocidib. In either human or mouse cells, alvocidib increased cell populations at the G_0/G_1 phase of cell cycle without increasing the sub-G_1 population (Fig. 1*B*). Although alvocidib has been reported to induce apoptotic death in some cell types (12–14), this result was not seen in synovial fibroblasts.

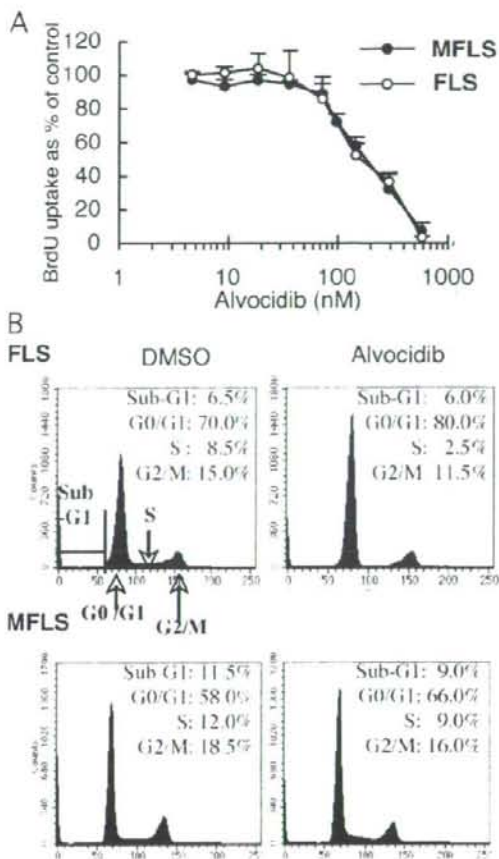

FIGURE 1. Alvocidib suppresses proliferation of synovial fibroblasts. *A*, Proliferation was assessed by BrdU incorporation. FLSs or MFLSs were stimulated with TNF-α and IL-1β for 24 h, and cultured for 24 h with indicated concentrations of alvocidib in the presence of TNF-α and IL-1β. The growth inhibitory effect as alvocidib concentrations increase is shown as declining BrdU incorporation relative to incorporation into cells incubated without alvocidib. DMSO, which was used to solubilize alvocidib, had no effects on incorporation at concentrations used in these experiments. Mean percentage is derived from three wells with error bars representing SD. Data are representative of three independent experiments. *B*, Cellular DNA content was assessed cytometrically as a means of cell cycle analysis. FLSs and MFLSs prestimulated as in *A* were cultured for 18 h with either 100 nM alvocidib or DMSO as a control before flow cytometry. Histograms of DNA content and percentage of population at each phase of the cell cycle are presented. Data shown are representative of three independent experiments. Two different cell lines of FLSs and MFLSs were tested for *A* and *B*. One line was tested twice in two independent experiments. The two lines responded quite similarly; calculated IC_{50} of alvocidib were 130 and 90 nM in one FLS sample and 75 nM in the other sample. Cells in the G_0/G_1 phase of cell cycle increased to 81 and 80% in one FLS sample and 72% in the other sample.

Alvocidib treatment suppresses arthritis in CIA mice

Next, mice with experimentally induced arthritis were treated with alvocidib. In the first series of experiments, the drug was given i.p. to DBA/1 mice with CIA. Treatment was started on day 25 after the first immunization, when joint swelling became evident according to examination by our protocol. Repeat injection of 1 or 2.5 mg/kg of alvocidib for 10 consecutive days significantly suppressed severity of arthritis in a dose-dependent manner (Fig. 2*A*). Therapeutic effect was seen even when the treatment was started 3 days later, showing that alvocidib suppressed ongoing disease (Fig. 2*B*). A similar effect was observed when alvocidib was given only twice weekly, starting at about the time of clinical disease onset (day 24). The effect persisted for as long as 5 wk (Fig. 2*C*).

Histologic analysis of synovial tissues from DMSO-treated control mice showed hyperplastic pannus tissues massively infiltrated by mononuclear cells, cartilage destruction, and bone erosion, which are all characteristic of the pathology of RA disease. These features were suppressed in synovial tissues from alvocidib-treated mice. Drug effects were dose-dependent; joints from most mice treated with 2.5 mg/kg of alvocidib appeared normal (Fig. 2,

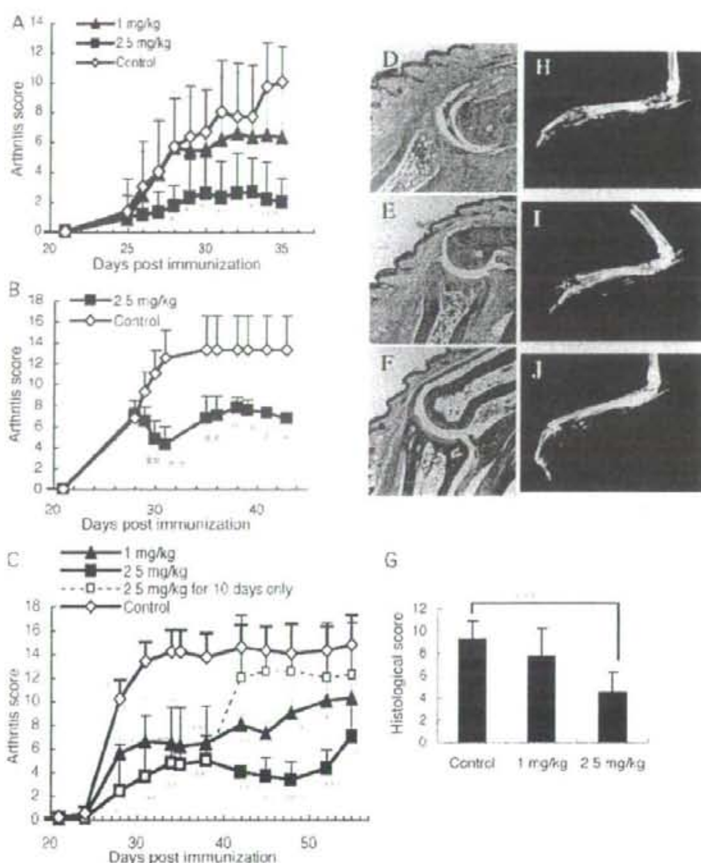

FIGURE 2. Alvocidib treatment suppresses arthritis in CIA mice. Mice with CIA were treated by i.p. injection of alvocidib at 1 mg/kg, or 2.5 mg/kg in saline containing 0.01% DMSO, or injection of only DMSO in saline, for 10 consecutive days (*A* and *B*) or twice weekly for 5 wk (*C*). Treatment was started 25 (*A*), 28 (*B*), or 24 (*C*) days after the initial immunization. Severity of arthritis was rated with an arthritis score. In other mice with CIA, twice-weekly treatment with 2.5 mg/kg of alvocidib was terminated after 10 days (*C*) (□). Mean score ± SD of seven mice per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *D–F*, H&E staining of joints. Original magnification, ×100. *G*, Histological score of the arthritis. Results are the mean ± SD from 10 paws. ***, $p < 0.001$. *H–J*, Specimen radiographs. Paws were examined at completion of the treatment for 10 consecutive days with DMSO in saline (control) (*D* and *H*) or 1 mg/kg alvocidib (*E* and *I*) and 2.5 mg/kg (*F* and *J*). Representative staining and radiograph are shown.

D–G). These results agreed well with specimen radiographic findings in the feet. DMSO-treated control mice displayed severe bone destruction, most characteristically as cystic changes and ankylosis of the articular bones. These changes were inhibited dose-dependently by alvocidib (Fig. 2, *H–J*).

Immune responses to CII are not impaired by alvocidib treatment

CIA is triggered by host immune responses to CII. Ab response is of central importance because B cell-deficient mice do not develop the disease, whereas transfer of mAbs against CII can induce full-blown arthritis (26). Immune responses require activation and proliferation of lymphocytes. Although peripheral lymphocyte counts were not affected by the dose of alvocidib used in the present study (data not shown), lymphocyte activation might be blocked by alvocidib. To assess immune suppression by alvocidib treatment, we determined serum concentrations of anti-CII Abs in the treated mice. When anti-CII-specific Abs of IgG1, IgG2a, and IgG2b subclasses were quantified separately with a specific ELISA, alvocidib-treated mice had serum IgG concentrations comparable to

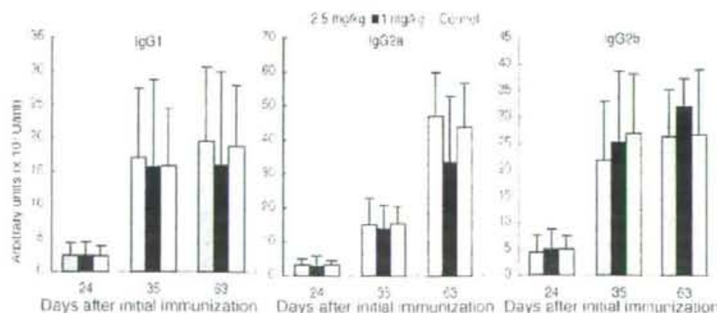

FIGURE 3. CII-specific IgG responses are maintained during alvocidib treatment. Serum concentrations of anti-CII Abs with IgG1, IgG2a, and IgG2b subclasses were determined by specific ELISA. Sera were collected 24, 35, and 63 days after the primary CII immunization in a series of experiments shown in Fig. 2C. Data are in arbitrary units for mean ± SD of 7 mice per group).

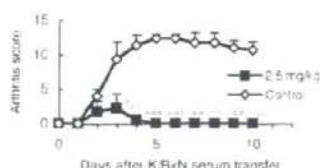

FIGURE 4. Alvocidib treatment suppresses arthritis in RAG2$^{-/-}$ mice induced by K/BxN serum transfer. Arthritis was induced in RAG2$^{-/-}$ mice by transfer of serum from arthritic K/BxN mice. Beginning on the day after serum transfer, mice were treated with 2.5 mg/kg of alvocidib for 10 consecutive days. Control mice were treated with saline containing DMSO. Severity of arthritis was assessed by an arthritis score. Mean score ± SD is shown (n = 5 mice per group). *, p < 0.05; **, p < 0.01; ***, p < 0.001

those in control mice (Fig. 3). No difference was seen in relation to dose or time during the experiment. We then studied effect of terminating alvocidib treatment, following the clinical disease. In a group of CIA mice given 2.5 mg/kg of alvocidib twice weekly, treatment was terminated after 10 days. Arthritis became evident ~8 days later, and then progressed rapidly until the disease score approached that in untreated mice (Fig. 2C). The results showed that the underlying immune reaction against CII was not impaired during alvocidib treatment.

Alvocidib shows efficacy in treating a lymphocyte-independent arthritis

To substantiate that alvocidib can inhibit arthritis without suppressing lymphocyte function, we investigated its effect on a K/BxN serum-induced arthritis model induced in lymphocyte-deficient RAG2$^{-/-}$ mice. K/BxN mice spontaneously develop arthritis similar to RA in several aspects (24). Transfer of serum from these arthritic mice induces arthritis depending upon the genetic background of recipient mice (27). Pathology in the joints is characterized by acute edema in synovial tissues followed by proliferative and erosive arthritis that can be induced in lymphocyte-deficient mice (28). Making use of this model, we induced arthritis in RAG2$^{-/-}$ mice and began i.p. administration of 2.5 mg/kg of alvocidib on the day after serum transfer, continuing for 10 days. Treatment reduced edematous swelling of the joins in the early phase, and then abrogated proliferative arthritis (Fig. 4). Thus, suppression of immune responses is not required for the antiarthritic effect of alvocidib.

A newly synthesized CDK4/6-selective inhibitor suppresses synovial fibroblasts growth and CIA

Alvocidib was tolerated well in clinical trials except for a few occurrences of diarrhea when administered as a prolonged infusion; its toxicity profile was dose- and schedule-dependent (11). This agent has broad activity, inhibiting all CDKs and some other kinases (29). To study further whether cell cycle inhibition by CDK4/6 inhibition had a important role in ameliorating arthritis, a selective inhibitor, compound A, was synthesized (20). It is an aminothiazole CDKI that is structurally unrelated to alvocidib (Fig. 5A). It selectively inhibited CDK4 and CDK6, both of which have been shown to have indistinguishable activities in vitro (20). The IC_{50} for CDK4 and CDK6 was 9.2 and 7.8 nM, respectively, which were at least 100-fold less than concentrations for other CDKs.

As alvocidib did, compound A inhibited growth of FLSs and MFLSs in a concentration-dependent manner (Fig. 5B) and induced cell cycle arrest at the G_1 phase (Fig. 5C). It suppressed RB

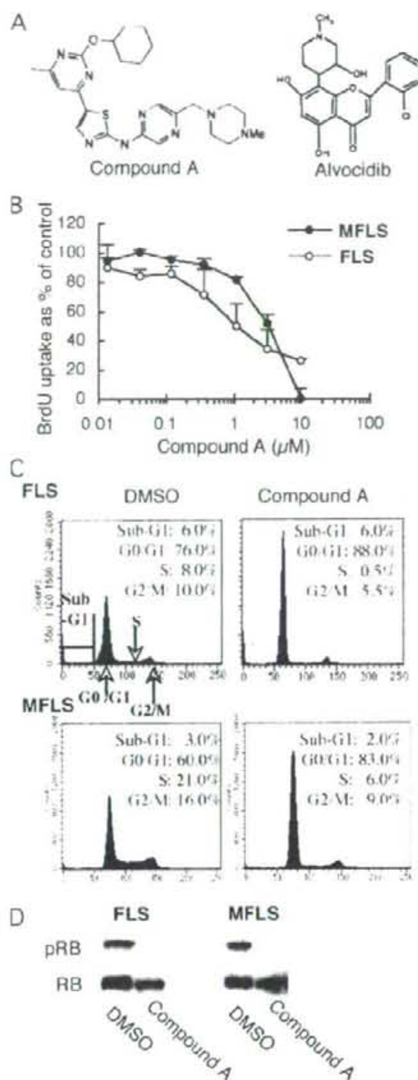

FIGURE 5. Compound A suppresses proliferation of synovial fibroblasts. *A*, Chemical structure of compound A and alvocidib. *B*, Proliferation was assessed as in Fig. 1. FLSs or MFLSs were stimulated with TNF-α and IL-1β for 24 h, and then cultured for 24 h with indicated concentrations of compound A in the presence of TNF-α and IL-1β. Shown are mean percentage ± SD of BrdU incorporation relative to uptake by control cells, derived from three wells. Data are representative of three independent experiments. *C*, DNA content of FLSs and MFLSs were analyzed as in Fig. 1. Instead of alvocidib, compound A (2.5 μM) was used. Histograms of DNA content and the percentage of population at each phase of the cell cycle are indicated. Data shown are representative of three independent experiments. Two different cell lines of FLSs and MFLSs were tested for *B* and *C* as was in Fig. 1. Again, they responded similarly; calculated IC_{50} of compound A were 1.0 and 0.6 μM in one FLSs sample and 1.8 μM in the other sample. Cells in the G_0/G_1 phase increased to 88 and 87% in one FLSs sample and 85% in the other sample. *D*, RB gene product phosphorylation of FLSs and MFLSs treated as in *C* were analyzed by Western blot using specific Ab to phospho-RB (pRB) or RB. The results are representatives of two samples.

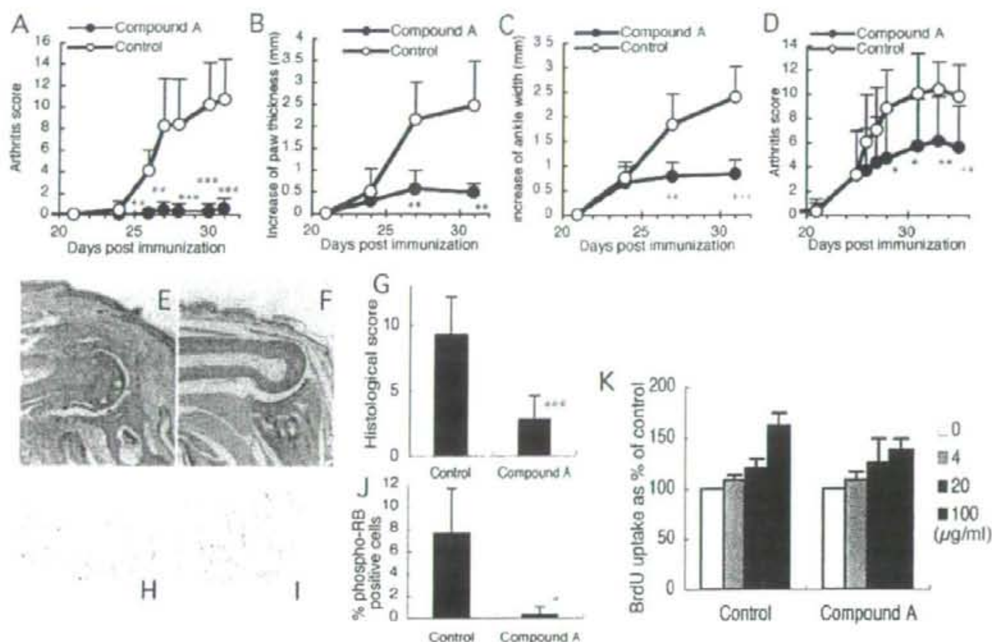

FIGURE 6. Treatment with compound A suppresses CIA in mice. *A–C*, Mice with CIA were treated with compound A orally (200 mg/kg) every 12 h for 7 days. Treatment was started when arthritis became evident, 24 days after the initial immunization. *D*, Other mice with CIA were treated i.p. with compound A (30 mg/kg for 5 days, repeated after a 1-day hiatus). Data are representative of two independent experiments. Control mice were treated with buffer alone. Severity of arthritis was assessed by an arthritis score (*A* and *D*), paw thickness (*B*), and ankle width (*C*). Data are the mean ± SD (n = 8 per group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *E* and *F*, H&E staining of joints at original magnification {times}100. Paws were examined at completion of the treatment with buffer alone (*E*) or compound A (*F*). Representative staining is shown. *G*, Histological abnormality of the mice treated as in *D* was scored. Results are mean + SD from 12 paws. ***, $p < 0.001$. *H* and *I*, Immunohistological staining of joints. Original magnification at ×200. The paw samples in *E* and *F* were stained with phospho-RB Ab. Representative staining is shown. *J*, Phospho-RB positive cells were semiquantified as percentage of positive nuclei in total nuclei. Data are mean ± SD from 5 paws. *, $p < 0.05$. *K*, LN cells from the mice treated as in *D* were isolated at completion of the treatment. Their proliferative responses to different concentrations of CII were assessed by BrdU incorporation. Data were displayed as BrdU incorporation relative to that in cells cultured without CII. Data are mean + SD from three mice.

phosphorylation at Ser^{807}/Ser^{811}, which are among residues specifically targeted by CDK4/6 in FLS and MFLS (Fig. 5*D*). Titration experiments showed that 0.25 μM compound A, which was below IC_{50} for other CDKs, started inhibiting the RB phosphorylation and also increasing cells at the G_0/G_1 phase; 83.0% in FLS and 72.0% in MFLS.

Compound A (200 mg/kg) was given orally twice daily for 7 days, beginning 24 days after the initial CII immunization. This treatment reduced arthritis score, ankle and paw swelling of the CIA mice (Fig. 6, *A–C*). The dose could be reduced to 30 mg/kg once daily i.p. injection without loss of therapeutic effects (Fig. 6*D*). This treatment was started after the onset of clinical arthritis. Five-day treatments repeated after a day hiatus suppressed ongoing arthritis. Histological analysis of the synovial tissues revealed that compound A, as well as alvocidib, suppressed characteristic features of pathology in the arthritis (Fig. 6, *E–G*). When nuclei with phosphorylated RB gene product in the synovial tissues were stained, their number was decreased in the compound A-treated mice (Fig. 6, *H* and *I*). Their ratio in the total nuclei was lower in the treated mice (Fig. 6*J*), showing that CDK4/6 was inhibited in vivo.

We found no abnormality by physical or behavioral observation in association with administration of compound A. At completion of the therapeutic experiment, inguinal LNs cells were isolated and stimulated with CII. No difference in proliferative response was observed between the compound A-treated and the nontreated mice (Fig. 6*G*). The preservation of T cell response was also observed in the alvocidib-treated mice (data not shown). Thus, inhibition of the lymphocytes did not play an important role in the therapeutic effects of the smCDK inhibitors.

Discussion

We have demonstrated that systemic administration of the two CDKIs exerted an antiarthritic effect without critical impairment of lymphocyte responses. Such an inhibitor proved effective in treating a lymphocyte-independent arthritis. Although alvocidib is known as a pan-CDKI, CDK4/6-selective inhibitor showed a comparable effect. This finding suggested that inhibition of CDK4/6 played an important role in the antiarthritic activity. It was reported that a CDK1/2/5 inhibitor, R-roscovitine, suppressed a passively induced arthritis model by promoting apoptosis of inflammatory cells (30). Alvocidib induces apoptotic cell death in various tumor cells (13, 14, 31, 32), likely by inhibition of other kinases than CDK (29). In this regards, TUNEL of the joint tissues treated with the two inhibitors did not show increase of apoptotic synovial cells (data not shown). This suggested that local induction of apoptosis in the joints was not responsible for the effect. Although we could not formally exclude effects of these agents on lymphocytes, the results argue that effects on nonlymphoid cells including synovial

fibroblasts and innate immune cells should account for the therapeutic activity.

A reported immunomodulatory action of endogenous CDKI does not depend upon CDK inhibition. This immune modulation could be at least partly responsible for therapeutic effects seen in CDKI gene therapy (18, 19). However, the present study demonstrated that inhibition of CDK activity alone is sufficient to ameliorate arthritis.

A major concern with systemic delivery of smCDK inhibitors is possible inhibition of normal cell turnover, which is essential for maintenance of organ homeostasis. Reassuringly, alvocidib, the prototype CDKI, was tolerated relatively well in clinical trials involving cancer (33). Diarrhea, the most frequent side effect in these trials, was not observed in any mice during our experiments. We suspect that proliferating cells involved in the arthritis were more sensitive than tumor cells because 2.5 mg/kg or less of alvocidib sufficed to treat arthritis, whereas at least 5 mg/kg was required to treat tumors (34, 35). Although alvocidib was injected i.v. for tumor treatment, oral administration of compound A was effective in treating arthritis. Although toxic effects might not be tolerated by patients without malignancies, route and dosage could be reconsidered in RA patients being treated in clinical trials. RA might respond to much lower doses than do tumors as is true in treatment with methotrexate.

Compound A is a more selective inhibitor acting primarily upon CDK4 and CDK6. Like alvocidib, it induced cell cycle arrest of synovial fibroblasts at the G_1 phase. The cell cycle arrest was accompanied by inhibition of RB phosphorylation. In selectivity testing of compound A against other serine/threonine or tyrosine kinases beyond those of the CDK family, only 6 of 45 kinases were inhibited >75% by compound A at 1 μM. No kinase among these six showed a IC_{50} comparable to those of CDK4 and CDK6. Partly because of structural irrelevance, alvocidib and compound A do not share the same kinases as off-target kinases. Accordingly, inhibition of cell cycling by CDK4/6 inhibition seemed important in suppressing arthritis. It has been proposed that MAPKs are potential therapeutic targets in RA (36). When their inhibitors showed efficacy, they suppressed Ab responses (37, 38). This was in contrast to the treatment with the CDKIs.

We assume that CDKIs might represent a new class of antirheumatic drugs. Based on the results of clinical trials of alvocidib as well as those of the present experiments, patients with RA easily should tolerate an antiarthritic dose of alvocidib because alvocidib seems to exert cytotoxic adverse effects primarily via inhibition of other kinases at antineoplastic doses (29). Alvocidib exhibited higher protein binding in human serum than in FBS, which led to development of an effective administration schedule (17). The optimal dose should be carefully determined for actual use in treating arthritic patients. In contrast, compound A needs to be modified to improve its pharmacokinetics. It was cleared quite rapidly in vivo, necessitating us using a relatively high dose as its present form.

The antiarthritic effect of these drugs did not require obvious suppression of the lymphocyte responses. Most of presently available antirheumatic treatments aim to inhibit harmful immune reactions. Combined use of conventional medication and smCDK inhibitors may well have a synergistic antiarthritic effect. As was revealed by our recent report and others (30, 39, 40), CDKI may have broader activity than anticipated. We found recently that both alvocidib and compound A suppressed matrix metalloproteinase-3 production by synovial fibroblasts and osteoclastogenesis of macrophages (data not shown). These effects may contribute to protection from joint damage.

The results of the present studies should encourage further development, clinical testing, and use of the smCDK inhibitors to treat human RA.

Acknowledgments

Institut de Génétique et de Biologie Moléculaire et Cellulaire in France provided KRN transgenic mice as our source of K/BxN mouse serum. We thank Dr. K. Goto (National Shimoshizu Hospital, Chiba, Japan) for providing patient samples and Dr. H. Iwai for radiographic examination in animal experiments.

Disclosures

The authors have no financial conflict of interest.

References

1. Bresnihan, B. 1999. Pathogenesis of joint damage in rheumatoid arthritis. *J. Rheumatol.* 26: 717–719.
2. Raza, K., C. E. Buckley, M. Salmon, and C. D. Buckley. 2006. Treating very early rheumatoid arthritis. *Best Pract. Res. Clin. Rheumatol.* 20: 849–863.
3. Arend, W. P. 2001. Physiology of cytokine pathways in rheumatoid arthritis. *Arthritis Rheum.* 45: 101–106.
4. Goldblatt, F., and D. A. Isenberg. 2005. New therapies for rheumatoid arthritis. *Clin. Exp. Immunol.* 140: 195–204.
5. Sherr, C. J., and J. M. Roberts. 1999. CDK inhibitors: positive and negative regulators of G_1-phase progression. *Genes Dev.* 13: 1501–1512.
6. Sherr, C. J., and J. M. Roberts. 1995. Inhibitors of mammalian G_1 cyclin-dependent kinases. *Genes Dev.* 9: 1149–1163.
7. Nasu, K., H. Kohsaka, Y. Nonomura, Y. Terada, H. Ito, K. Hirokawa, and N. Miyasaka. 2000. Adenoviral transfer of cyclin-dependent kinase inhibitor genes suppresses collagen-induced arthritis in mice. *J. Immunol.* 165: 7246–7252.
8. Nonomura, Y., H. Kohsaka, K. Nasu, Y. Terada, M. Ikeda, and N. Miyasaka. 2001. Suppression of arthritis by forced expression of cyclin-dependent kinase inhibitor $p21^{Cip1}$ gene into the joints. *Int. Immunol.* 13: 723–731.
9. Taniguchi, K., H. Kohsaka, N. Inoue, Y. Terada, H. Ito, K. Hirokawa, and N. Miyasaka. 1999. Induction of the $p16^{INK4a}$ senescence gene as a new therapeutic strategy for the treatment of rheumatoid arthritis. *Nat. Med.* 5: 760–767.
10. Verma, I. M., and M. D. Weitzman. 2005. Gene therapy: twenty-first century medicine. *Annu. Rev. Biochem.* 74: 711–738.
11. Senderowicz, A. M. 2003. Small-molecule cyclin-dependent kinase modulators. *Oncogene* 22: 6609–6620.
12. Li, Y., M. Bhuiyan, S. Alhasan, A. M. Senderowicz, and F. H. Sarkar. 2000. Induction of apoptosis and inhibition of c-erbB-2 in breast cancer cells by flavopiridol. *Clin. Cancer Res.* 6: 223–229.
13. Parker, B. W., G. Kaur, W. Nieves-Neira, M. Taimi, G. Kohlhagen, T. Shimizu, M. D. Losiewicz, Y. Pommier, E. A. Sausville, and A. M. Senderowicz. 1998. Early induction of apoptosis in hematopoietic cell lines after exposure to flavopiridol. *Blood* 91: 458–465.
14. Schrump, D. S., W. Matthews, G. A. Chen, A. Mixon, and N. K. Altorki. 1998. Flavopiridol mediates cell cycle arrest and apoptosis in esophageal cancer cells. *Clin. Cancer Res.* 4: 2885–2890.
15. Carlson, B. A., M. M. Dubay, E. A. Sausville, L. Brizuela, and P. J. Worland. 1996. Flavopiridol induces G_1 arrest with inhibition of cyclin-dependent kinase (CDK) 2 and CDK4 in human breast carcinoma cells. *Cancer Res.* 56: 2973–2978.
16. De Azevedo, W. F., Jr., H. J. Mueller-Dieckmann, U. Schulze-Gahmen, P. J. Worland, E. Sausville, and S. H. Kim. 1996. Structural basis for specificity and potency of a flavonoid inhibitor of human CDK2, a cell cycle kinase. *Proc. Natl. Acad. Sci. USA* 93: 2735–2740.
17. Byrd, J. C., T. S. Lin, J. T. Dalton, D. Wu, M. A. Phelps, B. Fischer, M. Moran, K. A. Blum, B. Rovin, M. Brooker-McEldowney, et al. 2007. Flavopiridol administered using a pharmacologically derived schedule is associated with marked clinical efficacy in refractory, genetically high-risk chronic lymphocytic leukemia. *Blood* 109: 399–404.
18. Nonomura, Y., H. Kohsaka, K. Nagasaka, and N. Miyasaka. 2003. Gene transfer of a cell cycle modulator exerts anti-inflammatory effects in the treatment of arthritis. *J. Immunol.* 171: 4913–4919.
19. Perlman, H., K. Bradley, H. Liu, S. Cole, E. Shamiyeh, R. C. Smith, K. Walsh, S. Fiore, A. E. Koch, G. S. Firestein, G. K. Haines, III, and R. M. Pope. 2003. IL-6 and matrix metalloproteinase-1 are regulated by the cyclin-dependent kinase inhibitor p21 in synovial fibroblasts. *J. Immunol.* 170: 838–845.
20. Shimamura, T., J. Shibata, H. Kurihara, T. Mita, S. Otsuki, T. Sagara, H. Hirai, and Y. Iwasawa. 2006. Identification of potent 5-pyrimidinyl-2-aminothiazole CDK4, 6 inhibitors with significant selectivity over CDK1, 2, 5, 7, and 9. *Bioorg. Med. Chem. Lett.* 16: 3751–3754.
21. Arnett, F. C., S. M. Edworthy, D. A. Bloch, D. J. McShane, J. F. Fries, N. S. Cooper, L. A. Healey, S. R. Kaplan, M. H. Liang, H. S. Luthra, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 31: 315–324.
22. Abad, C., C. Martinez, J. Leceta, R. P. Gomariz, and M. Delgado. 2001. Pituitary adenylate cyclase-activating polypeptide inhibits collagen-induced arthritis: an experimental immunomodulatory therapy. *J. Immunol.* 167: 3182–3189.

23. Nanki, T., K. Nagasaka, K. Hayashida, Y. Saita, and N. Miyasaka. 2001. Chemokines regulate IL-6 and IL-8 production by fibroblast-like synoviocytes from patients with rheumatoid arthritis. *J. Immunol.* 167: 5381–5385.
24. Kouskoff, V., A. S. Korganow, V. Duchatelle, C. Degott, C. Benoist, and D. Mathis. 1996. Organ-specific disease provoked by systemic autoimmunity. *Cell* 87: 811–822.
25. Schramm, C., J. Kriegsmann, M. Protschka, S. Huber, T. Hansen, E. Schmitt, P. R. Galle, and M. Blessing. 2004. Susceptibility to collagen-induced arthritis is modulated by TGFβ responsiveness of T cells. *Arthritis Res. Ther.* 6: R114–R119.
26. Holmdahl, R., R. Bockermann, J. Backlund, and H. Yamada. 2002. The molecular pathogenesis of collagen-induced arthritis in mice–a model for rheumatoid arthritis. *Ageing Res. Rev.* 1: 135–147.
27. Ji, H., D. Gauguier, K. Ohmura, A. Gonzalez, V. Duchatelle, P. Danoy, H. J. Garchon, C. Degott, M. Lathrop, C. Benoist, and D. Mathis. 2001. Genetic influences on the end-stage effector phase of arthritis. *J. Exp. Med.* 194: 321–330.
28. Korganow, A. S., H. Ji, S. Mangialaio, V. Duchatelle, R. Pelanda, T. Martin, C. Degott, H. Kikutani, K. Rajewsky, J. L. Pasquali, et al. 1999. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. *Immunity* 10: 451–461.
29. Sedlacek, H. H. 2001. Mechanisms of action of flavopiridol. *Crit. Rev. Oncol. Hematol.* 38: 139–170.
30. Rossi, A. G., D. A. Sawatzky, A. Walker, C. Ward, T. A. Sheldrake, N. A. Riley, A. Caldicott, M. Martinez-Losa, T. R. Walker, R. Duffin, et al. 2006. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. *Nat. Med.* 12: 1056–1064.
31. Bible, K. C., and S. H. Kaufmann. 1997. Cytotoxic synergy between flavopiridol (NSC 649890, L86–8275) and various antineoplastic agents: the importance of sequence of administration. *Cancer Res.* 57: 3375–3380.
32. Li, Y., S. R. Chinni, A. M. Senderowicz, and F. H. Sarkar. 2000. Induction of growth inhibition and apoptosis in prostate cancer cells by flavopiridol. *Int. J. Oncol.* 17: 755–759.
33. Senderowicz, A. M., D. Headlee, S. F. Stinson, R. M. Lush, N. Kalil, L. Villalba, K. Hill, S. M. Steinberg, W. D. Figg, A. Tompkins, et al. 1998. Phase I trial of continuous infusion flavopiridol, a novel cyclin-dependent kinase inhibitor, in patients with refractory neoplasms. *J. Clin. Oncol.* 16: 2986–2999.
34. Arguello, F., M. Alexander, J. A. Sterry, G. Tudor, E. M. Smith, N. T. Kalavar, J. F. Greene, Jr., W. Koss, C. D. Morgan, S. F. Stinson, et al. 1998. Flavopiridol induces apoptosis of normal lymphoid cells, causes immunosuppression, and has potent antitumor activity in vivo against human leukemia and lymphoma xenografts. *Blood* 91: 2482–2490.
35. Patel, V., A. M. Senderowicz, D. Pinto, Jr., T. Igishi, M. Raffeld, L. Quintanilla-Martinez, J. F. Ensley, E. A. Sausville, and J. S. Gutkind. 1998. Flavopiridol, a novel cyclin-dependent kinase inhibitor, suppresses the growth of head and neck squamous cell carcinomas by inducing apoptosis. *J. Clin. Invest.* 102: 1674–1681.
36. Sweeney, S. E., and G. S. Firestein. 2006. Mitogen activated protein kinase inhibitors: where are we now and where are we going? *Ann. Rheum. Dis.* 65(Suppl. 3): iii83–iii88.
37. Badger, A. M., J. N. Bradbeer, B. Votta, J. C. Lee, J. L. Adams, and D. E. Griswold. 1996. Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. *J. Pharmacol. Exp. Ther.* 279: 1453–1461.
38. Ohori, M., M. Takeuchi, R. Maruki, H. Nakajima, and H. Miyake. 2007. FR180204, a novel and selective inhibitor of extracellular signal-regulated kinase, ameliorates collagen-induced arthritis in mice. *Naunyn Schmiedebergs Arch. Pharmacol.* 374: 311–316.
39. Bradley, K., J. C. Scatizzi, S. Fiore, E. Shamiyeh, A. E. Koch, G. S. Firestein, L. J. Gorges, K. Kuntzman, R. M. Pope, T. L. Moore, J. Han, and H. Perlman. 2004. Retinoblastoma suppression of matrix metalloproteinase 1, but not interleukin-6, through a p38-dependent pathway in rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum.* 50: 78–87.
40. Nonomura, Y., K. Nagasaka, H. Hagiyama, C. Sekine, T. Nanki, M. Tamamori-Adachi, N. Miyasaka, and H. Kohsaka. 2006. Direct modulation of rheumatoid inflammatory mediator expression in retinoblastoma protein-dependent and -independent pathways by cyclin-dependent kinase 4/6. *Arthritis Rheum.* 54: 2074–2083.

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Ann Rheum Dis 2008;67;1153-1158; originally published online 30 Oct 2007;
doi:10.1136/ard.2007.072751

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Efficient management of rheumatoid arthritis significantly reduces long-term functional disability

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Accepted 13 October 2007
Published Online First 30 October 2007

ABSTRACT

Objectives: The aim of this study was to examine the effect of efficient management of rheumatoid arthritis (RA) in relation to disability levels in a large cohort of patients with RA over a period of 3 years.

Methods: We studied 2775 patients with RA who had continuous enrolment for at least 3 years from 7511 patients with RA enrolled in an observational cohort study (Institute of Rheumatology, Rheumatoid Arthritis (IORRA)) from October 2000 to April 2005. The 28-joint Disease Activity Scores (DAS28) were calculated at 6 month intervals for all the patients and a value <2.6 was considered as a tight control. We have set up a new variable for each patient, "Avg-Dscore", based on the transition of each patient's DAS28 value, taking the threshold level of 2.6 into consideration. The "Avg-DAS28" is the average of DAS28 values over all the phases. Functional disability status was assessed by J-HAQ, the validated Japanese version of the Health Assessment Questionnaire (HAQ). The relationship of "Avg-Dscore" and "Avg-DAS28" with the functional disability level was determined using Spearman correlation coefficients and multiple linear regression models.

Results: The baseline features of these 2775 patients were: female 83.7%, mean age 56.8 years, mean RA duration 9.5 years, mean initial DAS28 4.0, mean initial J-HAQ score 0.79, and mean final J-HAQ score 0.86. There was a statistically significant correlation between "Avg-DAS28" and final J-HAQ score ($r = 0.57$, $p<0.001$), indicating that tighter disease control has significant association with lower disability levels. A similar relationship was observed between "Avg-Dscore" and final J-HAQ score ($r = 0.47$, $p<0.001$). Multiple linear regression analysis, after adjusting for all the covariates, revealed that "Avg-Dscore" and "Avg-DAS28" were the most significant factors contributing to final J-HAQ score, and confirmed the strong relationship between disease activity and functional disability.

Conclusions: In patients with RA efficient disease management, by maintaining the DAS28 values at a level under 2.6, has significant association with improving functional capability. The threshold DAS28 level of 2.6 may be useful in developing targeted treatment guidelines for patients with RA.

The management of disease in patients with rheumatoid arthritis (RA) has dramatically altered in the last decade. The introduction of biologics has successfully modified the disease course of patients with RA with active disease that had not previously been controlled by conventional disease-modifying antirheumatic drugs (DMARDs).[1,2] Furthermore, the entire strategy for the treatment of RA has also been altered. Treatment with DMARDs should be aggressively initiated at an early stage of disease to improve prognosis,[3-5] and some large trials showed extensive suppression of disease activity led patients to a better functional capacity.[6,7] Although RA was regarded as a relentlessly progressive disease only 20 years ago, "remission" rather than "low disease activity" is achievable and a realistic therapeutic goal these days based on the development of RA treatment.

The ultimate goals in the treatment of RA generally are no disease activity, and no radiographic joint damage, and normalisation of functional disability. The Disease Activity Score (DAS),[8] which consists of the Ritchie score, swollen joint count, erythrocyte sedimentation rate (ESR) and the patient global assessment score, and its modified version, the 28-joint DAS (DAS28),[9] are commonly used for the assessment of RA disease activity in many clinical trials, while Health Assessment Questionnaire (HAQ)[10] is currently the most used, reliable instrument for assessing functional disability in patients with RA. Several studies have reported on the relationship between functional disability, disease activity, and radiographic assessment in RA.[11-27] Functional disability in patients with RA is associated with multiple variables such as disease activity, joint destruction, disease duration, age, gender, psychological state, educational level and comorbidities.[11-27] Among these variables, disease activity is thought to be the most important factor in the loss of functional capacity, especially in the earlier phase of RA disease course.[11-13]

Since maintenance of good functional capacity is the fundamental goal of RA treatment, "remission" is now an important concept and the accepted goal of management in RA. Despite this fact, RA suffers from the absence of a single "gold standard" quantitative measure, such as serum blood sugar and HbA1c levels in diabetes or blood pressure.[28] Although the definition of "remission" has been controversial and various types of definition of "remission" have been proposed,[29-31] DAS28 remission at a cut-off level of 2.6 is often used in clinical practice and clinical trials.[32-34] However, association between a threshold DAS28 level and long-term functional disability has not been well studied. In addition, it is not well known whether tight control of RA disease activity leads to prevention of long-term disability in clinical practice.

The IORRA (Institute of Rheumatology, Rheumatoid Arthritis) study is a prospective observational cohort study established at the Institute of Rheumatology, Tokyo Women's

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Medical University, Tokyo, Japan, since October, 2000.[38] For this cohort, physician assessments, patient assessments and laboratory data were supplemented with various types of patient information including disability index, disease activity, comorbidity, and medications used, collected from patient questionnaire sheets from approximately 5000 patients with RA every 6 months. More than 99% of patients with RA in our institute were enrolled, and more than 98% of patients answered and mailed their questionnaire back to us every time. This cohort database is a powerful resource not only for clinical research, but also for extensively evaluating therapeutic strategies under real-life conditions.[39-42]

In this study, we clarify the importance of suppression of disease activity in the prevention of long-term patient disability using a single-institute based large observational cohort in Japan.

PATIENTS AND METHODS

Patients

Among 7511 patients with RA (female 81.7%, mean age 55.8 years, mean RA duration 8.5 years) enrolled in an observational cohort study at our institute (IORRA) from October 2000 to April 2005, 2775 patients with RA (36.9%), who had continuous enrolment for at least 3 years from October 2000 to April 2005, were selected as the cohort of patients with RA for this study. All patients were of Japanese origin and were examined by rheumatologists, and had been diagnosed with RA according to the 1987 classification criteria by the American College of Rheumatology (ACR, formerly, the American Rheumatism Association).[43] DAS28 values (based on the tender joint counts, swollen joint counts, patient-oriented visual analogue scales (VAS) for general health, and erythrocyte sedimentation rate (ESR)) and the J-HAQ (Japanese Health Assessment Questionnaire, the validated Japanese version of HAQ[44]) were collected from all the patients at 6-month intervals (each phase). Functional disability status was assessed by J-HAQ.

"Avg-Dscore" and "Avg-DAS28"

DAS28 values were calculated at each phase (once every 6 months) for all patients and a value less than 2.6 was considered as a tight control. We set up two new variables for each patient, "Avg-Dscore" and "Avg-DAS28" (average of DAS28 values over all phases), based on the transition of each patient's DAS28 value. The Avg-Dscore was computed taking the threshold DAS28 level of 2.6 into consideration. The Avg-Dscore had a minimum score of 0 and a maximum score of 1; the DAS28 value of each patient at each phase (once every 6 months), was scored either as 0 if the DAS28 value was less than or equal to 2.6, or as 1 if it was greater than 2.6. The average of these scores over all the phases for each patient was calculated, and this gave the Avg-Dscore. The lower the Avg-Dscore, the higher the number of phases, in which the DAS28 value was under 2.6.

The Avg-DAS28 was the average of original DAS28 values, over all phases, computed for each patient. A larger Avg-DAS28 indicates a higher disease activity.

Statistical analysis

SAS software (V9.1; SAS Institute Inc., Cary, North Carolina, USA) was used for database management and statistical analysis. The relationship of "Avg-Dscore" and "Avg-DAS28" with the functional disability level ("Final J-HAQ score" and "Change in J-HAQ score" (final J-HAQ–initial J-HAQ)) was examined using Spearman correlation coefficients. Multiple linear regression analysis was carried out to investigate the relationship of DAS28 and functional disability. In models 1–6, the "Final J-HAQ score" was used as dependent variable, and in models 7–12 the "Change in J-HAQ score" was the dependent variable. The independent variable used in models 1, 2, 3, 7, 8, 9 was "Avg-Dscore" and in models 4, 5, 6, 10, 11, 12 it was "Avg-DAS28". Models 2, 5, 8 and 11 were adjusted for baseline J-HAQ, and models 3, 6, 9 and 12 were adjusted for baseline DAS. All the models were also adjusted for age, gender, RA disease duration, time of enrolment and seasonal effect. Since we found a definite seasonal fluctuation in disease activities of patients with RA, which tend to show improvement from Spring to Autumn while worsening from Autumn to Spring in our observational cohort study,[44] we adjusted for seasonal effect when performing multiple linear regression analysis. Considerable changes in RA therapy occurred during the 2000–2003 time period, so all the models were adjusted for time of enrolment. Differences in DAS28 and J-HAQ data between first phase and last phase observations were tested by Student paired t test or Wilcoxon signed-rank test where appropriate.

Based on the "Avg-DAS28", 2775 patients with RA in this cohort were divided into following three groups: "Under 2.6", "Between 2.6 and 3.2" and "Above 3.2". The significance of differences in DAS28 and J-HAQ between first and last phase observations were assessed individually in each of the above groups using Student paired t test and Wilcoxon signed-rank test as appropriate.

RESULTS

Baseline clinical features of 2775 patients with RA

The baseline characteristics of the 2775 patients with RA in this study sample are shown in table 1. Overall disease activities in these 2775 patients with RA were relatively low, as reflected in the mean tender joint count, swollen joint count, C-reactive protein (CRP) titre and ESR. The percentages of DMARD and steroid users during the follow-up period (average follow-up period 3.6 years, minimum 3 years, and maximum 5 years) in 2775 patients with RA were 97.3% and 65.5%, respectively. The mean DAS28 decreased from 4.0 at study entry to 3.7 at study exit ($p<0.001$ using paired t test), while median J-HAQ scores were 0.63 and 0.63 respectively. The means of "Avg-Dscore" and "Avg-DAS28", over all the patients, were 0.81 and 3.75, respectively.

We have also examined the baseline features of 4736 patients with RA who were not included in this study due to their lack of continuous follow-up for 3 years. The baseline features of these excluded patients with RA, such as age, RA duration, RA disease activity (mean tender joint count, swollen joint count, CRP titre and ESR, initial DAS28) and J-HAQ scores were quite similar to those of 2775 patients included in this study cohort (data not shown).

Relationship of RA disease activity with the functional disability by Spearman correlation coefficients

The relationship of disease activity ("Avg-Dscore" and "Avg-DAS28") with the functional disability level ("Final J-HAQ score" or "Change in J-HAQ score") using Spearman correlation coefficients is shown in table 2. There was a statistically significant correlation between "Avg-DAS28" and "Final J-HAQ score" ($r = 0.57$, $p<0.001$). A similar relationship was seen

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Table 1 Baseline features of 2775 patients enrolled continuously for at least 3 years in the IORRA cohort with rheumatoid arthritis (RA)

Demographic	Value
Number of patients	2775
Age, years*	56.8 (12.0) (58.0)
% Female	83.7
RA duration, years*	9.5 (8.4) (7.0)
Positivity for rheumatoid factor, %*	79.9
Swollen joints/tender joint counts per 67/68 joints*	3.6 (2.0)/4.2 (2.0)
Physician VAS score, mm*	25.0 (19.8) (20.0)
Patient general VAS score, mm*	35.3 (24.1) (33.0)
Patient pain VAS score, mm*	34.1 (25.5) (29.0)
CRP, mg/dl*	1.5 (2.0) (0.7)
ESR, mm/hr*	38.5 (24.7) (33.4)
DMARD use, %†	97.3
MTX use, %†	68.9
Dosage of MTX per week, mg†	6.3
Steroid use, %†	65.5
Dosage of steroid per day, mg†	2.8
Follow-up period, years	3.6 (0.8)
Initial DAS28 score*	4.0 (1.2) (4.0)
Final DAS28 score‡	3.7 (1.3) (3.7)
Avg-DAS28§	3.75 (1.00) (3.7)
Initial J-HAQ score*	0.79 (0.72) (0.63)
Final J-HAQ score‡	0.86 (0.79) (0.63)
Change in J-HAQ score•	0.06 (0.53) (0.0)
Avg-Dscore§	0.81 (0.28) (1.0)

Data shown is mean (SD) (median), unless otherwise stated.
*Observation in the first phase of study. †Drug usage during the follow-up period. ‡Observation in the last phase of study. §For explanation of Avg-DAS28 and Avg-Dscore, see Patients and methods section. • Change in J-HAQ score = final J-HAQ score–initial J-HAQ score.
CRP, C-reactive protein; DAS28, 28-joint Disease Activity Score; DMARD, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; IORRA, Institute of Rheumatology, Rheumatoid Arthritis; J-HAQ, the Japanese Health Assessment Questionnaire; MTX, methotrexate; NA, not applicable; VAS, visual analogue scale.

between "Avg-Dscore" and "Final J-HAQ score" ($r = 0.47$, $p<0.001$).

Relationship of RA disease activity with long-term functional disability by multiple linear regression analysis

The analysis was performed using six multiple linear regression models to examine the relationship of RA disease activity to "Final J-HAQ score" (table 3). For prediction of "Final J-HAQ score", after adjusting for all the covariates it was apparent that that "Avg-Dscore" and "Avg-DAS28" were the most significant factors contributing to "Final J-HAQ score", indicating a positive correlation.

For prediction of "Change in J-HAQ score", multiple linear regression analysis after adjusting for all the covariates also revealed that "Avg-Dscore" and "Avg-DAS28" were the factors most significantly contributing to "Change in J-HAQ score", indicating a positive correlation (table 4).

Relationship of RA disease activity with long-term functional disability individually in the three groups that were classified based on "Avg-Das28"

Of the 2775 patients with RA, the number of patients in the "Under 2.6", "Between 2.6 and 3.2" and "Above 3.2" groups were 346, 461 and 1968 respectively. The DAS28 values were significantly decreased during the follow-up period in all the three groups. The J-HAQ scores were significantly decreased only in the "Under 2.6" group. The difference in J-HAQ scores was not significant in "Between 2.6 and 3.2" group. However, the J-HAQ scores were significantly increased in the "Above 3.2" group.

DISCUSSION

Using a prospective observational large cohort in Japan, we clearly demonstrated that a tight control of RA disease activity has significant association with lower functional disability level after an average 3.6 years of follow-up when a DAS28 value of <2.6 was considered as a tight control.

In the cohort in this study, (table 2), there was a statistically significant correlation between "Avg-DAS28" or "Avg-Dscore" and "Final J-HAQ score", indicating a significant relationship between tighter disease control and lower disability levels. Combe *et al* found that 3- and 5- year HAQ disability could be predicted mainly by baseline HAQ score in 191 patients with early RA.[20] Other previous studies focusing on the predictive factors of HAQ disability have also identified baseline HAQ score as the best prognostic factor.[16–18 21 23] Hence, we also made multiple linear regression models (models 2, 5, 8 and 11) that were adjusted for age, gender, RA disease duration, time of enrolment, seasonal effect, and baseline J-HAQ score as an explanatory variable (tables 3 and 4). These multiple linear regression models also confirmed the strong relationship between "Avg-DAS28" or "Avg-Dscore" and "Final J-HAQ score" or "Change in J-HAQ score" after adjusting for all covariates including baseline J-HAQ score and baseline DAS28 (tables 3 and 4), indicating managing disease activity efficiently by maintaining DAS28 score values under 2.6 has significant association with reduced long-term disability.

Some prospective studies reported the relationship between functional capacity and disease activity of RA. Drossaers-Bakker *et al* found that functional capacity was strongly influenced by disease activity throughout the course of RA in 132 female patients with early RA over 12 years of cohort follow-up.[11] By contrast, Welsing *et al* reported that the effect of disease activity and joint destruction on functional capacity changed over the course of the disease in 378 patients with early RA with 9 years of cohort follow-up.[12] They concluded that the loss of

Table 2 Relationship of RA disease activity with the functional disability by Spearman correlation coefficient

	Final J-HAQ score	Change in J-HAQ score†	Avg-Dscore‡	Avg-DAS28‡
Final J-HAQ score	1.00	0.45*	0.47*	0.57*
Change in J-HAQ score†	0.45*	1.00	0.17*	0.21*
Avg-Dscore‡	0.47*	0.17*	1.00	0.83*
Avg-DAS28‡	0.57*	0.21*	0.83*	1.00

*p<0.001. †Change in J-HAQ score = final J-HAQ score – initial J-HAQ score. ‡For explanation of Avg-DAS28 and Avg-Dscore, see Patients and methods section.
DAS28, 28-joint Disease Activity Score; J-HAQ, Japanese Health Assessment Questionnaire RA, rheumatoid arthritis.

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Table 3 Relationship of rheumatoid arthritis (RA) disease activity to long-term disability using multiple linear regression analysis: prediction of "Final J-HAQ score"

	Explanatory variable	Linear regression coefficient (p value), n = 2775
Model 1	Avg-Dscore*	1.025 (<0.001)
Model 2	Avg-Dscore*, baseline J-HAQ	0.476 (<0.001)
Model 3	Avg-Dscore*, baseline DAS	0.712 (<0.001)
Model 4	Avg-DAS28*	0.414 (<0.001)
Model 5	Avg-DAS28*, baseline J-HAQ	0.202 (<0.001)
Model 6	Avg-DAS28*, baseline DAS	0.438 (<0.001)

All models were adjusted for age, gender, RA disease duration, time of enrolment and seasonal effect. Model 2 and model 5 were also adjusted for baseline (Initial) J-HAQ score as an explanatory variable. Model 3 and model 6 were also adjusted for baseline (Initial) DAS28 score as an explanatory variable.
*For explanation of Avg-DAS28 and Avg-Dscore, see Patients and methods section.

functional capacity was caused mainly by disease activity in early stages of the disease, while joint destruction was the main determinant for functional capacity later in the disease course. In our study, since mean RA duration of our cohort sample was 9.5 years at entry, most patients were categorised as having established RA. Although patient characteristics at entry of this study were different from the previous two reports in which their cohort was based on patients with early RA,[11 12] our findings are in concordance with the cohort study by Drossaers-Bakker *et al*, in which disease activity was the most important factor in determining the loss of functional capacity throughout the course of RA.[11]

Various definitions of "remission" in RA have been proposed, such as ACR remission criteria,[45] radiographic remission[14 46] and remission assessed by DAS,[29] DAS28,[13] simplified disease activity index (SDAI)[47 48] and clinical disease activity index (CDAI).[49 50] Although DAS28 remission at a cut-off level of 2.6 is often used in clinical practice and clinical trials[32–34] and we use this cut-off point in this study, many researchers have chosen their own favourable DAS28 cut-off points such as 2.66,[32] 2.81,[51] 2.32,[46] and 2.4.[51 48] Based on our analysis in the three groups that were classified by "Avg-DAS28", the disease activity was significantly reduced in all three groups. However, functional capability was significantly improved only in the "Under 2.6" group that had average DAS28 values under 2.6. The change in functional capability in the "Between 2.6 and 3.2" group with average DAS28 values between 2.6 and 3.2 was not significant and in the "Above 3.2" group with average DAS28 values above 3.2, functional capability significantly deteriorated. Hence, it can be suggested that we have to control the disease activity of patients with RA more strictly. A targeted treatment strategy should be undertaken in the management of RA in daily practice, and based on our results, the target DAS28 should be <2.6 to prevent progression of disability. The threshold DAS28 level of 2.6 may be useful in developing guidelines for targeted treatment strategies in patients with RA from a viewpoint of predicting patient functional disability. The definition of "remission" generally is the total absence of signs and symptoms of the disease: (1) no tender and swollen joints, (2) no increase in joint damage, and (3) full functioning (HAQ = 0). However, we have to acknowledge that a definition of remission according to DAS28 at a cut-off level of 2.6 may include many patients with a considerable number of tender and/or swollen joints,[51 48] and that a DAS28 cut-off level of 2.6 has to be used with caution in clinical practice and clinical trials of RA treatment.

In RA, disability consists of two major components, reversible components such as joint pain and swelling due to joint inflammation, and irreversible components such as joint destruction, deformity, and muscle weakness. Aletaha *et al* reported that average HAQ scores despite clinical remission increased progressively with duration of RA and the reversibility of HAQ scores decreased with the duration of RA in 295 patients in whom clinical remission was achieved in several clinical trials.[52] Hence, when we assess remission using DAS28 score, the cut-off point might not be limited to a single value for the whole population of patients with RA, and this may be considered separately for subgroups of patients with different disease duration. Further longer-term observational studies are required to examine a cut-off level of DAS28 remission.

The low continuation rate of our study and selection bias of patients with RA may be possible limitations. In fact, among 7511 patients with RA enrolled in IORRA from October 2000 to April 2005, only 2775 patients with RA (36.9%) had continuous enrolment for at least 3 years. Since our institute is located in the centre of Tokyo, many patients with RA may travel long distances. The purpose of their visit may be to obtain a second opinion for therapeutic strategy, and thus they may not keep visiting us thereafter. However, it is our principle to enrol all patients with RA into this cohort and thus we may lose many

Table 4 Relationship of rheumatoid arthritis (RA) disease activity to long-term disability using multiple linear regression analysis: prediction of "Change in J-HAQ score"

	Explanatory variable	Linear regression coefficient (p value)
Model 7	Avg-Dscore*	0.287 (<0.001)
Model 8	Avg-Dscore*, baseline J-HAQ	0.476 (<0.001)
Model 9	Avg-Dscore*, baseline DAS	0.598 (<0.001)
Model 10	Avg-DAS28*	0.099 (<0.001)
Model 11	Avg-DAS28*, baseline J-HAQ	0.202 (<0.001)
Model 12	Avg-DAS28*, baseline DAS	0.313 (<0.001)

All models were adjusted for age, gender, RA disease duration, time of enrolment and seasonal effect. Model 8 and model 11 were also adjusted for baseline (Initial) J-HAQ score as an explanatory variable. Model 9 and model 12 were also adjusted for baseline (Initial) DAS28 score as an explanatory variable. Change in J-HAQ score = final J-HAQ score–initial J-HAQ score.
*For explanation of Avg-DAS28 and Avg-Dscore, see Patients and methods section.

Ann Rheum Dis 2008;**67**:1153–1158. doi:10.1136/ard.2007.072751

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Table 5 Relationship of RA disease activity with long-term functional disability for each "Avg-DAS28" group

"Avg-DAS28" group*	Under 2.6	Between 2.6 and 3.2	Over 3.2
Number of patients	346	461	1968
Initial DAS28 score†	2.5 (0.8) (2.4)	3.3 (0.9) (3.2)	4.4 (1.1) (4.3)
Final DAS28 score‡	2.1 (0.7) (2.1)	2.9 (0.8) (2.9)	4.2 (1.1) (4.1)
Change in DAS28 score§	−0.40 (1.06), <0.001	−0.38 (1.37), <0.001	−0.29 (1.38), <0.001
Initial J-HAQ score†	0.32 (0.46) (0.13)	0.48 (0.55) (0.25)	0.95 (0.73) (0.88)
Final J-HAQ score‡	0.27 (0.45) (0.0)	0.44 (0.58) (0.25)	1.06 (0.79) (1.0)
Change in J-HAQ score¶	−0.05 (0.33), <0.01	−0.04 (0.48), 0.08	0.11 (0.56), <0.001

Data is mean (SD) (median) or mean (SD), p value, unless otherwise indicated.
*For explanation of Avg-DAS28, see Patients and methods section. †Observation in the first phase of study. ‡Observation in the last phase of study. §Change in DAS28 score = final DAS28 score–initial DAS28 score; the p value of this variable was calculated using the paired t test. ¶Change in J-HAQ score = final J-HAQ score–initial J-HAQ score; the p value of this variable was calculated using the sign test.
DAS28, 28-joint Disease Activity Score; J-HAQ, the Japanese Health Assessment Questionnaire; RA, rheumatoid arthritis.

patients during follow-up. Additionally, we sometimes refer patients with RA to their family doctors, who may lose them during follow-up. However, the baseline features of 2775 patients with RA included in this study sample were quite similar to the 4736 excluded patients with RA. Hence, we believe selection bias of patients with RA may be minimal.

In conclusion, efficient management of disease in patients with RA, by maintaining the DAS28 value at <2.6, has significant association with improved functional capability as assessed by J-HAQ over a long-term period. The threshold DAS28 level of 2.6 may be useful in developing guidelines for targeted treatment in patients with RA.

Acknowledgements: The authors would like to thank all members of the Institute of Rheumatology, Tokyo Women's Medical University, for the successful management of the IORRA cohort study. The IORRA cohort is supported by a consortium of 36 pharmaceutical companies.

Funding: This study was supported by a consortium of 36 pharmaceutical companies for the large observational cohort study of rheumatoid arthritis at our institute.

Competing interests: None declared.

REFERENCES

1. **Weaver AL.** The impact of new biologicals in the treatment of rheumatoid arthritis. *Rheumatology (Oxford)* 2004;**43**(Suppl 3):iii17–23.
2. **Furst DE,** Breedveld FC, Kalden JR, Smolen JS, Burmester GR, Bijlsma JW, *et al.* Updated consensus statement on biological agents, specifically tumor necrosis factor α (TNFα) blocking agents and interleukin-1 receptor antagonist (IL-1ra), for the treatment of rheumatic diseases, 2005. *Ann Rheum Dis* 2005;**64**(Suppl 4):iv2–14.
3. **Albers JM,** Paimela L, Kurki P, Eberhardt KB, Emery P, van't Hof MA, *et al.* Treatment strategy, disease activity, and outcome in four cohorts of patients with early rheumatoid arthritis. *Ann Rheum Dis* 2001;**60**:453–8.
4. **van Aken J,** Lard LR, le Cessie S, Hazes JM, Breedveld FC, Huizinga TW. Radiological outcome after four years of early versus delayed treatment strategy in patients with recent onset rheumatoid arthritis. *Ann Rheum Dis* 2004;**63**:274–9.
5. **Nell VP,** Machold KP, Eberl G, Stamm TA, Uffmann M, Smolen JS. Benefit of very early referral and very early therapy with disease-modifying anti-rheumatic drugs in patients with early rheumatoid arthritis. *Rheumatology (Oxford)* 2004;**43**:906–14.
6. **Puolakka K,** Kautiainen H, Mottonen T, Hannonen P, Korpela M, Hakala M, J *et al*; FIN-RACo Trial Group. Early suppression of disease activity is essential for maintenance of work capacity in patients with recent-onset rheumatoid arthritis: five-year experience from the FIN-RACo trial. *Arthritis Rheum* 2005;**52**:36–41.
7. **van der Heijde D,** Klareskog L, Rodriguez-Valverde V, Codreanu C, Bolosiu H, Melo-Gomes J, *et al.* Comparison of etanercept and methotrexate, alone and combined, in the treatment of rheumatoid arthritis: two-year clinical and radiographic results from the TEMPO study, a double-blind, randomized trial. *Arthritis Rheum* 2006;**54**:1063–74.
8. **van der Heijde DM,** van't Hof MA, van Riel PL, Theunisse LA, Lubberts EW, van Leeuwen MA, *et al.* Judging disease activity in clinical practice in rheumatoid arthritis: first step in the development of a disease activity score. *Ann Rheum Dis* 1990;**49**:916–20.
9. **Prevoo ML,** van't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum* 1995;**38**:44–8.
10. **Fries JF,** Spitz P, Kraines RG, Holman HR. Measurement of patient outcome in arthritis. *Arthritis Rheum* 1980;**23**:137–45.
11. **Drossaers-Bakker KW,** de Buck M, van Zeben D, Zwinderman AH, Breedveld FC, Hazes JM. Long-term course and outcome of functional capacity in rheumatoid arthritis: the effect of disease activity and radiologic damage over time. *Arthritis Rheum* 1999;**42**:1854–60.
12. **Welsing PM,** van Gestel AM, Swinkels HL, Kiemeney LA, van Riel PL. The relationship between disease activity, joint destruction, and functional capacity over the course of rheumatoid arthritis. *Arthritis Rheum* 2001;**44**:2009–17.
13. **Guillemin F,** Briancon S, Pourel J. Functional disability in rheumatoid arthritis: two different models in early and established disease. *J Rheumatol* 1992;**19**:366–9.
14. **van der Heijde DM,** van't Hof MA, van Riel PL, van Leeuwen MA, van Rijswijk MH, van de Putte LB. Validity of single variables and composite indices for measuring disease activity in rheumatoid arthritis. *Ann Rheum Dis* 1992;**51**:177–81.
15. **Jansen LM,** van Schaardenburg D, van Der Horst-Bruinsma IE, Bezemer PD, Dijkmans BA. Predictors of functional status in patients with early rheumatoid arthritis. *Ann Rheum Dis* 2000;**59**:223–6.
16. **Guillemin F,** Suurmeijer T, Krol B, Bombardier C, Briancon S, Doeglas D, *et al.* Functional disability in early rheumatoid arthritis: description and risk factors. *J Rheumatol* 1994;**21**:1051–5.
17. **Leigh JP,** Fries JF. Predictors of disability in a longitudinal sample of patients with rheumatoid arthritis. *Ann Rheum Dis* 1992;**51**:581–7.
18. **Sherrer YS,** Bloch DA, Mitchell DM, Young DY, Fries JF. The development of disability in rheumatoid arthritis. *Arthritis Rheum* 1986;**29**:494–500.
19. **Hakala M,** Nieminen P, Manelius J. Joint impairment is strongly correlated with disability measured by self-report questionnaires. Functional status assessment of individuals with rheumatoid arthritis in a population based series. *J Rheumatol* 1994;**21**:64–9.
20. **van Leeuwen MA,** van der Heijde DM, van Rijswijk MH, Houtman PM, van Riel PL, van de Putte LB, *et al.* Interrelationship of outcome measures and process variables in early rheumatoid arthritis. A comparison of radiologic damage, physical disability, joint counts, and acute phase reactants. *J Rheumatol* 1994;**21**:425–9.
21. **Smedstad LM,** Kvien TK, Moum T, Vaglum P. Life events, psychosocial factors, and demographic variables in early rheumatoid arthritis: relations to one-year changes in functional disability. *J Rheumatol* 1995;**22**:2218–25.
22. **Sokka T,** Kankainen A, Hannonen P. Scores for functional disability in patients with rheumatoid arthritis are correlated at higher levels with pain scores than with radiographic scores. *Arthritis Rheum* 2000;**43**:386–9.
23. **Eberhardt KB,** Fex E. Functional impairment and disability in early rheumatoid arthritis – development over 5 years. *J Rheumatol* 1995;**22**:1037–42.
24. **van der Heide A,** Jacobs JW, van Albada-Kuipers GA, Kraaimaat FW, Geenen R, Bijlsma JW. Physical disability and psychological well being in recent onset rheumatoid arthritis. *J Rheumatol* 1994;**21**:28–32.
25. **Uhlig T,** Smedstad LM, Vaglum P, Moum T, Gerard N, Kvien TK. The course of rheumatoid arthritis and predictors of psychological, physical and radiographic outcome after 5 years of follow-up. *Rheumatology (Oxford)* 2000;**39**:732–41.
26. **Wolfe F,** Sharp JT. Radiographic outcome of recent-onset rheumatoid arthritis: a 19-year study of radiographic progression. *Arthritis Rheum* 1998;**41**:1571–82.
27. **Combe B,** Cantagrel A, Goupille P, Bozonnat MC, Sibilia J, Eliaou JF, *et al.* Predictive factors of 5-year health assessment questionnaire disability in early rheumatoid arthritis. *J Rheumatol* 2003;**30**:2344–9.
28. **Smolen JS,** Aletaha D. What should be our treatment goal in rheumatoid arthritis today? *Clin Exp Rheumatol* 2006;**24**(Suppl 43):S7–13.
29. **Prevoo ML,** van Gestel AM, van T Hof MA, van Rijswijk MH, van de Putte LB, van Riel PL. Remission in a prospective study of patients with rheumatoid arthritis. American Rheumatism Association preliminary remission criteria in relation to the disease activity score. *Br J Rheumatol* 1996;**35**:1101–5.
30. **Makinen H,** Hannonen P, Sokka T. Definitions of remission for rheumatoid arthritis and review of selected clinical cohorts and randomised clinical trials for the rate of remission. *Clin Exp Rheumatol* 2006;**24**(Suppl 43):S22–8.

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31. **Makinen H,** Kautiainen H, Hannonen P, Sokka T. Is DAS28 an appropriate tool to assess remission in rheumatoid arthritis? *Ann Rheum Dis* 2005;**64**:1410–3.
32. **Fransen J,** Creemers MC, Van Riel PL. Remission in rheumatoid arthritis: agreement of the disease activity score (DAS28) with the ARA preliminary remission criteria. *Rheumatology (Oxford)* 2004;**43**:1252–5.
33. **van Riel PL,** van Gestel AM. Clinical outcome measures in rheumatoid arthritis. *Ann Rheum Dis* 2000;**59**(Suppl 1):i28–31.
34. **Sesin CA,** Bingham CO. Remission in rheumatoid arthritis: wishful thinking or clinical reality? *Semin Arthritis Rheum* 2005;**35**:185–96.
35. **Yamanaka H,** Tohma S. Potential impact of observational cohort studies in Japan on rheumatoid arthritis research and practice. *Mod Rheumatol* 2006;**16**:75–6.
36. **Matsuda Y,** Singh G, Yamanaka H, Tanaka E, Urano W, Taniguchi A, *et al.* Validation of a Japanese version of the Stanford Health Assessment Questionnaire in 3763 patients with rheumatoid arthritis. *Arthritis Rheum* 2003;**49**:784–8.
37. **Tanaka E,** Saito A, Kamitsuji S, Yamada T, Nakajima A, Taniguchi A, *et al.* Impact of shoulder, elbow, and knee joint involvement on assessment of rheumatoid arthritis using the American College of Rheumatology Core Data Set. *Arthritis Rheum* 2005;**53**:864–71.
38. **Furuya T,** Kotake S, Inoue E, Nanke Y, Yago T, Kobashigawa T, *et al.* Risk factors associated with incident clinical vertebral and nonvertebral fractures in Japanese women with rheumatoid arthritis: a prospective 54-month observational study. *J Rheumatol* 2007;**34**:303–10.
39. **Tanaka E,** Singh G, Saito A, Syouji A, Yamada T, Urano W, *et al.* Prevalence of *Helicobacter pylori* infection and risk of upper gastrointestinal ulcer in patients with rheumatoid arthritis in Japan. *Mod Rheumatol* 2005;**15**:340–5.
40. **Nakajima A,** Kamitsuji S, Saito A, Tanaka E, Nishimura K, Horikawa N, *et al.* Disability and patient's appraisal of general health contribute to depressed mood in rheumatoid arthritis in a large clinical study in Japan. *Mod Rheumatol* 2006;**16**:151–7.
41. **Iwatani M,** Inoue E, Nakamura T, Nakajima A, Hara M, Tomatsu T, *et al.* Efficacy profile of bucillamine in rheumatoid arthritis patients in a large observational cohort study, IORRA. *Mod Rheumatol* 2006;**16**:376–80.
42. **Tanaka E,** Inoue E, Kawaguchi Y, Tomatsu T, Yamanaka H, Hara M, *et al.* Acceptability and usefulness of mizoribine in the management of rheumatoid arthritis in methotrexate-refractory patients and elderly patients, based on analysis of data from a large-scale observational cohort study. *Mod Rheumatol* 2006;**16**:214–9.
43. **Arnett FC,** Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, *et al.* The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;**31**:315–24.
44. **Iikuni N,** Nakajima A, Inoue E, Tanaka E, Okamoto H, Hara M, *et al.* What's in season for rheumatoid arthritis patients? Seasonal fluctuations in disease activity. *Rheumatology (Oxford)* 2007;**6**:846–8.
45. **Pinals RS,** Masi AT, Larsen RA. Preliminary criteria for clinical remission in rheumatoid arthritis. *Arthritis Rheum* 1981;**24**:1308–15.
46. **van der Helm-van Mil AH,** Breedveld FC, Huizinga TW. Aspects of early arthritis. Definition of disease states in early arthritis: remission versus minimal disease activity. *Arthritis Res Ther* 2006;**8**:216.
47. **Smolen JS,** Breedveld FC, Schiff MH, Kalden JR, Emery P, Eberl G, *et al.* A simplified disease activity index for rheumatoid arthritis for use in clinical practice. *Rheumatology (Oxford)* 2003;**42**:244–57.
48. **Aletaha D,** Ward MM, Machold KP, Nell VP, Stamm T, Smolen JS. Remission and active disease in rheumatoid arthritis: defining criteria for disease activity states. *Arthritis Rheum* 2005;**52**:2625–36.
49. **Aletaha D,** Nell VP, Stamm T, Uffmann M, Pflugbeil S, Machold K, *et al.* Acute phase reactants add little to composite disease activity indices for rheumatoid arthritis: validation of a clinical activity score. *Arthritis Res Ther* 2005;**7**:R796–806.
50. **Aletaha D,** Smolen J. The Simplified Disease Activity Index (SDAI) and the Clinical Disease Activity Index (CDAI): a review of their usefulness and validity in rheumatoid arthritis. *Clin Exp Rheumatol* 2005;**23**(Suppl 39):S100–8.
51. **Balsa A,** Carmona L, Gonzalez-Alvaro I, Belmonte MA, Tena X, Sanmarti R, EMECAR Study Group. Value of Disease Activity Score 28 (DAS28) and DAS28-3 compared to American College of Rheumatology-defined remission in rheumatoid arthritis. *J Rheumatol* 2004;**31**:40–6.
52. **Aletaha D,** Smolen J, Ward MM. Measuring function in rheumatoid arthritis: identifying reversible and irreversible components. *Arthritis Rheum* 2006;**54**:2784–92.

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Functional SNPs in *CD244* increase the risk of rheumatoid arthritis in a Japanese population

Akari Suzuki[1], Ryo Yamada[1,2], Yuta Kochi[1], Tetsuji Sawada[3], Yukinori Okada[2], Koichi Matsuda[4], Yoichiro Kamatani[4], Mikako Mori[1,3], Kenichi Shimane[1,3], Yasuhiko Hirabayashi[5], Atsushi Takahashi[6], Tatsuhiko Tsunoda[7], Akihiko Miyatake[8], Michiaki Kubo[9], Naoyuki Kamatani[6], Yusuke Nakamura[4,10] & Kazuhiko Yamamoto[1,3]

Reprinted from *Nature Genetics*, Volume 40, October 2008

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Rheumatoid arthritis is a chronic autoimmune inflammatory disease with a complex genetic etiology. Members of the signaling lymphocyte activation molecule (SLAM) family carry out pivotal functions in innate immunity and in conventional lymphocytes. We identified a linkage disequilibrium block associated with rheumatoid arthritis in the chromosome 1q region containing multiple SLAM family genes. In this block, the association peaked at two functional SNPs (rs3766379 and rs6682654) in *CD244* in two independent rheumatoid arthritis cohorts from Japan ($P = 3.23 \times 10^{-8}$ and $P = 7.45 \times 10^{-8}$). We also identified a Japanese cohort with systemic lupus erythematosus that had a similar genotype distribution as the rheumatoid arthritis cohorts. We demonstrated that the rheumatoid arthritis–susceptible alleles of rs3766379 and rs6682654 and their haplotype increased their expression in luciferase and allele-specific transcript quantification assays. *CD244* is a genetic risk factor for rheumatoid arthritis and may have a role in the autoimmune process shared by rheumatoid arthritis and systemic lupus erythematosus.

Rheumatoid arthritis, which affects 0.5%–1% of the world's population, is a common inflammatory disease; both autoimmune and genetic factors are important in rheumatoid arthritis susceptibility[1–4]. The locus most conclusively associated with rheumatoid arthritis is the *HLA-DRB* locus, which accounts for about one-third of the genetic component of the disease[1,5]. Multiple genes, including *PTPN22, PADI4, FCRL3, SLC22A4, CTLA4* and *STAT4*, have been shown to have variants that increase susceptibility to rheumatoid arthritis (hereafter "rheumatoid arthritis–susceptible variants") in case-control association studies[6–11]. In particular, the associations of *PTPN22* and *PADI4* have been validated with multiple follow-up studies and meta-analyses[12,13]. These studies have clarified two important aspects of genetic factors in rheumatoid arthritis and autoimmune diseases. First, some rheumatoid arthritis–susceptible polymorphisms also increase the risks of other autoimmune diseases, as reported for *PTPN22* with type 1 diabetes mellitus, systemic lupus erythmatosus (SLE) and autoimmune thyroiditis (AIT)[13]; *FCRL3* with rheumatoid arthritis, AIT and SLE[9]; *CTLA4* with rheumatoid arthritis, type 1 diabetes mellitus and AIT[14]; *SLC22A4* and *SLC22A5* with Crohn's disease and rheumatoid arthritis[10] and, most recently, *STAT4* with rheumatoid arthritis and SLE[8]. The second factor that these studies highlight is the heterogeneity of risk variants between populations of different geographical origin. For example, the risk variant of *PTPN22* is not present in East Asians[15], although the association between *PTPN22* and rheumatoid arthritis is robust in individuals of European origin, as shown by a genome-wide association scan[16]. In addition, the relative risks of the risk variants of *PADI4* are different between individuals of East Asian and European origin, although the allele frequency of the variants was similar[12].

A large-scale, case-control association study of 830 cases and 658 controls (rheumatoid arthritis case-control cohort 1), which previously reported rheumatoid arthritis–susceptible variants of *PADI4, SLC22A4* and *FCRL3* (refs. 7,9,10), identified a positive signal in the region containing multiple SLAM family genes on chromosome 1, spanning more than 30 million base pairs. This region has also been implicated in previous linkage studies of rheumatoid arthritis and SLE[1,3,4,17]. We selected a 1.1-Mb linkage disequilibrium (LD) segment based on phase II HapMap data in which the initial association was detected. This segment contained 38 genes.

Along the 1.1-Mb core segment in the contig NT_004487.18 (**Fig. 1** and **Supplementary Table 1** online), we genotyped 91 SNPs and tested them for association with rheumatoid arthritis using rheumatoid

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Received 4 April; accepted 23 June; published online 14 September 2008; doi:10.1038/ng.205

arthritis case-control cohort 1 (830 cases and 658 controls). The central part of the segment, spanning 70 kb and containing *LY9*, *CD244* and *ITLN1*, was densely covered with 36 tagging SNPs that we selected from the HapMap project using the method of ref. 18. We compared genotype frequencies of 91 SNPs with the Cochran-Armitage trend test (**Fig. 1b,c** and **Supplementary Table 1**). In this segment, six SNPs were strongly associated with rheumatoid arthritis ($P < 0.001$); five of these were located in *CD244* and one in *LY9* (**Table 1**, meta-analysis of rheumatoid arthritis case-control cohorts 1 and 2). Because three SNPs in *LY9* (rs3817407, rs1333065 and rs509749) have been reported to be associated with SLE[19], the data count of these three SNPs is also shown in **Table 1**, although we did not note any significant associations in these three SNPs on *LY9*. We genotyped the six strongly associated SNPs from the first cohort in the second rheumatoid arthritis cohort (rheumatoid arthritis case-control cohort 2; 1,112 subjects with rheumatoid arthritis and 940 controls) to validate the association in the first cohort. We tested the results independently (**Table 1**) and combined them with results from the first cohort using a Mantel-Haenszel meta-analysis (**Table 1**). The second cohort validated the association of the five SNPs in *CD244* but not the SNPs in *LY9*. We observed the most significant association at rs3766379 ($P = 3.23 \times 10^{-8}$, odds ratio (OR) 1.31 (95% confidence interval (c.i.) 1.19–1.44); meta-analysis) in intron 5 of *CD244*. This association was statistically significant, even in genome-wide association studies[20]. Because the two independent rheumatoid arthritis cohorts indicated that the association peaked within *CD244*, and because some rheumatoid arthritis–associated genes, such as *PTPN22*, *FCRL3* and *STAT4*, are known to be associated with other autoimmune diseases, including SLE, we genotyped the five SNPs in *CD244* in a case cohort of 555 individuals with SLE and compared their genotype count with that of controls in the second rheumatoid arthritis case-control cohort. The genotype distribution in the SLE cohort was similar to that in the rheumatoid arthritis cohort, and it seems that polymorphisms in *CD244* increase the risk for developing SLE as well as rheumatoid arthritis (**Table 1**).

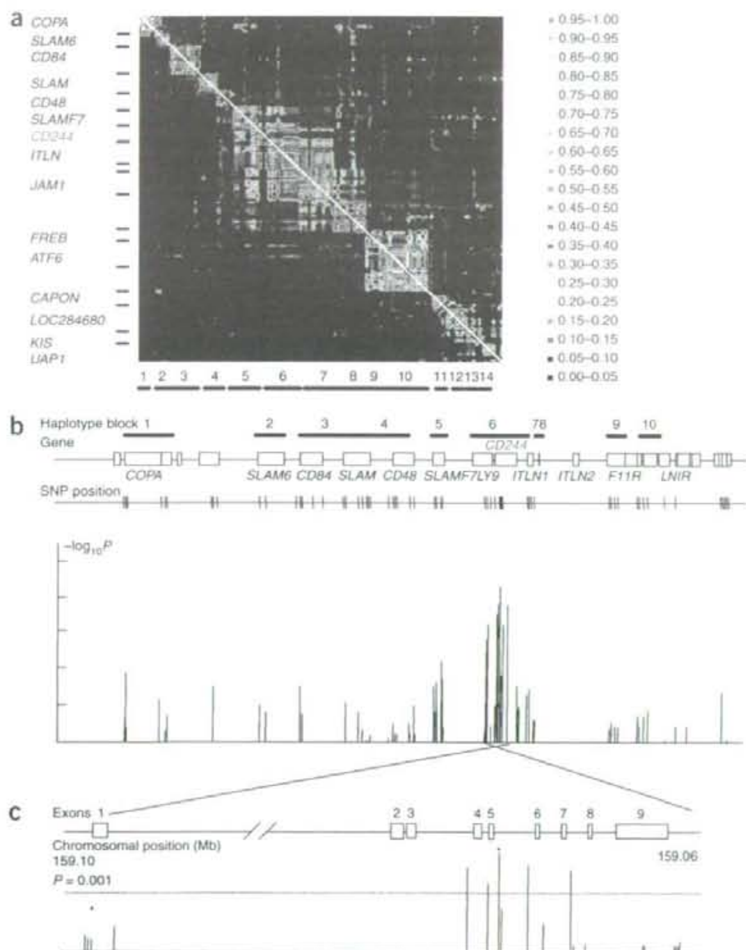

Figure 1 LD block and allelic association around *CD244*. (**a**) Pairwise LD in the 1.1-Mb region in 1q22 around the SLAM gene cluster. The index of pairwise LD, r^2, for individuals with rheumatoid arthritis and control individuals is presented in the upper right and the lower left, respectively. LD blocks (below) are indicated by thin lines, and the color gradient shows LD values. (**b**) The location of the genes around *CD244*, SNP positions and case-control association plots ($-\log_{10}(P)$). (**c**) Gene structure, SNP position and case-control association plots of *CD244*. * indicates the SNP that shows the strongest association in this region and the strongest association in the meta-analysis. Chromosomal positions are in NCBI build35 coordinates.

We evaluated the first cohort for variable inflation with 83,567 SNPs in autosomal chromosomes using the genomic control method[21], and λ_{GC} was 1.069. The smallest P value was corrected to 8.9×10^{-8} with the genomic control method. Because population stratification is known to have uneven effects over the genome, the correction for the stratification with λ_{GC} of 1.069 might not be conservative enough statistically. Thus, we calculated the value of λ_{GC} that would make the smallest P value, 3.23×10^{-8}, to $P = 5.0 \times 10^{-7}$, as 1.2, which was very different from 1.069. Therefore, we considered that the detected association was likely to be significant even after correction for population stratification with conservative consideration given to the unevenness of population stratification effects over the genome.

Because the SNPs in *CD244* associated with rheumatoid arthritis are located in its introns, we hypothesized that the variants affected the expression of *CD244*. From the Gene Expression Omnibus (GEO) database, we obtained a data set of whole-genome gene expression variation in lymphoblastoid cell lines derived from 210 unrelated HapMap individuals from three HapMap populations (CEPH (Centre d'Etude du Polymorphisme Humain) from Utah (CEU), Han Chinese from Beijing (CHB) and Japanese from Tokyo (JPT))[22]. We tested for correlation between *CD244* expression in the lymphoblastoid cell lines from 90 East Asian individuals (45 Han Chinese from Beijing and 45 Japanese from

Tokyo) and the genotypes of 802 SNPs in the core segment using a linear regression model. Ten SNPs had P values <0.0001, and eight of ten were located in *CD244*; rs6682654, which was also strongly associated with rheumatoid arthritis in our case-control test, was one of them (**Fig. 2** and **Supplementary Fig. 1** online). These results seem to support the hypothesis that genetic variants in *CD244* affect *CD244* expression, and the mechanism of this effect might explain the association between rheumatoid arthritis and SNP genotypes in *CD244*.

We performed a luciferase assay to test this hypothesis for the SNPs in *CD244* with the five smallest P values in the case-control association test: rs3766379 (intron 5), rs6682654 (intron 3), rs11265493 (intron 7), rs3753389 (intron 5) and rs1319651 (intron 4). We subcloned a nucleotide segment around each SNP into a vector containing an SV40 promoter. The allele-specific constructs containing the rheumatoid arthritis–susceptible allele of rs3766379 or rs6682654 showed approximately 1.5- to 1.7-fold higher transcriptional enhancer activity compared with the other constructs containing nonsusceptible alleles (**Fig. 3a,b**), indicating that the susceptible allele of each SNP upregulates the transcription of *CD244*. We did not observe any allele-specific difference in luciferase activity in the other SNPs.

Because rs3766379 and rs6682654 seemed to be functional, we evaluated their LD relation. D′ between them was 1, and we inferred three haplotypes. Among the three existing haplotypes, one haplotype was infrequent (frequency = 0.06), and there were two major haplotypes: haplotype_1 (rs3766379 T and rs6682654 C) and haplotype_3 (rs3766379 C and rs6682654 T). The haplotypes showed a significant association with rheumatoid arthritis, as seen with individual SNPs (**Table 2**). We evaluated the combined effect of these two SNPs with logistic regression analysis[23] but did not see any significant effect with the combination ($P = 0.39$). Because rs7528684 in *FCRL3* was associated with rheumatoid arthritis and SLE in our previous study[9], we also performed logistic regression analysis[24] for the effect of

Table 1 Genotype counts and case-control association test results

Gene	dbSNP ID	Allele 1	Allele 2	Case				Control				Frequency of allele 1		P^a	Odds ratio (allele)
				1/1	1/2	2/2	Sum	1/1	1/2	2/2	Sum	Case	Control		OR (95% c.i.)
RA case-control cohort 1				RA 1				Control 1							
LY9	rs1333065[b]	C	T	187	390	247	824	136	320	188	644	0.46	0.46	0.83294	1.02 (0.88–1.18)
	rs4017732	G	T	7	134	681	822	10	150	492	652	0.09	0.13	*0.00045*	0.66 (0.52–0.83)
	rs3817407[b]	A	G	101	328	395	824	79	307	272	658	0.32	0.35	0.07461	0.87 (0.74–1.01)
	rs509749[b]	A	G	446	321	51	818	344	255	51	650	0.74	0.73	0.32663	1.09 (0.92–1.28)
CD244	rs11265493	T	C	308	353	144	805	177	335	134	646	0.60	0.53	*0.00026*	1.32 (1.14–1.53)
	rs3753389	T	C	308	356	141	805	185	315	146	646	0.60	0.53	*0.00011*	1.35 (1.16–1.57)
	rs3766379	T	C	309	336	143	788	184	320	147	651	0.61	0.53	*0.00006*	1.37 (1.18–1.59)
	rs1319651	C	G	360	355	104	819	232	310	107	649	0.66	0.60	*0.00098*	1.29 (1.11–1.50)
	rs6682654	C	T	361	347	101	809	222	321	102	645	0.66	0.59	*0.00019*	1.34 (1.15–1.55)
RA case-control cohort 2				RA 2				Control 2							
LY9	rs4017732	G	T	17	187	908	1,112	11	169	759	939	0.10	0.10	0.80808	0.97 (0.79–1.20)
CD244	rs11265493	T	C	386	532	193	1,111	274	452	214	940	0.59	0.53	*0.00049*	1.25 (1.10–1.41)
	rs3753389	T	C	391	530	191	1,112	275	451	214	940	0.59	0.53	*0.00026*	1.26 (1.12–1.43)
	rs3766379	T	C	392	526	193	1,111	272	452	214	938	0.59	0.53	*0.00020*	1.27 (1.12–1.44)
	rs1319651	C	G	472	495	144	1,111	335	441	164	940	0.65	0.59	*0.00024*	1.27 (1.12–1.44)
	rs6682654	C	T	477	491	142	1,110	336	439	164	939	0.65	0.59	*0.00012*	1.29 (1.13–1.46)
Meta-analysis of RA case-control cohorts 1 and 2[c]				RA 1+ RA 2				Control 1 + Control 2							
LY9	rs4017732	G	T	24	321	1,589	1,934	21	319	1,251	1,591	0.10	0.11	0.01420	0.82 (0.70–0.96)
CD244	rs11265493	T	C	694	885	337	1,916	451	787	348	1,586	0.59	0.53	*0.00000041*	1.28 (1.16–1.41)
	rs3753389	T	C	699	886	332	1,917	460	766	360	1,586	0.60	0.53	*0.00000008*	1.30 (1.18–1.43)
	rs3766379	T	C	701	862	336	1,899	456	772	361	1,589	0.60	0.53	*0.00000003*	1.31 (1.19–1.44)
	rs1319651	C	G	832	850	248	1,930	567	751	271	1,589	0.65	0.59	*0.00000064*	1.28 (1.16–1.41)
	rs6682654	C	T	838	838	243	1,919	558	760	266	1,584	0.66	0.59	*0.00000007*	1.31 (1.19–1.44)
SLE case cohort[d]				SLE											
CD244	rs11265493	T	C	207	247	101	555					0.60		*0.00099*	1.30 (1.11–1.51)
	rs3753389	T	C	207	246	100	553					0.60		*0.00088*	1.30 (1.12–1.51)
	rs3766379	T	C	209	243	102	554					0.60		*0.00070*	1.31 (1.12–1.52)
	rs1319651	C	G	239	238	73	550					0.65		*0.00148*	1.29 (1.11–1.51)
	rs6682654	C	T	242	238	74	554					0.65		*0.00144*	1.29 (1.11–1.51)

P values <0.01 are italicized. RA, rheumatoid arthritis. RA1, rheumatoid arthritis case-control cohort 1. RA2, rheumatoid arthritis case-control cohort 2. OR, odds ratio.
[a]P values represent the Cochran-Armitage trend P for case-control comparisons. [b]Three SNPs that were reported to be associated with SLE. [c]Meta-analysis of cohorts 1 and 2 (Mantel-Haenszel). [d]Genotype counts were compared with controls in cohort 2.

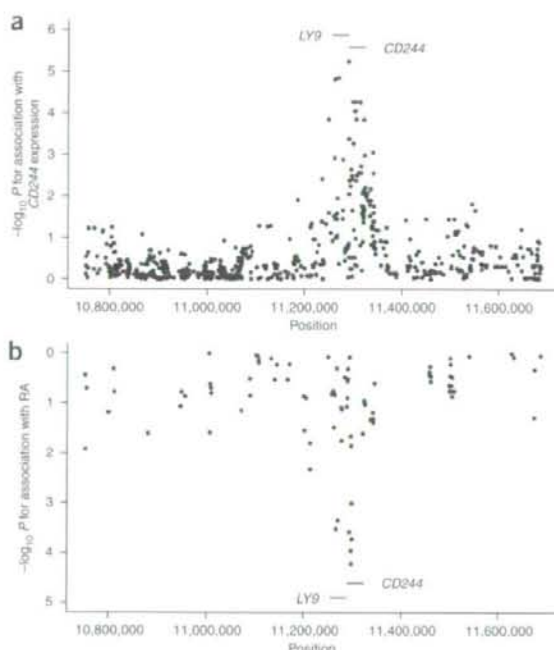

Figure 2 Association between *CD244* expression and SNPs in *CD244* and neighboring genes from ref. 22. (**a**) *P* values of linear regression test between *CD244* expression in lymphoblastoid cell lines from Asian populations and the number of alleles of individual SNPs, plotted in a logarithmic scale along the chromosomal location in the core segment. (**b**) *P* values of case-control association tests in the rheumatoid arthritis case cohort 1, for comparison with **a**. The horizontal axis represents the chromosomal location of SNPs, and the vertical axis represents the statistical significance of analysis in logarithmic scale.

the combined effect of the two loci (two SNPs in the *CD244* locus and one SNP in the *FCRL3*). We did not find any significant effects of combinations of any pair or of all three combined ($P > 0.1$).

We subsequently looked for nuclear transcription factor(s) that might bind to oligonucleotide sequences containing these SNPs using TRANSFAC. We found that rs3766379 and rs6682654 were located in a heat-shock transcription factor (HSF)-1/HSF-2/early growth response (EGR)-2 binding site and an upstream transcription factor 1 (USF-1) binding site. We examined the allelic differences in the binding of nuclear proteins between a susceptible allele and a nonsusceptible allele by electrophoretic mobility shift assay (EMSA). The signal intensities of the DNA-protein complex from the susceptible alleles of rs3766379 and rs6682654 were higher than those from the nonsusceptible alleles in the presence of Jurkat E6-1 nuclear extract (**Fig. 3c,d**). In the EMSA of rs6682654, the band of DNA-protein complex disappeared with the addition of antibody against USF-1 to the reactant (**Fig. 3c**). We then cotransfected an expression vector expressing USF-1 with a luciferase construct containing rs6682654 into Jurkat E6-1 cells. Overexpression of USF-1 significantly increased luciferase activity of rs6682654 ($P < 0.01$, Student's *t*-test, **Fig. 3e**). However, HSF-1, EGR-1 and EGR-2 were not related to the transcription enhancement in the DNA region, including rs3766379. It is possible that the combination of USF-1 and unidentified nuclear factor(s) interact with each regulatory region of gene expression in *CD244* and influence rheumatoid arthritis susceptibility. USF-1 interacts with its target sequence, E-box, resulting in transcriptional activation in response to various stimuli[25].

To further investigate the effect of the two major haplotypes of *CD244* (haplotype_1 and haplotype_3) on transcription, we performed allele-specific quantitative PCR using a TaqMan probe on Epstein-Barr virus (EBV)–transformed human lymphoblastoid cell lines with heterozygous genotypes of rs3766379 and rs6682654. We used these genotypes because they are in complete LD, and the cell lines that were heterozygous with respect to rs6682654 and rs3766379 had haplotype_1 or haplotype_3. In these cell lines, the susceptible haplotype, haplotype_1, showed significantly higher expression than the nonsusceptible haplotype, haplotype_3 (**Fig. 4**, $P < 0.0001$, Student's *t*-test).

CD244, also known as 2B4, is one of the NK cell–activating or –inhibitory molecules in mice and humans, and recent findings on the molecular mechanisms of SLAM family members, including *CD244*, have indicated their important roles in the immune system[26,27]. Therefore, we do not find it surprising that genetic polymorphisms in *CD244* are associated with susceptibility to autoimmune diseases such as rheumatoid arthritis. We have demonstrated that two SNPs in

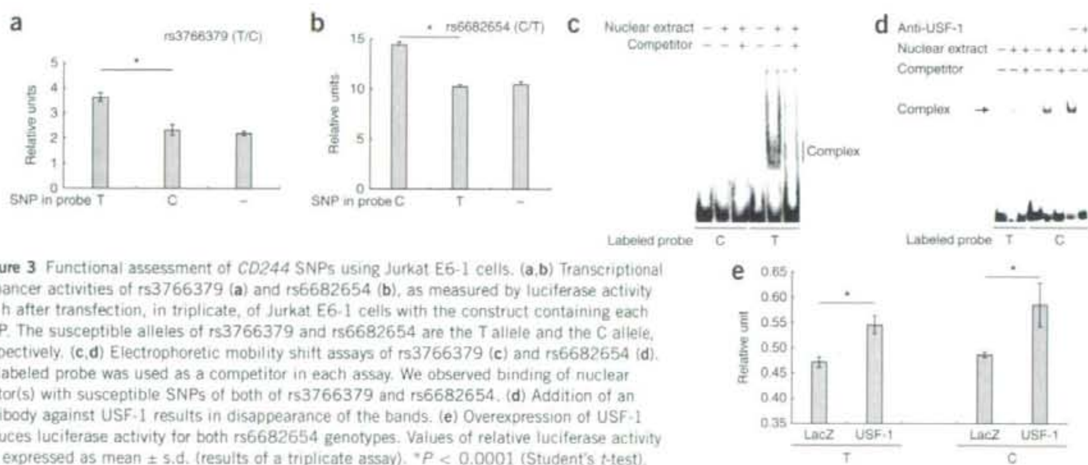

Figure 3 Functional assessment of *CD244* SNPs using Jurkat E6-1 cells. (**a**,**b**) Transcriptional enhancer activities of rs3766379 (**a**) and rs6682654 (**b**), as measured by luciferase activity 24 h after transfection, in triplicate, of Jurkat E6-1 cells with the construct containing each SNP. The susceptible alleles of rs3766379 and rs6682654 are the T allele and the C allele, respectively. (**c**,**d**) Electrophoretic mobility shift assays of rs3766379 (**c**) and rs6682654 (**d**). Unlabeled probe was used as a competitor in each assay. We observed binding of nuclear factor(s) with susceptible SNPs of both of rs3766379 and rs6682654. (**d**) Addition of an antibody against USF-1 results in disappearance of the bands. (**e**) Overexpression of USF-1 induces luciferase activity for both rs6682654 genotypes. Values of relative luciferase activity are expressed as mean ± s.d. (results of a triplicate assay). $^{*}P < 0.0001$ (Student's *t*-test).