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Expression of extra trinucleotide in CD44 variant of rheumatoid arthritis patients allows generation of disease-specific monoclonal antibody

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Abstract

Selective targeting of cells engaged in pathological activities is a major challenge for medical research. We generated monoclonal antibodies (mAbs) that exclusively bind, at concentrations ranging from 2 to 100 μg/ml, to a modified CD44 variant (designated CD44vRA) expressed on synovial fluid cells from joints of rheumatoid arthritis (RA) patients. These mAbs cross-reacted with keratinocytes expressing wild type CD44vRA (CD44v3−v10) only at a relatively high concentration (200 μg/ml). Sequence analysis of CD44vRA cDNA revealed, in 33 out of 43 RA and psoriatic arthritis patients, an extra intron-derived trinucleotide, CAG, which allows translation of an extra alanine. This insertion imposes a configurational change on the cell surface CD44 of RA synovial fluid cells, creating an immunogenic epitope and potentiating the ability to produce disease-specific antibodies. Indeed, the anti-CD44vRA mAbs (designated F8:33) were able to induce apoptosis in synovial fluid cells from RA patients, but not in peripheral blood leukocytes from the same patients, in keratinocytes from normal donors or in synovial fluid cells from osteoarthritis patients. Furthermore, injection of anti-CD44vRA mAbs reduced joint inflammation in DBA/1 mice with collagen-induced arthritis. These findings show that anti-CD44vRA mAbs are both bioactive and RA-specific.

Keywords: Anti-CD44 antibody; Apoptosis; Collagen-induced arthritis; Inflammation; Rheumatoid arthritis; Synovial fluid cells

1. Introduction

Selective eradication of cells involved in pathological activities, such as cancer cells or inflammatory cells mediating tissue destruction, is a major challenge for modern medicine. Most drugs and technologies (e.g., radiotherapy or chemotherapy) used to destroy cancer cells or cells involved in damage related to inflammatory reactions (e.g., those occurring in autoimmune diseases such as juvenile diabetes, multiple sclerosis, rheumatoid arthritis and ulcerative colitis) may also destroy cells essential to the normal physical functions of the individual. The development of drugs or technologies capable of targeting cells involved in pathological activities, while leaving the normal cells intact and functioning, would be a stunning victory for medical science.

One way to cope with this challenge is to screen cancer or inflammatory cells for cell surface structural entities expressed on the cells engaged in pathological functions, but not on

Abbreviations: CD44s, standard CD44; CD44v, CD44 variant; CD44vRA, CD44 variant of RA patients; CIA, collagen-induced arthritis; ECM, extracellular matrix; HA, hyaluronic acid; mAb, monoclonal antibody; OA, osteoarthritis; RA, rheumatoid arthritis; PBLs, peripheral blood leukocytes; PSA, psoriatic arthritis; RA, rheumatoid arthritis.

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normal cells involved in physiological activities. Specific targeting agents (e.g., antibodies or competitive peptides) recognizing such hypothetical structures or their counter molecules, should selectively neutralize the cells implicated in pathological functions, with minimal side effects. Although in the past three decades efforts have been made to identify such disease-specific cell surface entities, the results are disappointing, especially from the patient's view.

The targeting of CD44 molecule and its ligand provides, however, new opportunities in establishing specific therapies for cancer and inflammatory diseases. CD44 is a cell surface glycoprotein involved in many vital normal bioactivities, including interaction between cells and extracellular tissues, support of cell migration in the blood vessels and inside the tissues, presentation of growth factors, cytokines, chemokines and enzymes to other cells or to the surrounding tissues, as well as signal transmission from the cell surface to the interior of the cell, leading to programmed cell death or cell survival and proliferation [1,2]. While hyaluronic acid (HA) is the principal ligand of CD44, this receptor can interact with many other cell surface and extracellular matrix (ECM) components [1–3]. Cells involved in pathological activities use CD44 to maintain at least some of the above-mentioned functions, but with destructive outcomes. For example, in a normal setting, cell surface CD44 supports the migration of inflammatory cells from the blood circulation toward sites of bacterial infection [4], resulting in the killing of the bacteria. Under pathological conditions, CD44 can support the migration of metastatic cells from the site of the primary tumor growth to remote organs or the migration of auto-destructive inflammatory cells to potential sites of inflammation [1,5]. We and others have shown in animal models that monoclonal antibodies (mAbs) against CD44 can markedly reduce the pathological activities of malignant lymphoma [6], juvenile diabetes [5], collagen-induced arthritis [7,8], experimental colitis [9] and experimental allergic encephalomyelitis (analogous to human multiple sclerosis) [10], possibly by interfering with cell migration or inducing apoptosis in the target cells. However, such anti-CD44 mAbs can simultaneously target normal cells expressing CD44, causing damage to essential biological functions.

CD44, however, is not a single molecule. The genomic structure of CD44 includes five constant exons at the 5′ end, five (or four) constant exons at the 3′ end and 10 (mouse) or 9 (man) variant exons inserted between the two constant exons (Fig. 1A). Alternative splicing, can theoretically generate by differential utilization of the 10 (or 9) variant exons over 800 isoforms [11], of them a few dozen have been already discovered. Direct splicing of constant exon 5 to constant exon 16 (Fig. 1A), skipping the entire variable region, generates the most ubiquitous isoform-standard CD44 (CD44s), predominantly expressed on hematopoietic cells [3]. Modifications in the alternative splicing regulation, either genetically inherited, or induced, for example, in the stressed pathological site, may further enrich the molecular diversity of CD44 by giving rise to CD44 variants (CD44v) with extra sequences illegitimately transcribed from the end of an intron adjacent to junction sites of the alternative splicing [12]. Such a mechanism may generate exclusive CD44 variants expressed on cells engaged in pathological activities, i.e., disease-specific CD44 molecules. The present communication describes disease-specific CD44 variant expressed on synovial fluid cells of rheumatoid arthritis patients (designated CD44vRA). The inclusion of extra trinucleotide in CD44vRA allows translation of an extra alanine, resulting in the generation of novel immunogenic epitope, which enabled the production of functional anti-CD44vRA mAb. This anti-CD44vRA mAb can induce apoptosis in synovial fluid cells from rheumatoid arthritis (RA) patients, but not in peripheral blood leukocytes (PBLs) from the same patients.

2. Materials and methods

2.1. Patients

Fifty-five patients (30–75 years old) were included in our study, of them 35 with RA, eight with psoriatic arthritis (PSA) and 12 with osteoarthritis (OA). The patients had been diagnosed with RA, PSA or OA according to the American College of Rheumatology (ACR) criteria. Patients treated with anti-TNFα reagents were excluded from this study. All patients in this study underwent an arthrocentesis of the joint, owing to acute synovitis. Institutional ethics committee approval and written informed consent were obtained before any protocol specific procedures were undertaken.

2.2. Cloning and transfection of human CD44vRA, CD44v3–v10 and CD44s

For cloning human CD44vRA cDNA, the total synovial fluid cell population of RA patients undergoing joint aspiration was isolated. RNA was separated with a commercial kit (Promega, Madison, WI), CD44vRA cDNA was prepared by RT-PCR (PTC-100™ Programmable Thermal Controller; MJ Research, Watertown, MA), using primers representing the constant coding regions of CD44 (see Fig. 1A): Ex1-sense, 5′-GAATTCGCCGCCACCATGGACAAGTTTTGGTGG-3′; Ex20-antisense, 5′-TCTAGATTACACCCCAATCTTCATG-3′.

PCR product size was confirmed by agarose gel electrophoresis, sequencing (ABI PRISM 310, Perkin-Elmer, Wellesley, MA) or *Pst*I (New England BioLabs, Beverly, MA) digestion (the nucleotide insertion in CD44vRA introduces a *Pst*I digestion site). The PCR product was excised from the gel, purified and subcloned into the pGEM vector (Promega). Positive clones were selected by white/blue screening. Plasmids were purified with a commercial kit (Promega), subjected in *Eco*RI/*Xba*I-double digestion and cloned into the pcDNA3.1 vector (Invitrogen, Paisley, UK) in which the gene product was expressed. The plasmid was transfected into the CD44-negative Namalwa Burkett lymphoma cell line (ATCC, Manassas, VA) as described [13]. For cloning of human CD44v3–v10, RNA was isolated from human keratinocytes (a gift from Dr. Ben-Basat, Hadassah University Hospital, Jerusalem), and for cloning of human CD44s, RNA was isolated from the HeLa cervical cancer cell line

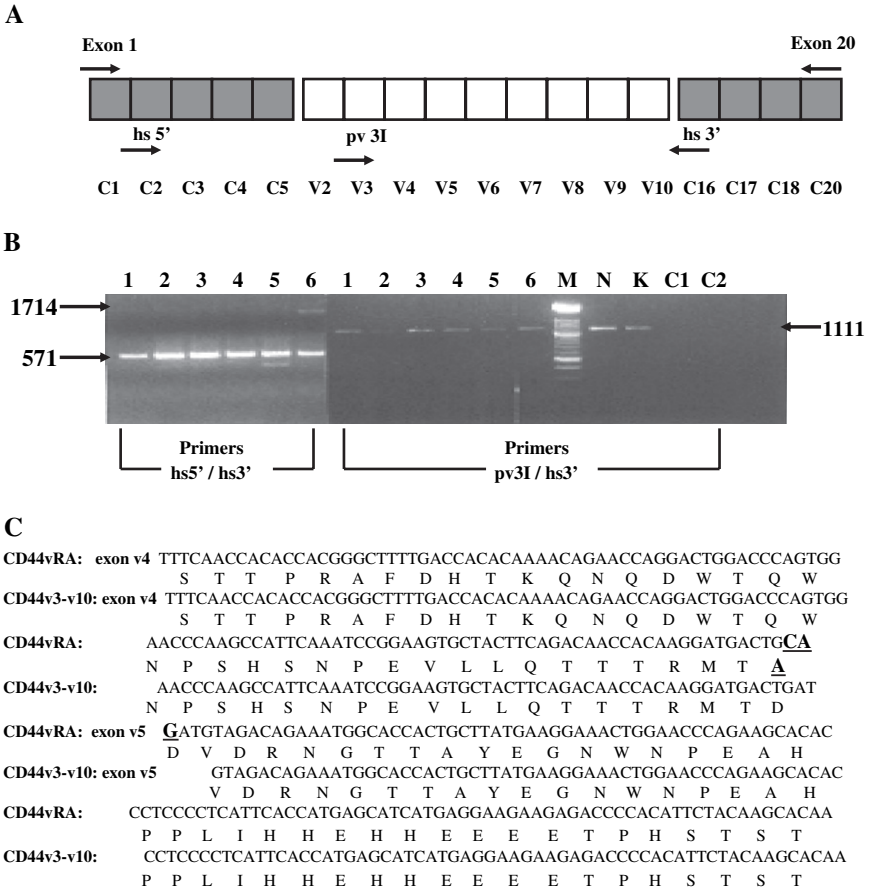

Fig. 1. Schematic description of the CD44 molecule and the trinucleotide CAG inclusion in the CD44v3−v10 variant of RA patients. (A) A schematic diagram of the CD44 genomic map. The black squares represent the constant exons (designated C1, C2, etc.) at the two ends of the molecule. The white squares represent the variant exons (designated v2, v3, etc.) subjected to alternative splicing. Differential utilization of the variant exons generates the different CD44 isoforms, e.g., utilization of exons v3−v10 in tandem forms the CD44v3−v10. Note that exon v1 is not included in human CD44. Arrows mark the positions and directions of exon 1 sense and exon 20 antisense primers used to isolate the CD44 transcripts, as well as the hs5′, hs 3′ and pv3I primers (for sequence, see Section 2) used in the RT-PCR described in B. (B) RT-PCR analysis of synovial fluid cells from RA patients. RT-PCR of total RNA derived from synovial fluid cells of six RA patients and keratinocytes from one donor (marked with K). Primers hs5′/hs3′ were used to detect the CD44s transcripts (571 bp). The faint signals at 1714 bp represent the CD44v3−v10 transcripts, which are dominated by CD44s transcripts. To see the CD44v3−v10 transcripts (1111 bp) more clearly we used the pv3I/hs3′ primers (see A). N-positive control: Namalwa cells expressing CD44vRA. C1- and C2-negative control: samples without RNA and cDNA, respectively. M − markers' ladder. (C) The entire nucleotide and amino acid sequences of CD44vRA exons v4 and v5 with alignment to the corresponding CD44v3−v10 wild type sequences derived from normal human primary keratinocytes. CAG and alanine inclusion in CD44vRA sequence are shown in bold letters.

(obtained from ATCC), using the above-described protocol. Transfection of CD44vRA, CD44v3−v10 and CD44s cDNAs as well as of the pcDNA3.1 vector (''empty vector'') was performed as described above. Accordingly, the transfected Namalwa cells were designated Namalwa-CD44vRA, Namalwa-CD44v3−v10, Namalwa-CD44s and Namalwa-Neo, respectively.

2.3. Preparation of soluble hCD44v3−v10, hCD44vRA and hCD44s plasmids

The soluble CD44v3−v10 cDNA was cloned from total RNA of primary human keratinocyte by RT-PCR amplification, using two primers assigned from the published CD44 sequence: Ex1s, 5′-TATCTAGAGCCGCCACCATGGACAAGT TTTGGTGG-3′; Ex16/17as, 5′-TATCTAGAGCCATTCTGGA ATTTGGGGTGT-3′.

Both primers contained a *Xba*I recognition site. The PCR products were digested with *Xba*I enzyme and pCXFc zeovector was digested with *Nhe*I enzyme. After digestion, the PCR products were ligated into the pCXFc zeovector to generate CD44v3−v10-immunoglobulin (Ig)-Fc recombinant. Using the same protocol, the soluble CD44vRA and soluble CD44s cDNAs were cloned from synovial cells of a rheumatoid arthritis patient. The soluble CD44 fragments were assigned from the published sequence of CD44 (1−1824 bases) [14].

2.4. Transient transfection of the soluble CD44 plasmids into 293T cells

A quantity of 3 μg of each one of the above-indicated Fc containing plasmids was incubated for 20 min with 12 μl of FuGene 6 (Hoffmann-La Roche, Basel, Switzerland). The mixture was added into 15 cm cell plates containing 70%

confluent of 293T cells. Supernatant was collected after 48 h and 72 h. The CD44-IgFc fragmented proteoglycans were purified on protein-G column and analyzed for correctness by SDS-PAGE and immunoblotting with antibody recognizing a constant epitope shared by all CD44 isoforms, designated anti-pan-CD44 (or anti-CD44s) mAb (Hermes-3 derived from American Type Culture Collection, ATCC).

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was extracted from synovial fluid cells of RA patients, primary human keratinocytes or Namalwa-CD44vRA cells, using RNA-BEE reagent (RNA isolation solvent, Tel-Test, Friendswood, TX) according to the manufacturer's instructions. Reverse transcription was performed with 5 units of AMV reverse transcriptase (Promega) in a 20 μl reaction volume containing 50 mM Tris–HCl, pH 8.3, 50 mM KCL, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT) and 20 units RNasin (Promega), using 500 ng of RNA and 100 ng of oligo d(T)18 primer (Promega). Reaction samples were incubated for 1 h at 41 °C and then the reverse transcriptase was inactivated by heating the mixture for 10 min at 65 °C. The amplification was performed in a micro-processor-controlled incubator (MiniCycler™, MJ Research), using 0.5 μl of the reverse transcriptase reaction product (cDNA) in a final volume of 50 μl containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0, 250 μM dNTPs and 2.5 units *Taq* DNA polymerase (Promega). The following primers (see Fig. 1A) were added to reaction mixture: hs 5′ sense, 5′-GATGGAGAAAGCTCTGAGCATC-3′; pv3I sense, 5′-ACGTCTTCAAATACCATCTC-3′; hs 3′ antisense, 5′-TTTGCTCCACCTTCTTGACTCC-3′.

The CD44 amplification was carried out for 30 cycles with denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 2 min, followed by 10 min final extension at 72 °C. The amplified products were resolved on 1.5% agarose gel. Determination of the cellular CD44 isoform transcripts was based on the position of the bend in relation to the markers' ladder, and on the expected bp size of the different CD44 variants.

2.6. Generation of monoclonal antibody secreting hybridomas

Thirty mer CD44vRA peptide (SNPEVLLQTTTRMTANPSHSNPEVLLOTTT) obtained from Corixa (Seattle, WA) and/or 100 μg/ml soluble CD44vRA produced in our laboratory, both emulsified in complete Freund's adjuvant (CFA) (Sigma, St. Louis, MD), were used to immunize subcutaneously or intramuscularly 8-week-old female C57BL/6 mice. The immunization was repeated on days 14 and 28 and 2 weeks later the mice were bled and their sera were tested by flow cytometry for their ability to bind to Namalwa cells expressing CD44. The animals with highest polyclonal anti-CD44 antibody titers were selected and boosted intraperitoneally (i.p.) with 10^8 Namalwa-CD44vRA cells. After 72 h, spleen cells were harvested and fused with SP 2/0 myeloma cells according to Kohler and Milstein [15]. After 1 day of incubation in enriched RPMI 1640 (Sigma) containing L-glutamine, Pen–Strep solution, sodium pyruvate and MEM-Eagle non-essential amino acids (Biological Industries, Bet-Haemek, Israel) and 20% fetal bovine serum (FBS) (Sigma) the hybridomas were grown in ClonaCell™-HY Hybridoma Selection Medium (medium D, StemCell Technologies, Vancouver, BC). Between days 10–14, separated hybridoma colonies were collected from the semi-solid agar and grown in 96-well plates (Costar, Milpitas, CA) in enriched RPMI 1640 medium containing HAT media supplement (Sigma) and 20% of FBS. At day 7, the supernatants from isolated hybridoma clones were screened by flow cytometry for their ability to bind to Namalwa-Neo, Namalwa-CD44v3–v10 or Namalwa-CD44vRA cells. Hybridoma whose supernatants bound selectively or preferentially to Namalwa-CD44vRA were cloned by limiting dilution and then recloned for additional three cycles. The isolated hybridoma was maintained in enriched RPMI 1640 containing HAT media supplement and 20% FBS. The isotype of the CD44vRA-positive hybridoma supernatant was determined by enzyme-linked immunosorbent assay (ELISA) using Clonotype System-HRP (Southern Biotechnology Associates, Birmingham, Alabama). Monoclonal antibodies were partially purified from hybridoma supernatants, using G-protein column.

2.7. Flow cytometry analysis

A quantity of 10^6 cells were incubated with 3G5 anti-pan-CD44s (Hermes 3, IgG1; ATCC) F-10-44-2 anti-pan-CD44 (known also as anti-CD44s) mAb (IgG2b, Serotec, Oxford, UK), VFF7 anti-CD44v6 mAb (IgG1, Bender MedSystem, Vienna, Austria), anti-CD44v9 mAb (ATCC) or anti-CD44vRA for 45 min on ice. After extensive washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary anti-Ig antibody (Jackson ImmunoResearch, West Grove, PA) for 30 min on ice. The cells were then washed and analyzed with a Flow Cytometry (Becton Dickinson, San Jose, CA).

2.8. Enzyme-linked immunosorbent assay (ELISA)

Polystyrene plates of 96 wells (Nunc, Roskilde, Denmark) were coated with purified CD44vRA-IgFc, CD44v3–v10-IgFc, CD44s-IgFc soluble proteins or with BSA (100 μg/ml/well diluted in 100 μl sodium acetate buffer, pH 7.0). After overnight incubation at 4 °C, the plate was washed three times with phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20 (PBS/T). Following blocking with 10% milk in PBS at 37 °C for 2 h, different concentrations of F8:33 anti-CD44vRA mAb or anti-pan-CD44 (Hermes 3) mAb were added to the wells. The plates were incubated at 37 °C for 1 h, washed and a secondary goat anti-mouse polyvalent peroxidase-conjugated antibody (Jackson ImmunoResearch, Palo-Alto, CA) was added for an additional 1 h. The enzyme reaction was developed with 0.04% H_2O_2 and 0.04% *o*-phenylenediamine in phosphate–citrate buffer, pH

5.0 and stopped after 20 min by addition of 25 μl 4N H_2SO_4. The optical density (OD) was measured at 405 nm on a microplate reader MRX (Dynatech Laboratories, Chantilly, VA) and values above 0.1 OD were considered positive.

2.9. Western blot analysis

Cells were lyzed in NP-40 buffer and 100 μg of protein, run on denaturing SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). Blots were blocked with 1% BSA in PBS containing 0.1% Tween-20 (PBS-T), and incubated for 1 h with 1 μg/ml Hermes-3 anti-pan-CD44 mAb, which was obtained from ATCC hybridoma supernatant and purified on a protein-G column. The blots were rewashed in PBS-T, incubated with the appropriate HRP-conjugated anti-Ig secondary antibody (1:10,000 dilution) (Jackson ImmunoResearch) for 45 min, rewashed in PBS-T and developed with ECL reagent (Amersham Biosciences, Buckinghamshire, UK).

2.10. Flow cytometry analysis of apoptosis

Apoptosis was induced by culturing 2×10^6 synovial fluid cells from RA patients with different concentrations of anti-CD44vRA mAb or with commercial anti-pan-CD44 mAb diluted in the serum-free RPMI 1640 for 16 h at 37 °C in CO_2 incubator. Synovial fluid cells incubated with 5 μg/ml doxorubicin served as a positive control. Peripheral blood leukocytes (PBLs) from the same patients, as well as from synovial fluid cells from OA patients underwent identical treatment with antibody. After the antibody treatment the cells were washed once in PBS and resuspended in 100 μl of annexin V binding buffer (Pharminogen, San Diego, CA). Five microliters of fluorescein isothiocyanate (FITC)-conjugated annexin V (Pharmigen) and 5 μg/ml propidium iodide (Sigma) were added to the cells. The cells were mixed gently and incubated for 15 min at room temperature in the dark, then diluted with 400 μl binding buffer and analyzed by two color flow cytometry (Becton Dickinson Inc, Franklin Lakes, NJ). Apoptotic cells were distinguished from normal cells by labeling with annexin V and inclusion of propidium iodide. Events accumulated in the lower right hand side quadrate of the panel represent cells in early apoptosis, those accumulated in the upper right hand side quadrate represent cells in late apoptosis, and those at the lower left hand side quadrate represent survived cells.

2.11. Collagen-induced arthritis

Generation of collagen-induced arthritis (CIA) in mice and assessment of inflammation by measuring footpad swelling were performed as we described previously [7]. Briefly, Type II collagen was injected twice 3 weeks apart, into DBA/1 mice. The development of joint inflammation was assessed by measuring footpad swelling with a micro-caliper in mice marked by numbers, decoded only at the end of the experiment, and then assigned to the different groups. Anti-CD44 antibodies (150 μg/injection) were injected at onset of disease (when the footpad swelling measurement was >1.7 mm) and then every other day for 12 days. This protocol was approved by the institute's Animal Ethics Committee.

2.12. Statistical analysis

Data were analyzed using microcomputer programs for one-way ANOVA, followed by Student's *t*-test for unpaired values. $P < 0.05$ was considered significant. The results are expressed as the mean ± s.e.m. Each experiment was repeated at least three times, all showing similar results.

3. Results

3.1. The mRNA of CD44 expressed on synovial fluid cells of RA patients contains intron-derived extra trinucleotide

Synovial fluid cells from RA patients (comprising approximately 70% polymorphonuclears and macrophages; the remaining are T and B lymphocytes, Ref. [12]) were isolated following joint aspiration. Their total RNA was reversed transcribed and subjected to PCR, using primers representing the constant coding regions of CD44 (hs 5′ and hs 3′ in Fig. 1A). Fig. 1B shows the RT-PCR of samples from synovial fluid cells derived from the joint of six representative RA patients. Two major signals were detected: a fast-migrating band (571 bp) corresponds to CD44s, and a slow-migrating band (1714 bp) corresponds to CD44v3−v10, which is also expressed on keratinocytes (Fig. 1B). As the 1714 bp bands are faint (they are dominated by the CD44s transcripts), we included in the RT-PCR a third primer (pv 3I in Fig. 1A), representing a coding region from v3 variant exon, coupled to the antisense constant region primer (hs 3′). The 1111 bp bands represent the CD44v3−v10 transcripts (Fig. 1B). These findings were confirmed by direct sequencing (not shown).

The CD44 variant (CD44vRA or CD44v3−v10) was detected in samples of synovial fluid cells derived from all 35 RA and eight PSA patients, who were included in the project. Five of 12 samples from synovial fluid cells of OA patients displayed the CD44v3−v10 variant. Extra trinucleotide (CAG) was detected between exon v4 and exon v5 (Fig. 1) in 27 of 35 RA patients and 6 of 8 PSA patients following computerized alignment versus the wild type variant-CD44v3−v10 described in Ref. [14] (reported in Ref. [12] and confirmed in Fig. 1C). The wild type variant, CD44v3−v10, was detected in synovial fluid cells of the remainder patients, i.e. 8 of 35 RA patients and 2 of 8 PSA patients. The CAG trinucleotide was transcribed from the extreme end of the intron bridging exon v4 to exon v5, precisely at the splicing junction. This trinucleotide allows the translation of alanine without interfering with the reading frame of CD44 transcript. Rheumatoid arthritis-derived CD44v3−v10 with extra CAG was designated CD44vRA.

3.2. Production of anti-CD44vRA mAb

Expression of extra alanine in CD44vRA may induce the inclusion of a novel immunogenic epitope, allowing the

generation of mAbs able to discriminate between the RA variant and the wild type isoform-CD44v3−v10 expressed on keratinocytes. CD44vRA, CD44v3−v10 (derived from human keratinocytes) and CD44s (derived from HeLa cells) cDNAs were transfected into CD44-negative Namalwa Burkitt lymphoma cell line. CD44 transfectants expressing high levels of CD44s, as well as CD44v3−v10 and CD44vRA transfectants expressing equal levels of v6-containing CD44 were selected (Fig. 2A). They were designated Namalwa-CD44s, Namalwa-CD44v3−v10 and Namalwa-CD44vRA, respectively. Namalwa cells transfected with the empty vector were designated Namalwa-pcDNA3.1 or Namalwa-Neo. C57BL/6 mice were immunized with synthetic peptide or soluble CD44vRA, both incorporated into CFA and challenged with Namalwa-CD44vRA cells (for details, see Section 2). Splenocytes from mice showing the highest concentration of polyclonal anti-CD44v antibodies in their serum were fused with SP 2/0 myeloma cells. Hybridoma cell clones were selected according to the ability of their supernatants to bind to Namalwa-CD44vRA, but not or much less, to Namalwa-CD44v3−v10, Namalwa-CD44s or to Namalwa-Neo, as indicated by flow cytometry. Clones and subclones were established from a positive hybridoma, and they were stable in culture for over 8 months. Anti-CD44vRA mAbs from supernatants of positive hybridomas,

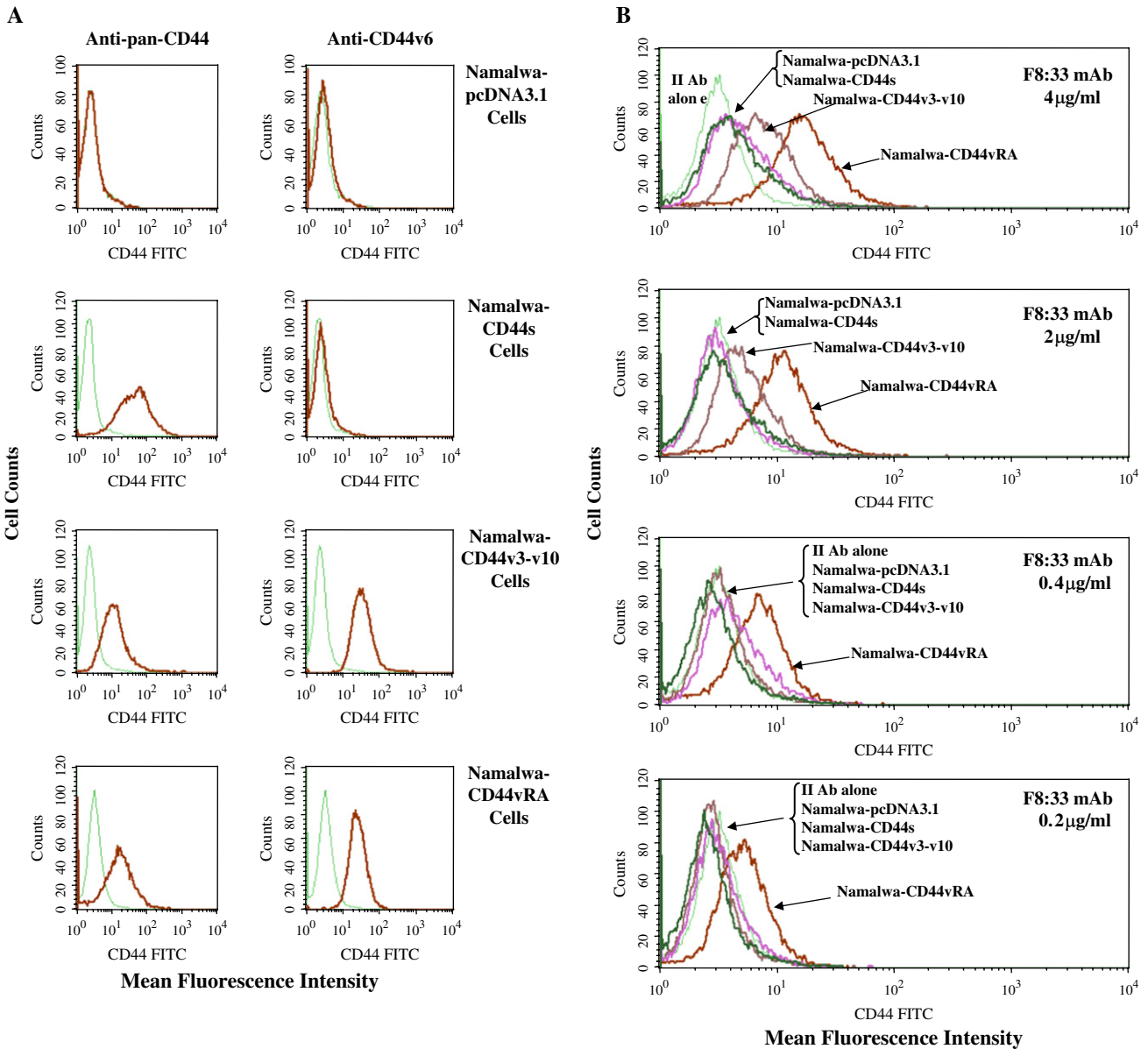

Fig. 2. Selective binding of F8:33 anti-CD44vRA mAb to Namalwa cells transfected with CD44vRA (Namalwa-CD44vRA). (A) Binding of commercial antibodies. Binding of commercial anti-pan and anti-variant CD44 (anti-CD44v6) mAbs to Namalwa-Neo (transfected with empty vector-pcDNA3.1) and Namalwa-CD44s (transfected with standard CD44 cDNA) as well as to Namalwa-CD44v3−v10 and Namalwa-CD44vRA (transfected with CD44v3−v10 and CD44vRA cDNAs, respectively) was detected with fluorescein-labeled anti-mouse Ig (second antibody). First histogram (faint line) in each panel: binding of second antibody only. (B) Binding of anti-CD44vRA mAb: binding of F8:33 anti-CD44vRA to Namalwa transfectants was detected with fluorescein-labeled anti-mouse IgG (second antibody). First histogram in each panel: binding of the second antibody (IIAb) only. A representative finding of at least 10 different experiments.

designated F8:33 or MFI-16-11, were purified on G-protein column. Flow cytometry further revealed that at concentrations of 0.2 μg/ml or 0.4 μg/ml (Fig. 2B, Table 1), F8:33 anti-CD44vRA mAb interacts with Namalwa-CD44vRA, but not with the other transfectants, including Namalwa cells transfected with the wild type CD44v3–v10 cDNA. At a concentration of 2 μg/ml or higher F8:33 cross-reacts with Namalwa-CD44v3–v10 (Table 1) and at higher concentrations (>100 μg/ml) – with Namalwa-CD44s and Namalwa-Neo cells as well (not shown). Isotype-matched control antibody did not interact, at these concentrations, with the Namalwa transfectants (not shown). This finding was confirmed by ELISA: F8:33 mAb bound, in a dose-dependent manner, to CD44vRA-coated microwells at higher rates than to CD44v3–v10 or CD44s-coated microwells (Fig. 3A). In contrast, anti-pan-CD44 mAb similarly binds to CD44vRA and CD44v3–v10 (Fig. 3B), while it does not bind to CD44s, presumably due to its inability to recognize the relevant epitope. The molecular masses of the soluble CD44 proteins were confirmed by Western blot (Fig. 3C). The isotype of F8:33 is IgG2a, as determined by ELISA.

3.3. Exclusive targeting of RA synovial fluid cells by F8:33 anti-CD44vRA mAb

The Namalwa transfectants are not, however, the authentic targets for F8:33 anti-CD44vRA mAb. Rather, the cells donating the transfected CD44vRA and CD44v3–v10 transcripts, i.e., synovial fluid cells and keratinocytes, respectively, should be tested for their ability to interact with this antibody. Flow cytometry analysis revealed slightly higher expression of CD44s on synovial fluid cells of an RA patient than on keratinocytes derived from two donors, with variations in expression of v6-containing CD44. Note that keratinocytes expressing v9-containing CD44 (which is included in CD44v3–v10) at much higher level than RA synovial fluid cells (Fig. 4A). However, at even as wide a range of concentrations as 2–100 μg/ml, F8:33 anti-CD44vRA mAb interacts with synovial fluid cells from RA patients (Fig. 4B and Table 1), but neither with keratinocytes (Fig. 4B and Table 1) nor or hardly with synovial fluid cells from OA patients (Fig. 5 and Table 1), as indicated by flow cytometry. Even at as high a concentration as 100 μg/ml F8:33 selectively bound to RA synovial fluid cells, whereas at a concentration of 200 μg/ml F8:33 cross-reacts with keratinocytes and synovial fluid cells from OA patients (Table 1). Note that the synovial fluid cells were identified as CD44vRA-positive cells by *Pst*I digestion of their DNA. Keratinocytes constitutively express CD44v3–v10 (3 and our own observations). Next we found that anti-CD44vRA mAbs from two different clones (F8:33 and MFI-16-11) bound, at a concentration of ≈10 μg/ml, to synovial fluid cells from an RA patient, but they only marginally bound to PBL from the same patient or to synovial fluid cells from an OA patient (Fig. 6). Anti-pan-hCD44 mAb (Hermes 3) bound to all three types of cells and anti-hCD44v6 mAb (recognizing CD44 containing v6 epitope), bound exclusively to synovial fluid cells of OA patients (Fig. 6), emphasizing the specificity of F8:33 and MFi-16-11 binding.

3.4. Anti-CD44vRA mAb induces apoptosis in synovial fluid cells of RA patients

Does the exclusive binding of anti-CD44vRA mAbs deliver any biological signal into synovial fluid cells of RA patients? To challenge this question, we analyzed by two-dimensional flow cytometry the ability of F8:33 anti-CD44vRA mAb to induce apoptosis in synovial fluid cells and PBLs of RA patients. Increasing the dose of F8:33 anti-CD44vRA, gradually increased the apoptosis in synovial fluid cells of RA patients, but not in the PBL of the same patients, as indicated by flow cytometry double staining with annexin v and propidium iodide in samples of one patient (Fig. 7A) and by graphical description of such a double staining analysis in samples of a different patient (Fig. 7B). Note that the level of apoptosis differs from one patient to another, and that the anti-pan-hCD44 mAb (Hermes 3) induces apoptosis in synovial fluid cells of RA patients at the highest concentrations only (Fig. 7). Similar

Table 1
Summary of the binding capability of F8:33 anti-CD44vRA mAb to different types of cells

F8:33 mAb (μg/ml)	Ability to bind to[a]					
	Namalwa cells expressing CD44vRA	Namalwa cells expressing CD44v3–v10	SFC expressing CD44vRA from RA patient	PBL expressing CD44s from RA patient	SFC expressing CD44v3–v10 from OA patient	Human keratin[b] expressing CD44v3–v10
0.2	+	–	–	–	–	–
0.4	+	–	–	–	–	–
2	+	+	+	–	–	–
4	+	+	+	–	–	–
20	+	+	+	–	–	–
40	+	+	+	–	–	–
100	+	+	+	–	–	–
200	+	+	+	–	+	+

SFC, synovial fluid cells; RA, rheumatoid arthritis; OA, osteoarthritis; and PBL, peripheral blood leukocytes.
[a] +, Indicates binding of antibody above 10^1 MFI.
[b] Human keratinocytes from healthy donors.

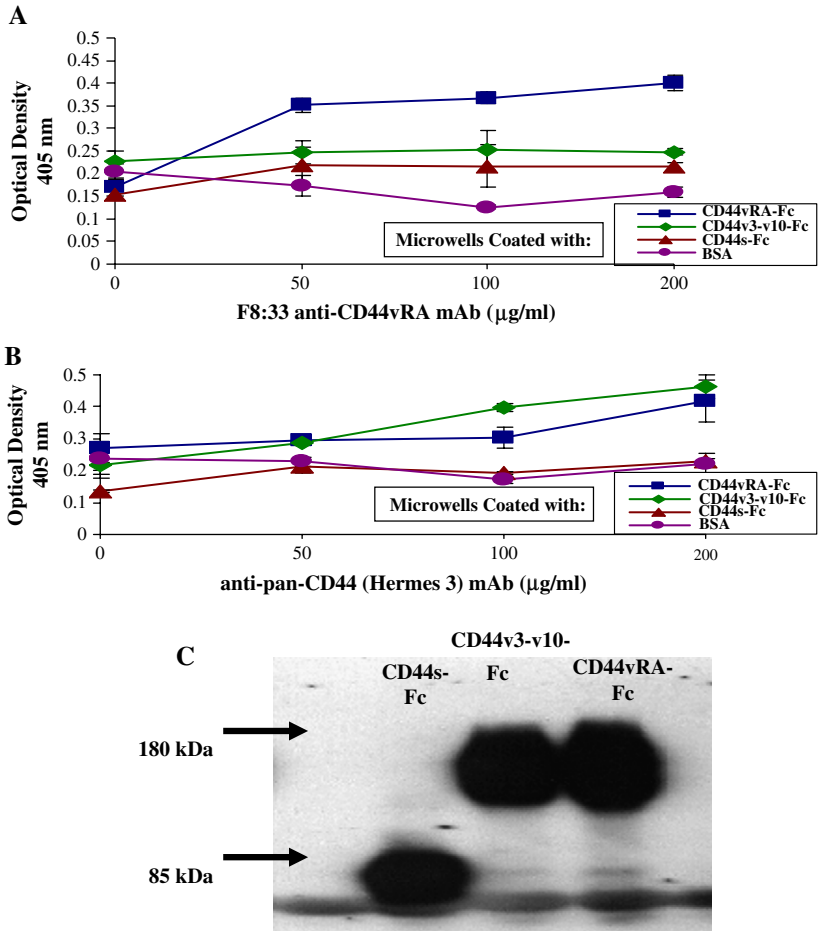

Fig. 3. Analysis of F8:33 anti-CD44 mAb by ELISA. F8:33 anti-CD44vRA mAb (A) and anti-pan-CD44 mAb (Hermes-3) (B) were tested for their ability to bind to microwells coated with soluble CD44vRA, CD44v3−v10, CD44s and CD44-Neo. The binding was detected by a colorimetric assay at optical density of 405 nm. (C) Western blot analysis of CD44s-Fc, CD44v3−v10-Fc and CD44vRA-Fc confirms the molecular mass of these soluble proteins.

results were detected in samples of at least additional 30 patients. While increasing the doses of F8:33 anti-CD44 mAb (but not anti-pan-hCD44 mAb) gradually enhanced the apoptosis in synovial fluid cells of a still another patient (Fig. 8A), the same antibody failed to enhance, or enhanced less significantly, apoptosis in synovial fluid cells of two OA patients (Fig. 8B), further stressing the specific apoptotic effect of F8:33. Note that the synovial fluid cells of the patients depicted in Fig. 8 display relatively high levels of spontaneous apoptosis.

3.5. Injection of anti-CD44vRA mAbs attenuates collagen-induced arthritis in DBA/1 mice

Although the anti-CD44vRA mAbs are human-not mouse-specific and despite the fact that we did not detect CD44vRA in the joints of arthritic DBA/1 mice, we decided to verify the ability of F8:33 and MF1-16-11 anti-human CD44vRA mAbs to reduce joint inflammation in DBA/1 mice displaying collagen-induced arthritis (CIA). Such mice were named also arthritic mice. We were prompted to perform this *in vivo* experiment, because anti-human F8:33 bound to spleen cells of arthritic mice, significantly increasing apoptosis above the spontaneous level. In contrast, this antibody failed to bind to and increase apoptosis in spleen cells from normal mice (results not shown). In this context it should be stressed that spleen cells from arthritic mice express both CD44s and CD44v3−v10, whereas spleen cells from normal mice express CD44s only. First, we confirmed our previous finding [7,8] showing that injection of KM81 anti-mouse pan-CD44 mAb at the onset of CIA and then every other day for 12 days, reduced joint inflammation versus that of similarly treated mice administered isotype-matched control antibody. Interestingly, injection, under the same experimental protocol, of F8:33 or MFI-16-11 anti-human CD44vRA mAbs reduced also joint inflammation in the arthritic mice, albeit slightly less effectively (Fig. 9). Further on, we shall discuss our explanation for this cross reactivity, showing that the anti-CD44vRA mAbs are effective in the animal model of CIA.

4. Discussion

We previously showed that the CD44v3−v10 transcript of synovial fluid cells from the joints of RA patients (designated CD44vRA) contains an extra trinucleotide, CAG, which is not

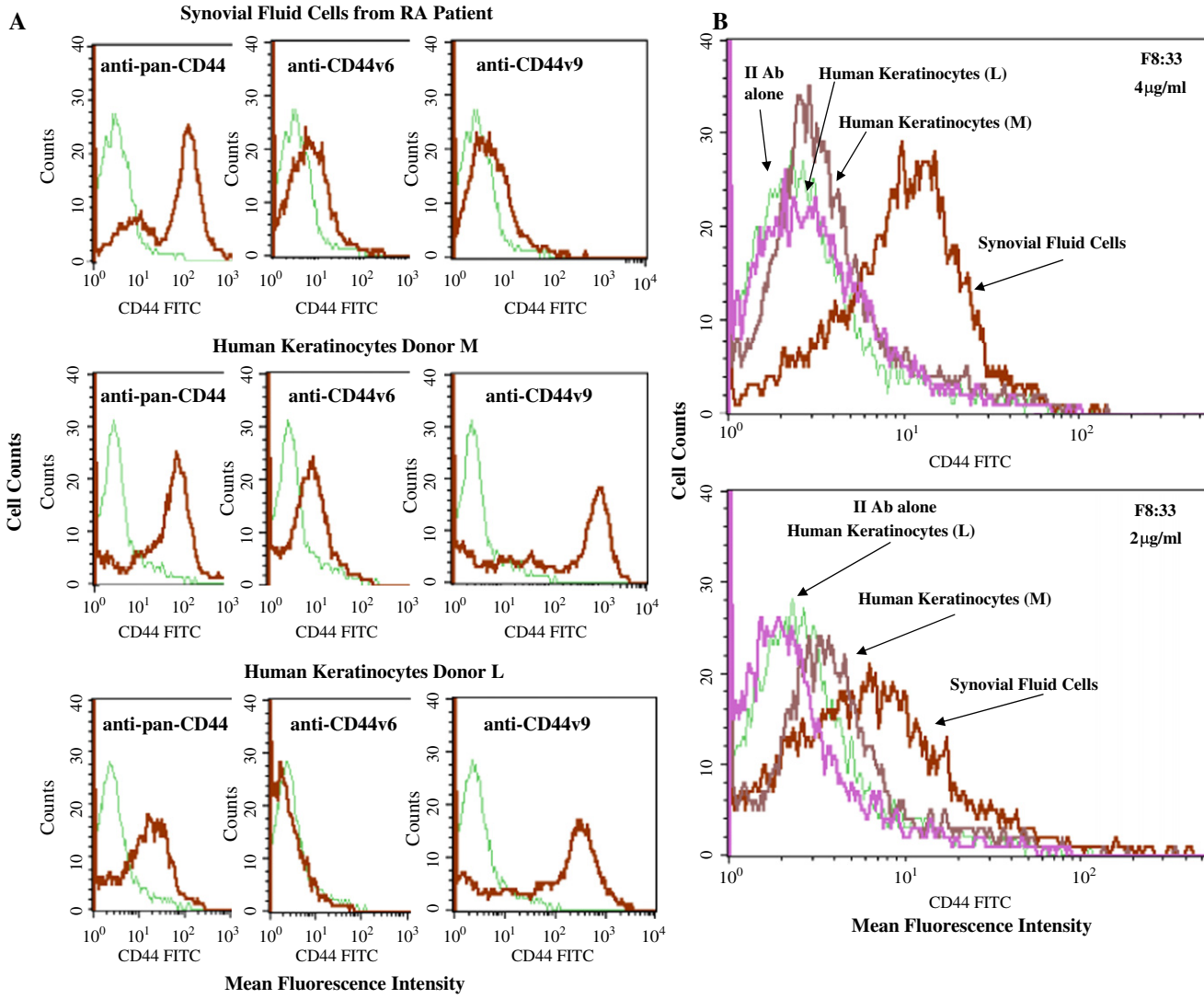

Fig. 4. Exclusive binding of F8:33 anti-CD44vRA mAb to CD44vRA-positive (detected by *Pst*I digestion) synovial fluid cells from an RA patient. (A) Binding of commercial anti-pan-CD44 and anti-variant (anti-CD44v6, anti-CD44v9) mAbs to synovial fluid cells from the joint of an RA patient and to primary human keratinocytes. First histogram in each panel: binding of second antibody only. (B) Binding of F8:33 anti-CD44vRA to synovial fluid cells from an RA patient and primary keratinocytes derived from L and M donors. Binding of F8:33 anti-CD44vRA mAb was detected with fluorescein-labeled anti-mouse-Ig antibody (second antibody). The faint histogram depicts binding of second antibody (II Ab) only. A representative finding of samples from at least five patients.

included in the corresponding wild type molecule, CD44v3−v10, predominantly expressed on primary keratinocytes [12]. We now describe the generation of mAbs exclusively recognizing CD44vRA and inducing apoptosis in synovial fluid cells from RA patients, but not in their PBLs.

The "illegitimate" transcription of the intronic CAG flanking exon v5 of CD44vRA is presumably a consequence of misregulation of the splicing machinery in this molecule. Unknown genetic or environmental factors or a combination of both may modify the relative abundance, tissue distribution or activity of serine−arginine (SR) or of heterogeneous nuclear ribonucleoprotein (hnRNP) splicing factors that antagonistically control the differential splicing [16]. The CAG-containing splicing junction may be particularly susceptible to such changes, resulting in the inclusion of CAG in the CD44 mRNA sequence of RA patients. It should be mentioned that mutations located in non-coding regions, such as those affecting 5′ and 3′ splice sites, branch sites or polyadenylation signals, are frequently (∼15%) the cause of genetic diseases [17].

The inclusion of CAG in the CD44 mRNA allows insertion of alanine into the translated cell surface CD44 glycoprotein without interfering with the reading frame of the entire molecule. This creates a novel immunogenic epitope that is sufficient to allow the production of mAbs (e.g., F8:33 and MFI-16-11) recognizing CD44vRA expressed on synovial fluid cells of RA patients. The antibodies bound to CD44vRA, coated on microplates or expressed on Namalwa cells, at higher intensity than to the corresponding wild type molecule CD44v3−v10 or CD44s. Notably, CD44vRA and CD44v3−v10 are expressed to a similar extent on Namalwa cells, whereas CD44s is expressed on these cells at an even higher level, indicating that the

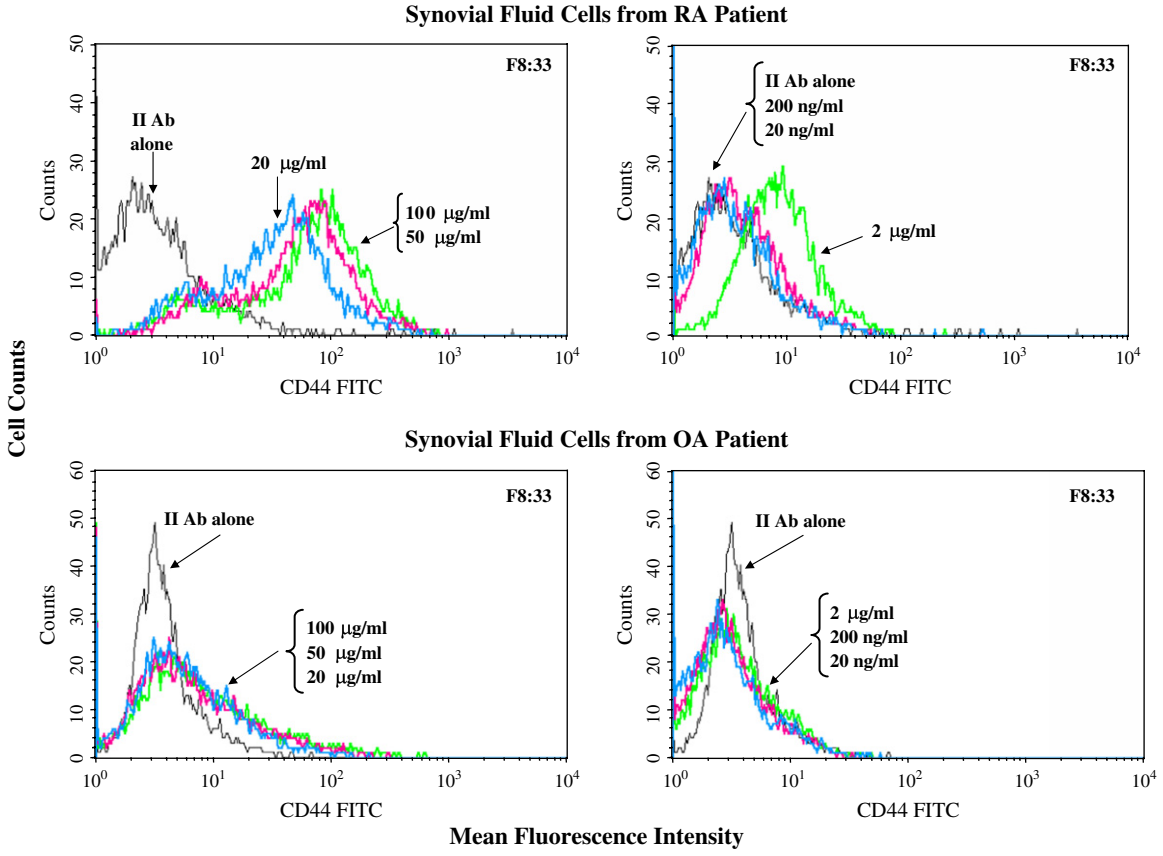

Fig. 5. Anti-CD44vRA mAb discriminates between synovial fluid cells from rheumatoid arthritis and osteoarthritis patients. Binding of different concentrations of F8:33 anti-CD44vRA mAbs to synovial fluid cells isolated from the joints of RA or OA patients was detected by fluorescein-labeled anti-mouse Ig (second antibody). The faint histogram in each panel depicts binding of second antibody only.

preferential binding of F8:33 to Namalwa-CD44vRA is not quantitatively dictated. The selective binding of F8:33 anti-CD44vRA mAb to Namalwa-CD44vRA was detected at concentrations ≤ 0.4 μg/ml. At increasing concentrations, F8:33 first cross-reacts with Namalwa-CD44v3–v10 (Table 1) and then with Namalwa-CD44s (not shown), implying the existence of a hierarchy in binding affinity, the highest to cell surface CD44vRA and the lowest to cell surface CD44s.

However, the Namalwa transfectants are, in fact, an artificial model for evaluating the binding capacity and bioactivity of anti-CD44vRA mAbs. To obtain a more realistic assessment, the interaction of F8:33 with authentic RA synovial fluid cells and primary keratinocytes was examined. We chose keratinocytes as a reference group, because they are known expressors of CD44v3–v10 [3,18], the wild type of CD44vRA. Owing to the low frequency of CD44v3–v10-positive RA patients, it is impractical to use synovial fluid cells from such donors. Interestingly, the concentration of F8:33 anti-CD44vRA that was able to discriminate between RA synovial fluid cells and keratinocytes was much higher than that discriminating between Namalwa-CD44vRA and Namalwa-CD44v3–v10. Even at concentrations as high as 100 μg/ml, F8:33 bound exclusively to RA synovial fluid cells. This concentration is close to the anti-arthritic "therapeutic window" of infliximab anti-TNF mAb [19], i.e., the highest blood concentration of the antibody, which elicits a therapeutic response in a patient, but is not toxic. Anti-TNF mAb is widely used in the therapy of RA patients [19], but its possible long-range deleterious effects are not known.

Why does F8:33 discriminate, at high concentrations, between RA synovial fluid cells and keratinocytes, but not between Namalwa-CD44vRA and Namalwa-CD44v3–v10 model cells? Conceivably, the F8:33 recognizing-epitope, exposed to the relevant antibody, is differentially expressed on the authentic cells and on model cells, resulting in distinct binding affinities to these two sets of cells. Does expression of cell surface CD44vRA entail a functional meaning with physiological or pathological consequences? Namalwa cells and RA synovial fluid cells expressing CD44vRA and immobilizing FGF-2 activate FGF-receptor bearing cells more intensively than corresponding cells expressing the wild type CD44v3–v10 [12]. As activation of the FGF-receptor on fibroblasts and endothelial cells in inflammatory joints of RA patients is an important factor in the pathology of the disease [20–22], it is conceivable that CD44vRA expression contributes to the RA inflammatory process.

Can the exclusive structure of CD44vRA, expressed on inflammatory cells of RA patients, be exploited for selective

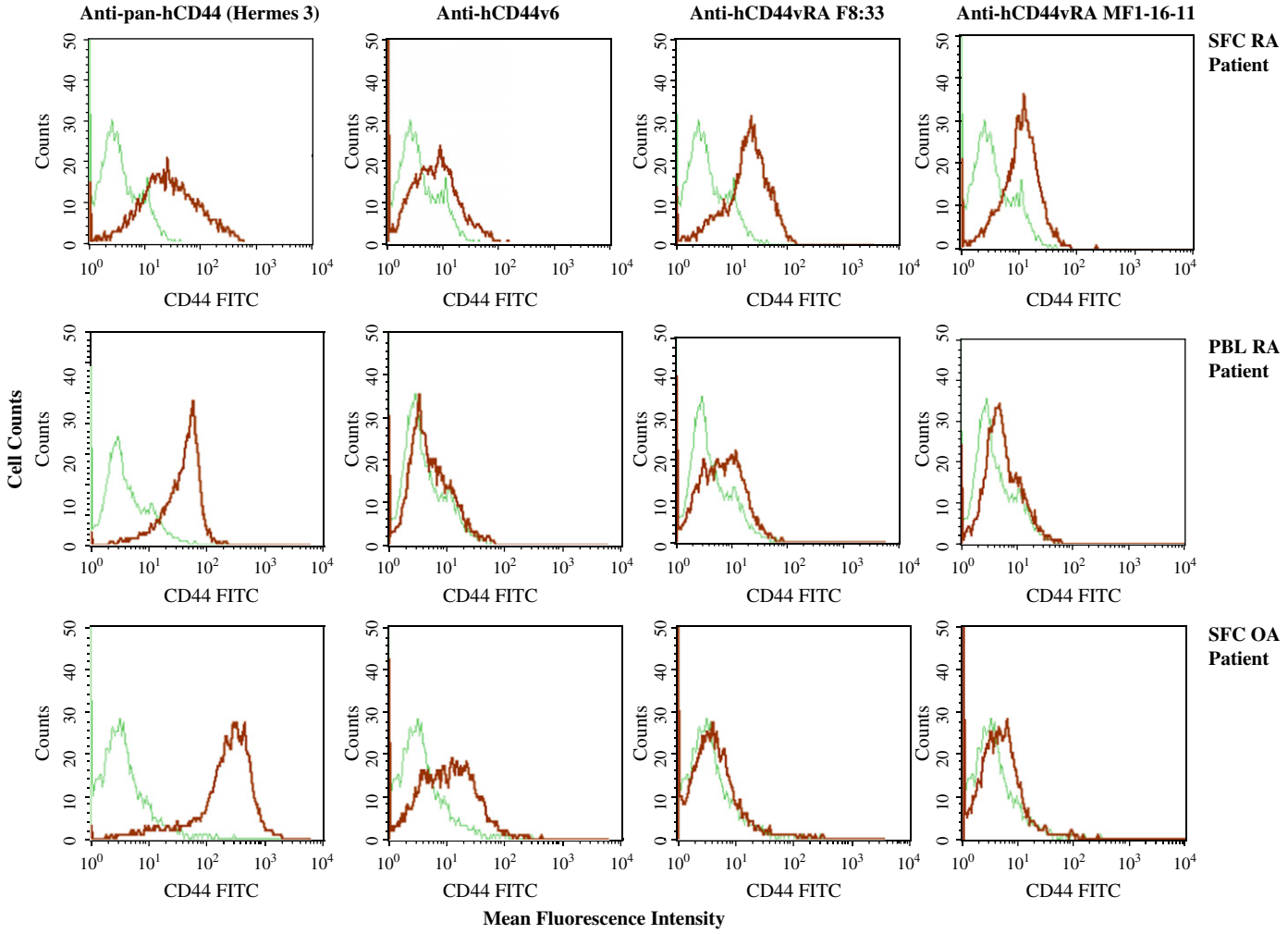

Fig. 6. Anti-CD44vRA mAbs bind to synovial fluid cells of an RA patient but not to PBLs from the same patient. The binding of anti-pan-hCD44 mAb, anti-hCD44v6 mAb and anti-CD44vRA mAbs from two clones (F8:33 and MFI-16-11) to synovial fluid cells (SFCs) and peripheral blood leukocytes (PBLs) of a rheumatoid arthritis (RA) patient as well as to synovial fluid cells of an osteoarthritis (OA) patient was detected with fluorescein-labeled anti-mouse Ig (second antibody). The faint histogram in each panel depicts binding of second antibody only. A representative finding of samples from at least 30 patients.

therapeutic targeting of these cells? To address this issue, we generated anti-CD44vRA mAbs that bound at much higher intensity to synovial fluid cells from RA patients than to PBLs from the same patients, synovial fluid cells from OA patients or keratinocytes from normal donors. Furthermore, the anti-CD44vRA mAb interaction with the CD44vRA receptor of RA synovial fluid cells delivers apoptotic signals to the cell interior, leading to programmed cell death of those cells involved in the destructive inflammatory cascade of the joint. Since the PBLs of the same patients were not affected by this antibody treatment, a selective therapeutic effect might be obtained *in vivo* as well. This challenge will be the thrust of our future studies.

It has been well established that the CD44 receptor can directly or indirectly deliver apoptotic [23–25] or survival [26] signals. The nature of the signal is possibly dependent on the isoform of cell surface CD44 (standard or variant) as well as on the type of receptor ligand and its concentration or on the components of the extra-vascular tissue associated with the ligand. The presence or absence (e.g., in CD44 knock out mice) of cell surface CD44 or the conditions of receptor activation dictate the upregulation or the downregulation of pro-apoptotic (Apaf-1, Bak, Bax, Bid, Hrk, CRADD, caspase-1, caspase-3, caspase-8 and caspase-9) or anti-apoptotic (A1, phosphorylated BAD, Bcl-2, Bcl-xl, phosphoinositide 3-kinase, Akt, TANK, OX40, OX40L, TRAF-2 and TRAF-3) genes, leading to programmed cell death or cell survival [27–30]. Apoptotic [23–25,27] or anti-apoptotic (survival) [26] signals can be delivered following engagement of cell surface CD44 with anti-pan-CD44 mAb [23–27] as well as with its ligand – hyaluronan [23,26]. In addition, anti-CD44 mAb or hyaluronan can inhibit apoptosis induced by other stimuli, including anti-integrin mAb and therapeutic drugs [31,32]. The apoptotic [23] or anti-apoptotic [26] signals delivered by anti-CD44 mAb or hyaluronan may be mediated by Fas upregulation or downregulation, respectively.

Targeting of CD44 can attenuate inflammatory-mediated diseases, owing to apoptosis of CD44-positive inflammatory

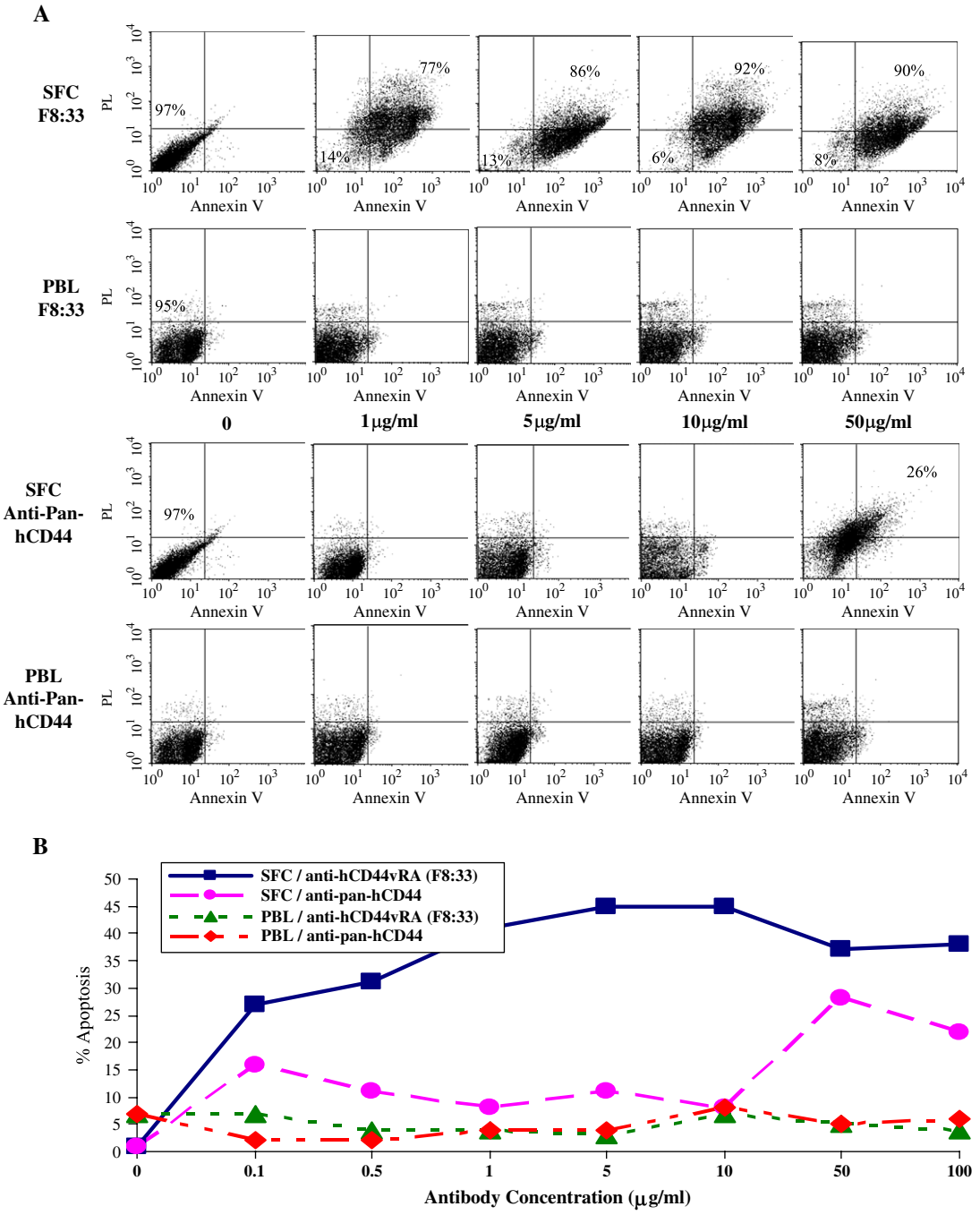

Fig. 7. Anti-CD44vRA mAb-induced apoptosis in synovial fluid cells (SFCs) from a rheumatoid arthritis patient, but not in peripheral blood leukocytes (PBLs) of the same patient. The apoptosis-inducing capacity of F8:33 anti-CD44vRA mAb and anti-pan-hCD44 mAb was analyzed by two-dimensional flow cytometry, using annexin V (*x*-axis) and propidium iodide (*y*-axis). (A) Two-dimensional flow cytometry analysis of synovial fluid cells and PBL from an RA patient. Events accumulating at the bottom left hand side quadrate simulate surviving cells. Events accumulating at the bottom and top right hand side quadrates simulate cells in early and late apoptosis, respectively. (B) Graphical description of two-dimensional flow cytometry analysis of apoptosis in synovial fluid cells and PBL from a different RA patient. A representative finding of samples from at least 30 patients.

cells. In contrast, in the absence of CD44 (e.g., in CD44 knock out mice), the disease is aggravated because the inflammatory cells are not exposed to CD44-transmitted death signals [33]. Conversely, when cell surface CD44 or one of its variants delivers survival signals, the absence of CD44 allows induction of apoptosis in the inflammatory cells and disease attenuation. On the other hand, in the presence of CD44 or CD44 variants, the inflammatory cells are preserved and the disease is exacerbated [34].

We suggest that the CD44 receptor of synovial fluid cells from different RA patients (CD44vRA), is committed, owing to a relevant setting (as discussed above) to transmit apoptotic signals after interaction with anti-CD44vRA mAbs or with corresponding, yet unknown, natural ligand. Under such

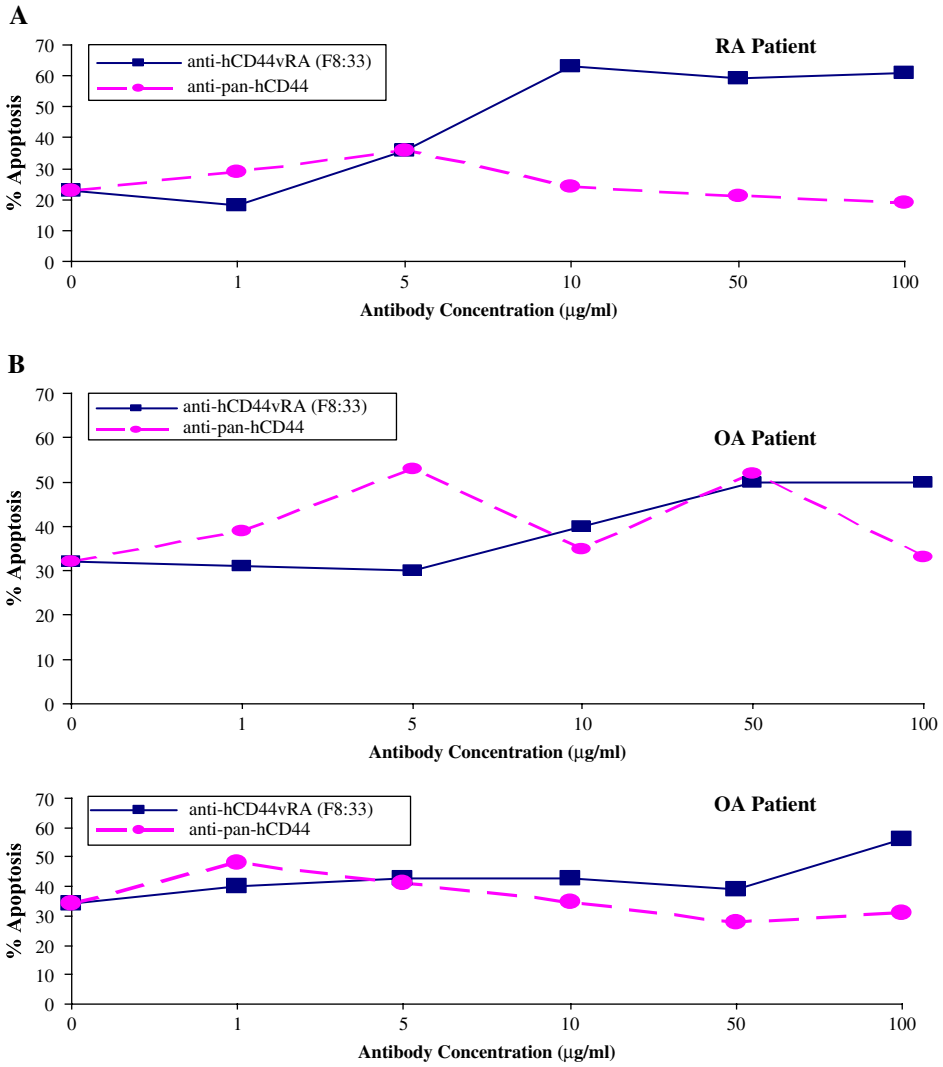

Fig. 8. Anti-CD44vRA enhanced apoptosis in synovial fluid cells of a rheumatoid arthritis (RA) patient but not, or less, in corresponding cells of two osteoarthritis (OA) patients. The apoptotic-inducing capacity of F8:33 anti-CD44vRA mAb and anti-pan-hCD44 mAb was analyzed by two-dimensional flow cytometry as indicated in Fig. 7.

circumstances, the concentrations of anti-CD44vRA mAbs that induced programmed cell death in synovial fluid cells of RA patients failed to induce or enhance apoptosis in the PBLs of the same patients, while anti-pan-CD44 mAbs-induced apoptosis in RA synovial fluid cells only at high concentrations. Notably, the apoptotic effect induced in RA synovial fluid cells with anti-CD44vRA mAb was detected at a concentration as low as 1 μg/ml, whereas at least 10 times higher concentrations were required to induce apoptosis with anti-pan CD44 mAbs, as shown by other investigators [23,24,27] and ourselves (Fig. 7).

Our anti-human CD44vRA mAb (designated F8:33) enhances spontaneous apoptosis in spleen cells from arthritic mice, but not in spleen cells from normal mice (not shown). The same F8:33 kills by apoptosis synovial fluid cells from RA patients, but not PBLs from the same patient (Fig. 7). In addition, injection of F8:33, at the onset of CIA, markedly reduced joint inflammation in arthritic DBA/1 mice (Fig. 9). Obviously this begs the question: how does the anti-human CD44vRA mAb recognize the CD44 of the arthritic mouse? This enigma appears to be even more complicated, given that we were unable to detect the arthritic CD44 variant (CD44vRA) in the joints of arthritic mice (not shown). However, we have found that the spleen cells of arthritic mice express CD44v3−v10, whereas spleen cells of normal non-arthritic mice do not express this isoform (but they do express standard CD44). The CD44v3−v10 isoform is the wild type molecule of CD44vRA, i.e. they are identical in sequence, but CD44vRA includes an extra alanine between exon v4 and v5, without any change in the rest of the reading frame. We compared the sequence of the splicing junction ends of v4 and v5 exons in human CD44vRA (where alanine is inserted), human CD44v3−v10 and mouse CD44v3−v10. Surprisingly we found that the sequence of mouse wild type CD44v3−v10, which is expressed in arthritic mice, but not in non-arthritic mice, contains alanine like human CD44vRA

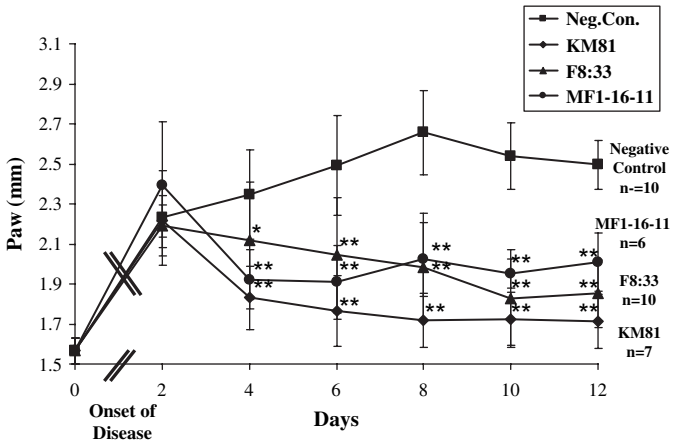

Fig. 9. Anti-human CD44vRA mAbs reduce joint inflammation in mice with collagen-induced arthritis (CIA). KM81 anti-mouse pan CD44 mAb (ATCC) and anti-human CD44vRA mAbs from two clones (F8:33, MF-1-16-11) were injected into DBA/1 mice with CIA (see Section 2) at disease onset and then every other day for 12 days. Isotype-matched anti-mouse mAb (4D2; five mice) or anti-human mAb (Serotec nonfunctional anti-CD44 mAb; five mice) were injected, using the same experimental protocol, into a control group. Joint inflammation was assessed by measuring footpad swelling with a micro-caliper. *, $P < 0.05$; **, $P < 0.01$, by Student's *t*-test for unpaired values when compared with the control mouse group. $n =$ Number of mice in each group.

(but unlike human CD44v3−v10) in the junction between v4 and v5 (see underlined A in the sequences) as shown:

Mouse CD44v3−v10; v4/v5 junction: NPRVLLQTTTRM AD IDRISTSAHGENWT,
Human CD44v3−v10; v4/v5 junction: NPEVLLQTTTRM TD VDRNGTTATHGNWNP,
Human CD44vRA; v4/v5 junction: NPEVLLQTTTRMT AD VDRNGTTATHGNWNP.

The remainder of the sequence of human CD44vRA and mouse CD44v3−v10 is similar (but not identical) on both sides flanking their alanines (see sequences above). This sequence comparison can explain why anti-human CD44vRA mAb was able to enhance apoptosis in spleen cells from arthritic mice and to induce at least partial resistance to CIA in DBA/1 mice.

We [5,8] and others [9,10] have shown that a considerable number of pathological activities in inflammatory and cancer experimental diseases are CD44-dependent. Furthermore, there is circumstantial evidence that CD44 is an important factor in the corresponding human diseases as well [1,35]. The intensive alternative splicing machinery of the CD44 molecule provides opportunities for creating disease-specific targeting structures that cannot be detected in other pro-inflammatory or pro-cancerous molecules that are far less subjected to diversification mechanism. If sequence variations generated in CD44 alternative splicing junctions are a ubiquitous phenomenon in CD44-dependent pathologies, specific targeting of these modified structures, e.g., by mAbs, may open up new avenues for specific therapy not only in RA, but also in other cancer and inflammatory diseases.

Acknowledgments

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