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(54) Title: ENHANCED FC RECEPTOR-MEDIATED TUMOR NECROSIS FACTOR SUPERFAMILY MRNA EXPRESSION IN PERIPHERAL BLOOD LEUKOCYTES IN PATIENTS WITH RHEUMATOID ARTHRITIS

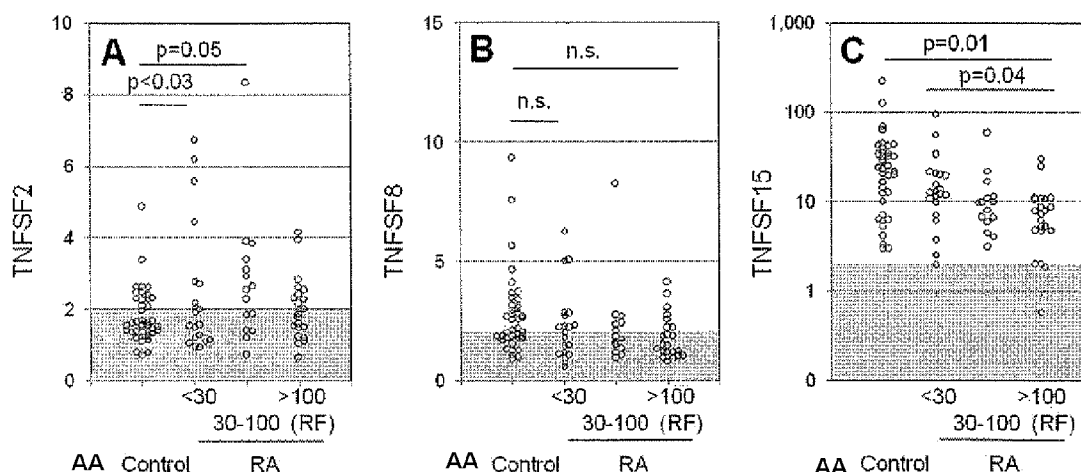


FIGURE 2

AA Groupe témoin

(57) Abstract: A method for predicting patient responsiveness to rheumatoid arthritis treatments involving altering expression of tumor necrosis factor superfamily ("TNFSF")-2, TNFSF-8, or TNFSF-15 is disclosed. A method for monitoring the effectiveness of such therapy is also disclosed. Furthermore, a method of screening compounds for use in the treatment of rheumatoid arthritis is disclosed. A method of monitoring the disease state over time in rheumatoid arthritis patients is also disclosed.

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**ENHANCED FC RECEPTOR-MEDIATED TUMOR NECROSIS FACTOR
SUPERFAMILY MRNA EXPRESSION IN PERIPHERAL BLOOD LEUKOCYTES IN
PATIENTS WITH RHEUMATOID ARTHRITIS**

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a non-provisional of U.S. Provisional Patent Application No. 61/002,967 filed November 14, 2007 and is a continuation-in-part of U.S. Patent Application No. 12/296,423 filed October 7, 2008, which is the National Phase of International Application No. PCT/US2007/008559 filed April 5, 2007, which in turn claims priority to U.S. Provisional Patent Application No. 60/790,511 filed April 7, 2006.

BACKGROUND

Field

[0002] The disclosure relates to a method for predicting patient responsiveness to treatments for rheumatoid arthritis involving a tumor necrosis factor superfamily member or a cytokine, to a method of monitoring the effectiveness of such therapy, and to a method for screening compounds for use in the treatment of rheumatoid arthritis. The disclosure also relates to a method for monitoring the disease state in rheumatoid arthritis patients.

Description of the Related Art

[0003] Autoimmune disease is characterized by production of either antibodies that react with host cells or immune effector T cells that are autoreactive. Autoantibodies are frequently identified in certain types of autoimmune disease, such as anti-acetylcholine receptor antibodies in myasthenia gravis and anti-DNA antibodies in systemic lupus erythematosus. However, such autoantibodies are not seen in many types of autoimmune disease. Moreover, autoantibodies are often detected among healthy individuals, but such antibodies do not induce autoimmune disease. Thus, beside autoantibodies, additional yet-to-be identified mechanisms are evidently involved in the pathogenesis of autoimmune disease.

[0004] Once autoantibodies bind to the target host cells, the complement cascade is thought to be activated to form the C5-9 membrane attack complex on the target cell membranes, which leads to the death of host cells (see Esser, *Toxicology* 87, 229 (1994)). Byproduct chemotactic factors, such as C3a, C4a, or C5a recruit more leukocytes to the lesion (see Hugli, *Crit. Rev. Immunol.* 1, 321 (1981)). Recruited leukocytes or naturally present leukocytes at the lesion recognize antibody-bound cells (immune complexes) via Fc receptors ("FcR"). Once the

FcR is cross-bridged by the immune complex, leukocytes release TNF- α (see Debets *et al.*, *J. Immunol.* 141, 1197 (1988)), which binds to specific receptors present on the surface of host cells, and induces apoptosis or cell damage (see Micheau *et al.*, *Cell* 114, 181 (2003)). Activated FcR also initiates the release of chemotactic cytokines to recruit different subsets of leukocytes to the lesion (see Chantry *et al.*, *Eur. J. Immunol.* 19, 189 (1989)). This is an overall hypothesis of the molecular mechanism of FcR-related autoimmune disease.

[0005] Rheumatoid arthritis ("RA") is an immune disease involving inflammation of the gastrointestinal tract. Although it is well characterized clinically, its pathogenesis is poorly understood. RA is characterized by persistent inflammatory synovitis, usually involving peripheral joints in a symmetric distribution. This may lead to cartilage destruction, bone erosion, and changes in joint integrity. The cause of RA remains unknown, but it is strongly suspected that CD4+ T-cells play a role in the disease because of the predominance of such cells in the synovium, the increase in soluble IL-2 receptors (produced by activated T-cells) in the blood and serum of RA patients, and the noted amelioration of the disease by removal of T-cells. RA is associated with a buildup of TNF- α (also known as TNFSF-2) in the joints. TNF- α normally serves to mobilize white blood cells to fight infections and other invaders, causing inflammation in the affected area. A healthy body can rid itself of excess TNF- α , but the body of a patient with rheumatoid arthritis cannot. As a result, more and more white blood cells travel to the affected area. The build up of TNF- α , particularly in the rheumatoid joint, causes inflammation, pain and tissue damage.

[0006] IgG Fc receptors (Fc γ R) are known to react with immune complexes (ICs) (the combination of an epitope with an antibody directed against that epitope) to elicit various inflammatory reactions. ICs are frequently identified at joint lesions in patients with RA, although specific antigens have not been not fully characterized. ICs are also known to activate complement cascades to establish inflammation, as well as antibody-dependent cell mediated cytotoxicity (ADCC) by binding to the Fc γ R of various leukocytes. Locally infiltrating leukocytes have been collected from synovial fluids and studied previously. However, because these collections contain both newcomer and exhausted cells, the results have been difficult to interpret. Since peripheral blood leukocytes play a major role in the pathogenesis of RA when they migrate to disease sites, numerous experiments have been conducted to simulate such functions *in vitro*. Typically, mononuclear leukocytes are isolated, suspended in culture media, and incubated in a CO₂ incubator with various stimulants or effector cells. However, the conditions under which such assays are conducted do not approximate physiological conditions, due to a lack of communication among different cell populations, oxygen supply from

erythrocytes, as well as complex interactions with plasma proteins and other components. Secondary reactions may occur during the lengthy incubation period. Moreover, due to labor-intensive techniques and substantial experiment-to-experiment variations, these *in vitro* tests have less application in diagnostic testing.

[0007] Treatment of RA focuses on pain relief, reduction of inflammation, protection of articular structures, maintenance of function, and control of systemic involvement. Options include: aspirin and other nonsteroidal anti-inflammatory drugs; antirheumatic drugs such as methotrexate, gold compounds, D-penicillamine, the antimalarials, and sulfasalazine; glucocorticoids; TNF- α neutralizing agents such as infliximab and etanercept; and immunosuppressive drugs such as azathioprine, leflunomide, cyclosporine, and cyclophosphamide. Because the choice of therapeutic options depends on an assessment of the disease state in RA patients, it would be desirable to develop new methods of evaluating the disease state and monitoring the progression of the disease.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Figures 1A-1C show the results of quantification of Fc γ R-mediated gene expression of various TNFSF mRNAs in human leukocytes in peripheral whole blood.

[0009] Figures 2A-2C show the fold increases in various TNFSF mRNA levels induced by heat aggregated IgG (HAG) stimulation in the whole blood of rheumatoid arthritis patients and control patients as compared to unstimulated whole blood from said patients.

[0010] Figures 3A-3C show comparative fold increases in various TNFSF mRNA levels induced by heat aggregated IgG (HAG) stimulation in the whole blood of rheumatoid arthritis patients and control patients as compared to unstimulated whole blood from said patients.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0011] The present disclosure relates to the use of differential mRNA transcription patterns in leukocytes in response to specific cellular stimuli in assessing whether RA patients are good candidates for specific therapies. The present disclosure also relates to the use of such differential transcription patterns in assessing whether therapy administered to an RA patient is effective. The present disclosure also relates to the use of such differential transcription patterns in screening candidate agents for use in treating RA. The present disclosure also relates to the use of such differential transcription patterns in evaluating the state of RA in patients over time and monitoring the progression of the disease.

[0012] As described above, the pathology of RA may be related to the interaction between Fc γ R in the immune cells of an RA patient and the IC. In order to further assess this possibility, heat aggregated IgG (HAG, a classic model of the IC) was used to stimulate Fc γ R in human whole blood in both healthy adult controls and patients with RA. Other stimuli that can be used include phorbol myristate acetate (PMA), phytohemagglutinin (PHA), wheat germ agglutinin (WGA), concanavalin-A (ConA), lipopolysaccharides (LPS), jacalin, fucoidan, heat-aggregated IgE, heat-aggregated IgA, and heat-aggregated IgM.

[0013] HAG was added directly into heparinized whole blood, and the changes in the mRNA level of various members of the tumor necrosis factor superfamily (TNFSF) were assessed. Although multiple Fc γ Rs exist, such as Fc γ RI, IIa, IIb, and III (GeneBank UniGene database), HAG acts as a universal stimulus that can react with all FcR subtypes. The changes in the mRNA level of members of the TNFSF mRNA (see, for example, the GeneBank UniGene database) resulting from the stimulus with HAG were quantified.

[0014] As stated, Fc γ R-mediated functions consist of four subclasses of the Fc portions of IgG (IgG1-4), multiple classes of Fc γ R (Fc γ RIa-c, Fc γ RIIa-c, and Fc γ RIIIa-b), different subsets of Fc γ R-bearing leukocytes, and various downstream intracellular signalling cascades. Moreover, these proteins have multiple transcript variants and various genetic polymorphisms. Altered functions of Fc γ R in disease states may happen at any level. However, it may be impractical to characterize each factor in each individual. The present disclosure contemplates use of whole blood (with its attendant *ex vivo* conditions) as a screening tool to identify individuals who would benefit from various downstream assays, such as individual cell analysis, genotyping of various genes, and variation of intracellular signalling cascades. All of the tumor necrosis factor superfamily (TNFSF) members were screened in this way, as IC is known to induce TNFSF-2 (=TNF- α) and TNFSF-15 (=TL1A) mRNA.

[0015] The method employed was as follows. Nucleotide sequences for various TNFSF genes were retrieved from the UniGene database in the GenBank. PCR primers for each gene were designed by Primer Express (Applied Biosystem, Foster City, CA) and HYBsimulator (RNAture, Irvine, CA) (see Mitsuhashi *et al.*, *Nature* 367, 759 (1994); Hyndman *et al.*, *BioTechniques* 20, 1090 (1996)). The sequences are summarized in Table I below. Oligonucleotides were synthesized by IDT (Coralville, IA), Tsukuba Oligo Service (Tsukuba, Japan), Nippon EGT (Toyama, Japan), and Hokkaido System Science (Sapporo, Japan).

Table 1. Primer Sequences

Target mRNA	Forward(3'-5')	SEQ ID NO	Reverse (3'-5')	SEQ ID NO
TNFSF-1	CAGCTATCCACCCACACAGATG	1	CGAAGGCTCCAAAGAAGACAGT	2
TNFSF-2	CGAAGGCTCCAAAGAAGACAGT	3	CAGGGCAATGATCCCAAAGT	4
TNFSF-3	AGGGTGTACGTCAACATCAGTCA	5	CACGGCCCCAAAGAAGGT	6
TNFSF-4	GCCCCTCTTCCAACCTGAAGAA	7	GGTATTGTCAGTGGTCACATTCAAG	8
TNFSF-5	CCACAGTTCGCCAAACCT	9	CACCTGGTTGCAATTCAAATACTC	10
TNFSF-6	TGGCAGCATCTTCACTTCTAAATG	11	GAAATGAGTCCCCAAAACATCTCT	12
TNFSF-7	CACACTCTGCACCAACCTCACT	13	TGCACTCCAAAGAAGGTCTCATC	14
TNFSF-8	ACCACCATATCAGTCAATGTGGAT	15	GAAGATGGACAACACATTCTCAAGA	16
TNFSF-9	AGCTACAAAGAGGACACGAAGGA	17	CGCAGCTCTAGTTGAAAGAAGACA	18
TNFSF-12	TACTGTCAGGTGCACTTTGATGAG	19	CGCAGTGGCTGAGAATTCCT	20
TNFSF-13	ATATGGTGTCCGAATCCAGGAT	21	CCTGACCCATGGTGAAAGTCA	22
TNFSF-13B	ATGCCTGAAACACTACCCAATAAT	23	GCAAGTTGGAGTTCATCTCCTTCT	24
TNFSF-14	CGTCCGTGTGCTGGATGA	25	CATGAAAGCCCCGAAGTAAGAC	26
TNFSF-15	TGCGAAGTAGGTAGCAACTGGTT	27	CCATTAGCTTGCCCCTTCTTG	28

[0016] Heat aggregated IgG (HAG) was prepared by heating 20 mg/mL human IgG (Sigma, St. Louis) in PBS at 63°C for 15 min (see Ostreiko *et al.*, *Immunol. Lett.* 15, 311 (1987)). In 8-well strip microtubes, 1.4 µl of HAG or control (phosphate buffered saline) were added, and stored at -20°C until use. Seventy µl of fresh heparinized whole blood (which was kept at 4°C until stimulation with IC) was added into each well in triplicate, and incubated at 37°C for 4 hours with the cap closed. The blood samples were treated on the same day as the blood draw. After treatment, each blood sample was stored frozen at -80°C until use.

[0017] The mRNA and cDNA were prepared from whole blood following the method set forth in Mitsunashi *et al.*, *Clin. Chem.* 52, 634 (2006). The method disclosed in U.S. Patent Application No. 10/796,298, which is incorporated here by reference, may also be employed. In brief, 50 µl of whole blood was transferred to 96-well filterplates in order to trap the leukocytes therein; these filterplates were placed over collection plates, and 150 µl 5 mM Tris, pH 7.4, was applied. Following centrifugation at 120 xg for 1 min at 4°C, 50 µl of blood sample was applied to each well and immediately centrifuged at 120 xg for 2 min at 4°C, followed by washing of each well with 300 µl PBS once with centrifugation at 2000 xg for 5 min at 4°C. Then, 60 µl stock lysis buffer, supplemented with 1% 2-mercaptethanol (Bio Rad, Hercules, CA, USA), 0.5 mg/ml proteinase K (Pierce, Rockford, IL, USA), 0.1 mg/ml salmon sperm DNA (5 Prime Eppendorf/Brinkmann, Westbury, NY, USA), 0.1 mg/ml *E. coli* tRNA (Sigma), a cocktail of 10 mM each of specific reverse primers, and standard RNA34 oligonucleotides, were applied to the filterplates, followed by incubation at 37°C for 10 min. The filterplates were then placed over oligo(dT)-immobilized microplates (GenePlate, RNAture) (see Mitsunashi *et al.*, *Nature* 357, 519 (1992); Hamaguchi *et al.*, *Clin. Chem.* 44, 2256 (1998)

(both incorporated herein by reference)), and centrifuged at 2000 xg for 5 min at 4°C. Following overnight storage at 4°C, the microplates were washed with 100 µl plain lysis buffer three times, followed by 150 µl wash buffer (0.5 M NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA) three times at 4°C. The cDNA was directly synthesized in 30 µl solutions in each well by adding buffer containing 1x RT-buffer, 1.25 mM each of dNTP, 4 units rRNasin, and 80 units of MMLV reverse transcriptase (Promega) (without primers), and incubating at 37°C for 2 hours. The specific primer-primed cDNA existed in solution, and oligo(dT)-primed cDNA stayed immobilized in the microplate (see Hugli, *Crit. Rev. Immunol.* 1, 321 (1981)). For SYBR Green PCR (see Morrison *et al.*, *Biotechniques* 24, 954 (1998) (incorporated herein by reference)), cDNA was diluted 4-fold in water, and 4 µl of cDNA solution was directly transferred to 384-well PCR plates, to which 5 µl iTaq SYBR master mix (BioRad, Hercules, CA) and 1 µl oligonucleotide cocktail (15 µM each of forward and reverse primer) were applied, and PCR was conducted in PRISM 7900HT (ABI), with one cycle of 95°C for 10 min followed by 45 cycles of 95°C for 30 sec and 60°C for 1 min. TaqMan PCR could also be employed; in such a case, the cDNA solution is directly transferred to 384-well PCR plates, to which 5 µl of TaqMan universal master mix (ABI) and 1 µl oligonucleotide cocktail (15 µM each of forward and reverse primer, and 3-6 µM TaqMan probe) are applied, and PCR is conducted in PRISM 7900HT (ABI), with one cycle of 95°C for 10 min followed by 45 cycles of 95°C for 30 sec, 55°C for 30 sec, and 60-65°C for 1 min. Each gene was amplified individually.

[0018] The 1x RT buffer was used as a negative control to confirm that no primer dimer was generated under SYBR Green PCR conditions. Moreover, the melting curve was analyzed in each case to confirm that PCR signals were derived from a single PCR product. The cycle threshold (Ct), which was the number of PCR cycles required to generate certain amounts of PCR products (fluorescence), was determined by analytical software (SDS, ABI). The Ct values of HAG-treated triplicate samples were reduced by the mean Ct values of PBS controls individually to calculate ΔCt , and the fold increase for stimulated samples compared to unstimulated samples (hereinafter simply referred to as the “fold increase”) was calculated as $2^{(-\Delta Ct)}$, by assuming that the efficiency of each PCR cycle was 100%.

[0019] Figures 1A-C show FcγR-mediated mRNA expression in peripheral blood leukocytes. Each data point represents the mean+standard deviation (Figure 1A) or mean (Figures 1B, 1C) from triplicate aliquots of whole blood.

[0020] Figure 1A shows the results of an analysis of the kinetics of the expression. Triplicate aliquots of 70 µl each of heparinized whole blood were mixed with PBS or 200 µg/ml of heat aggregated IgG (HAG) and incubated at 37°C for 0-8 hours. TNFSF-2 (=TNF- α , ●),

TNFSF-8 (▲), TNFSF-15 (=TL1A, ◆), and β -actin (Δ) mRNA were then quantified and the fold increase (y-axis) was calculated as described above. As shown in Figure 1A, the induction of TNFSF-2 was very rapid with a peak around 30 min followed by a large sustained peak around 4 hours. In contrast to TNFSF-2, TNFSF-8 (Fig. 1A, ▲) and TNFSF-15 (=TL1A) (Fig. 1A, ◆) increased slowly with a peak around 4 hours. A housekeeping gene (β -actin) was not induced during incubation for 8 hours with HAG (Fig. 1A, Δ). HAG incubation was accordingly fixed at 4 hours to analyze TNFSF-2, -8 and -15 mRNA. This short incubation (4 hours) is one of the advantages of an mRNA-based assay, because protein detection requires at least overnight incubation to identify drug-induced changes.

[0021] Figure 1B shows the dose response. Heparinized whole blood was incubated with 0-800 μ g/ml HAG at 37°C for 4 hours, then various mRNAs were quantified (each symbol is defined as in Figure 1A). HAG-induced TNFSF-2, TNFSF-8, and TNFSF-15 were confirmed from 10 μ g/ml of HAG in a dose dependent manner and saturated at 100-200 μ g/ml, whereas β -actin was unchanged.

[0022] Figure 1C shows the results obtained when blood was withdrawn from the same individuals twice within 1 week, and HAG-induced TNFSF-2 (●), TNFSF-8 (▲), TNFSF-15 (◆) mRNA and an external control mRNA (synthetic RNA34) (○) were quantified. As shown in Figure 1C, the induction was reproducible and the r^2 value between Day 1 and Day 2 among 8 healthy individuals were 0.927 (n=32, p<0.001) (Fig. 1C). The fold increase of external synthetic RNA spiked into lysis buffer (RNA34, Fig. 1C, ○) was always less than 1.5, suggesting that the assay was performed appropriately in each experiment.

[0023] Although the actual blood volume used for mRNA analysis was 50 μ l, the assay used 70 μ l of whole blood per reaction (20 μ l is a reserve during the transfer from 8-well strips to filterplates). Thus, each test consumed as little as 420 μ l (=70 μ l/well x 2 (HAG and PBS) x 3 (triplicate)) of whole blood. From 50 μ l of whole blood/well, 30 different mRNAs were quantified by RT-PCR (30 μ l cDNA plus 90 μ l water (1:4 dilution), 4 μ l cDNA/PCR). Even from 1:4 dilution of cDNA, measurement of various TNFSF mRNAs was achieved. Unlike serum-based tests, where serum volume is unpredictable due to the variation of hematocrit among individuals, whole blood is easy to manipulate.

[0024] Table 2 shows the results of an analysis of the FcR-mediated gene expression of TNFSF mRNA in human leukocytes in peripheral whole blood. The results shown are expressed as a percentage of "responder" subjects, defined as those subjects that exhibit a fold increase of greater than 2 in response to HAG stimulation. The χ^2 test was used to compare the incidence of positive responses between healthy subjects and RA patients for each TNFSF

mRNA. Since there were two populations (positive and negative responses), the t-test was only applied to the subjects with a fold increase of greater than 2.

Table 2. Immune complex-induced TNFSF mRNA expression.

TNFSF	Control		RA		p value
	No. positive*/n	%	No. positive/n	%	
TNFSF-1	7/39	18	2/16	13	n.s.
TNFSF-2	15/40	38	30/59	51	n.s.
TNFSF-3	2/40	5	4/60	7	n.s.
TNFSF-4	1/40	3	4/57	7	n.s.
TNFSF-5	0/40	0	0/61	0	n.s.
TNFSF-6	1/40	3	7/61	11	n.s.
TNFSF-7	8/40	20	6/58	10	n.s.
TNFSF-8	25/40	63	25/61	41	n.s.
TNFSF-9	7/36	19	7/52	13	n.s.
TNFSF-12	0/40	0	1/26	4	n.s.
TNFSF-13	0/40	0	0/26	0	n.s.
TNFSF-13B	1/40	3	0/24	0	n.s.
TNFSF-14	19/40	48	20/57	35	n.s.
TNFSF-15	38/38	100	58/61	95	n.s.

*:Fold increase >2.0

[0025] As shown in Table 2, HAG induced TNFSF-15 (=TL1A) mRNA (fold increase >2) in all cases of healthy donors, and 59 out of 61 RA patients. These results replicated, under *ex vivo* conditions, recently published reports (see Prehn *et al.*, The T cell costimulator TL1A is induced by FcγR signaling in human monocytes and dendritic cells, *J. Immunol.* 178, 4033 (2007); Cassatella *et al.*, Soluble TNF-like cytokine (TL1A) production by immune complexes stimulated monocytes in rheumatoid arthritis, *J. Immunol.* 178, 7325 (2007) (both incorporated herein by reference)). HAG is also known to induce TNFSF-2 (=TNF-α) mRNA (see Satoh *et al.*, Endogenous production of TNF in mice with immune complex as a primer, *J. Biol. Response Mod.* 5, 140 (1986); Chouchakova *et al.*, Fc gamma RIII-mediated production of TNF-alpha induces immune complex alveolitis independently of CXC chemokine generation, *J. Immunol.* 166, 5193 (2001) (both incorporated herein by reference)). However, as shown in Table 2, TNFSF-2 was not found to be induced in all individuals, and more than half of the subjects failed to respond. Furthermore, HAG stimulation induced TNFSF-8 (=CD153, CD30 ligand) and TNFSF-14 (=LIGHT) mRNA in more than 1/3 of controls and RA patients, and TNFSF-1 (=lymphotoxin α, LTA), TNFSF-3 (=lymphotoxin β, LTB), TNFSF-4 (=CD252, CD134 ligand), TNFSF-6 (=Fas ligand), TNFSF-7 (=CD70, CD27 ligand), and TNFSF-9 in some cases, whereas TNFSF-5 (=CD154, CD40 ligand), TNFSF-12 (=TWEAK), TNFSF-13 (=CD256), and TNFSF-13B (=CD257) showed little or no induction. This individual-to-

individual variation, or the presence of responders and non-responders at 4 hours from the start of stimulation was meaningful because, as shown in Figures 1A and 1B, saturation was achieved at incubation for 4 hours with 200 $\mu\text{g/ml}$ HAG. This *ex vivo* assay is expected to be useful as a screening platform for various downstream assays.

[0026] A test for rheumatoid factor (RF) (either Type 2 (monoclonal IgM to polyclonal IgG) or 3 (polyclonal IgM to polyclonal IgG) cryoglobulin) is a standard diagnostic method in cases of suspected RA. The results of TNFSF-2, TNFSF-8, and TNFSF-15 induction resulting from HAG stimulation were broken out by RF levels to look for correlations. The results are shown in Figures 2A-2C. HAG-induced TNFSF-2 (A), TNFSF-8 (B), and TNFSF-15 (C) mRNA were quantified from 40 healthy adult volunteers (control), and 61 RA patients with RF <30, 30-100, and >100 IU/ml, respectively. Because two populations were observed (responders with fold increase >2, and non-responders), the t-test was only applied to the responder population. Analysis by χ^2 test revealed no significant difference among these four groups.

[0027] As shown in Figure 2, classification of RA patients by amount of RF revealed significant differences in TNFSF induction. As shown in Figure 2C, the fold increase of TNFSF-15 in RA patients with RF >100 IU/ml was significantly less than that of control ($p=0.01$) and RA patients with RF <30 IU/ml ($p=0.04$), respectively. This probably results from the fact that RF is a native form of IC and was present in the whole blood when HAG was added *ex vivo*. The majority of RA patients maintained the response to HAG stimulation. This indicates that circulating leukocytes in peripheral blood of RA patients are still capable of activating an IC despite a long period of exposure to RF. Reduced TNFSF-15 responses shown in RA patients with high RF may indicate that TNFSF-15 receptor function in peripheral blood leukocytes is somehow down-regulated.

[0028] In view of the activation of Fc γ R by RF, an assessment was made of whether the baseline levels of TNFSF-15 mRNA are elevated by the continuous exposure to RF. The results were measured in terms of Ct by calculating the relative expression of TNFSF-15 over TNFSF-5, -13, and -13B, because these three TNFSF mRNAs were not induced by HAG, as shown in Table 2. However, the basal levels of TNFSF-15 in RA patients were not found to be significantly different from control subjects in all three calculations.

[0029] Figure 3 shows the results obtained when the results shown in Figure 2 were transformed to an x-y graph by comparing TNFSF-2 and -8 (A), TNFSF-2 and -15 (B), and TNFSF-8 and -15 (C), with control (\circ) and RA (\bullet), respectively. As shown in Figure 3C, the fold increase of TNFSF-15 was well correlated with that of TNFSF-8 in both RA (\bullet) and healthy

subjects (○) with r^2 values of 0.48 (n=61, $p<0.001$) and 0.27 (n=38, $p<0.001$), respectively. However, because the fold increase of TNFSF-8 was lower and the population of positive responders was smaller than that of TNFSF-15, a significant difference was not observed for TNFSF-8 between control and RA, even when RA patients were classified by the amount of RF (Fig. 2B). However, in contrast to TNFSF-15, the fold increase of TNFSF-2 in RA patients with RF <30 and <100 was significantly ($p<0.03$, 0.05) higher than that of healthy subjects, as shown in Figure 2A. The fold increase of TNFSF-2 was not correlated to that of TNFSF-8 (Fig. 3A) ($r^2=0.03$ and 0.02 for control and RA, respectively) and TNFSF-15 (Fig. 3B) ($r^2=0.11$ and 0.003 for control and RA, respectively), suggesting that TNFSF-2 and TNFSF-8/-15 are derived from distinct pathways. This was further confirmed by the evidence shown in Figure 1A, where the kinetics between TNFSF-2 and TNFSF-8/-15 were different.

[0030] TNFSF-2 (=TNF- α) is one of the inflammatory cytokines involved in the pathogenesis of RA, and is present in synovial fluid in RA (see Saxne *et al.*, Detection of tumor necrosis factor alpha but not tumor necrosis factor beta in rheumatoid arthritis synovial fluid and serum, *Arthritis Rheum.* 31, 1041 (1988) (incorporated herein by reference)). It has been shown by *in situ* hybridization that TNF- α transcripts were present in synovial tissue macrophages (see MacNaul *et al.*, Analysis of IL-1 and TNF-alpha gene expression in human rheumatoid synoviocytes and normal monocytes by in situ hybridization, *J. Immunol.* 145, 4154 (1990) (incorporated herein by reference)). Moreover, anti-TNF- α monoclonal antibodies (infliximab, Remicade) and soluble TNF receptors (etanercept, Enbrel) which block the action of TNF- α have demonstrated clinical efficacy in patients with RA (see Weaver, The impact of new biologicals in the treatment of rheumatoid arthritis, *Rheumatology* (Oxford) 43 Suppl. 3:iii17-iii23 (2004) (incorporated herein by reference)). Thus, the elevated HAG-induced TNF- α induction shown in RA patients in our *ex vivo* assay is quite reasonable. mRNA induction does not, of course, always correspond to protein synthesis and subsequent biological and clinical outcomes due to altered splicing, post-translational modification, and co-activation of inhibitory cascades. However, this *ex vivo* simulation will be effective as a starting point screening tool for various downstream molecular assays, such as genetic polymorphisms of receptors and associated proteins.

[0031] We also compared patients' clinical characteristics between responders (fold increase ≥ 2) and non-responders (fold increase < 2) of HAG-induced TNFSF-2 mRNA. The results are shown in Table 3.

Table 3. Characteristics of responders and non-responders on HAG-induced TNFSF-2

		HAG-induced TNFSF-2		p value
		FI* \geq 2	FI* $<$ 2	
RF<30	N	9	11	
	Age (years)	54.6 \pm 12.1	66.5 \pm 11.8	0.04
	Male:Female	0:9	1:10	0.35**
	Duration (years)	2.4 \pm 1.1	6.2 \pm 7.5	0.2
	CRP	0.1 \pm 0.1	0.5 \pm 0.8	0.17
	No. swelling/joint	2.9 \pm 3.8	3.7 \pm 4.2	0.7
	No. pain/joint	1.7 \pm 3.1	0.7 \pm 1.6	0.4
	Treatment with Remicade/Enbrel	3	1	0.2**
RF 30-100	N	9	6	
	Age (years)	64.2 \pm 6.0	62.0 \pm 18.3	0.74
	Male:Female	2:7	1:5	0.79**
	Duration (years)	8.9 \pm 7.8	6.7 \pm 4.6	0.5
	CRP	1.0 \pm 1.8	0.2 \pm 0.3	0.28
	No. swelling/joint	1.9 \pm 2.2	2.0 \pm 3.2	0.9
	No. pain/joint	0.8 \pm 2.1	0.5 \pm 1.2	0.8
	Treatment with Remicade/Enbrel	2	0	0.2**
RF >100	N	10	11	
	Age (years)	56.6 \pm 11.5	54.5 \pm 16.5	0.75
	Male:Female	2:8	1:10	0.4**
	Duration (years)	9.0 \pm 17	5.6 \pm 2.9	0.5
	CRP	1.4 \pm 2.2	1.6 \pm 2.6	0.9
	No. swelling/joint	5.0 \pm 7.3	8.3 \pm 7.3	0.3
	No. pain/joint	2.9 \pm 5.1	5.6 \pm 5.3	0.2
	Treatment with Remicade/Enbrel	2	0	0.1**
All	N	28	28	
	Age (years)	58.4 \pm 1.07	60.9 \pm 15.6	0.5
	Male:Female	4:24	3:25	0.7**
	Duration (years)	6.9 \pm 11.0	6.1 \pm 5.3	0.7
	CRP	0.9 \pm 1.7	0.9 \pm 1.7	0.9
	No. swelling/joint	3.4 \pm 5.2	5.2 \pm 6.0	0.3
	No. pain/joint	1.9 \pm 3.8	2.7 \pm 4.3	0.5
	Treatment with Remicade/Enbrel	7	1	0.02**

*:FI: fold increase, **: χ^2 test

[0032] As shown in Table 3, the responder population in the RF <30 IU/ml group was significantly ($p=0.04$) younger than that of non-responders. Other clinical parameters, such as age, gender, disease duration, CRP, number of swelling joints, and number of tender joints were not significantly different between responders and non-responders in all RF groups (Table 3). Interestingly, when all patients were combined, the number of patients treated with biological agents such as an anti-TNF- α monoclonal antibody or a soluble TNF receptor were significantly ($p=0.02$) higher in the responder population than in the non-responder population (Table 3). Use of these anti-TNF- α agents may enhance Fc γ R-mediated TNF- α function through an unidentified

negative feedback mechanism, or such patients may be good candidates for biological therapy. RA patients that show response in HAG-induced TNF- α mRNA are more likely to have a significant TNF- α participation in the RA pathology, and therefore are more likely to be good candidates for these biological agents. Since biological drugs are very expensive, not effective in all patients, and occasionally show undesirable side effects, the identification of patients likely to respond to these drugs will have clinical utility as personalized medicine.

[0033] Cytotoxic assays have generally been used to study actual cell death resulting from the activity of the immune system, such as that which is believed to occur in RA. Cytotoxic assays are generally conducted by incubating ^{51}Cr -loaded target cells with effector cells at various ratios, and quantifying the amounts of ^{51}Cr radioactivity released from the dead or damaged cells (see Dunkley *et al.*, *J. Immunol. Methods* 6, 39 (1974)). Radioactive materials have been replaced with non-radioactive materials, such as fluorometric materials, in some cases (see Kruger-Krasagakes *et al.*, *J. Immunol. Methods* 156, 1 (1992)), but the basic principle is unchanged. The results of cytotoxic assays are thus reflective of actual cell death.

[0034] However, cytotoxic assays are performed under non-physiological experimental conditions, and complex cell-to-cell and cell-to-plasma interactions are difficult to assess in the course of such studies. Furthermore, cytotoxic assays do not indicate which TNFSF member is responsible for cell death. Once effector cells recognize the target cells, the effector cells' function is not only to kill the target, but also to recruit other effector cells, because a single effector cell is not enough to kill many target cells. This recruitment function is thought to be represented by the release of chemotactic factors. The identity of such chemotactic factors released by effector cells would not be revealed by classic cytotoxic assays. The assay system set forth in this disclosure is, however, capable of identifying many classes of gene expression in effector cells simultaneously.

[0035] The use of whole blood is preferable to using isolated leukocytes in culture media, because the former is more physiological than the latter, and whole populations of leukocytes can be screened. Longer incubation of whole blood may produce additional artifacts. Thus, the ideal way is to identify early signals of killer and recruitment signals in whole blood during a short period of incubation by switching *in vitro* to *ex vivo*. The transcription of mRNA is an earlier event than either protein synthesis or the final biological outcomes. Thus, mRNA is a logical target.

[0036] Since the present method uses whole blood, it may be used as a diagnostic test for RA to evaluate possible responsiveness to TNFSF-inactivating therapy, and to monitor the therapeutic response. Specifically, in a preferred embodiment of a method for determining

whether a human having RA is likely to respond to a therapy targeting an mRNA transcribed in response to T-cell stimulation, whole blood is obtained from an RA patient and samples of the blood are subjected to HAG stimulation and optionally to control stimulation (PBS), as described above. The amount of TNFSF-2, TNFSF-8, or TNFSF-15 mRNA may be measured in the samples as described above. An RA patient having a significantly elevated level of one or more of these mRNAs after stimulation with HAG (as indicated, for example, by a fold increase of greater than 2) is a good candidate for therapy targeting these mRNAs.

[0037] Furthermore, in a preferred embodiment of a method of evaluating the effectiveness of RA treatment targeting one or more of the TNFSF-2, TNFSF-8, or TNFSF-15 mRNAs in a patient, a first ratio of the amount of the mRNA in whole blood after HAG stimulation to the amount after control stimulation is obtained prior to the initiation of the treatment. A second ratio of the amount of the mRNA in whole blood after HAG stimulation to the amount after control stimulation is obtained after the initiation of the treatment. A significant difference in the ratios, such as where the first ratio is larger than the second ratio, can indicate the effectiveness of the therapy. Such therapies could include, for example, the administration of infliximab or etanercept.

[0038] Importantly, this *ex vivo* method can be used for the screening of compounds which inhibit anti-FcR-mediated expression of one or more of the TNFSF-2, TNFSF-8, or TNFSF-15 mRNAs, and particularly TNFSF-2, which is known to participate in disease pathology. Such compounds will be interesting drug targets, because these new drug candidates will block mRNA production in leukocytes at the transcription level. This will provide a new strategy for drug development against RA.

[0039] In an embodiment of a method of screening drug compounds using the disclosed system and thereby identifying a putative agent for treating RA, whole blood is obtained from RA patients that are responders, wherein responders are individuals whose leukocytes exhibit at least a 2-fold increase in the level of an RA-associated mRNA when exposed to a T-cell stimulation such as HAG. A first ratio of the amount of the mRNA in whole blood of the subjects after HAG stimulation to the amount after control stimulation is calculated. Further whole blood samples from the subjects are exposed *in vitro* to the drug compound, and then differentially stimulated as described above. A second ratio of the amount of the mRNA in whole blood after HAG stimulation to the amount after control stimulation of these exposed samples is then calculated. A significant difference in the two ratios, such as where the first ratio is larger than the second ratio, can indicate that the drug compound is a candidate for further investigation as a potential therapeutic for RA.

[0040] Additionally, in a preferred embodiment of a method of monitoring the state of the disease in a RA patient by measuring levels of one or more of TNFSF-2, TNFSF-8, or TNFSF-15 mRNAs in samples comprising leukocytes obtained from the patient, a first ratio of the amount of the mRNA in whole blood after T-cell stimulus using heat-aggregated IgG antibody or another stimulus *in vitro* to the amount after control stimulation *in vitro* is obtained at a first time. At a second time subsequent to the first time, a second ratio of the amount of the mRNA in whole blood after the T-cell stimulus *in vitro* to the amount after control stimulation *in vitro* is obtained. A significant difference in the ratios can indicate a change in the disease state. For example, when the second ratio is larger than the first, this can indicate disease progression, while a larger first ratio can indicate that the disease has regressed.

WHAT IS CLAIMED IS:

1. A method of determining whether a patient having rheumatoid arthritis is likely to respond to anti-TNF therapy, comprising:
 - stimulating the Fc receptors of leukocytes *in vitro* in a first sample from said patient;
 - measuring the amount of an mRNA encoding a tumor necrosis factor superfamily (“TNFSF”) protein in the first sample after the stimulation;
 - exposing leukocytes *in vitro* in a second sample to a control stimulus;
 - measuring the amount of said mRNA in the second sample;
 - determining a ratio of the amount of mRNA in the first sample to the amount of mRNA in the second sample; and
 - determining that the patient is likely to respond to the therapy if the ratio is about 2:1 or greater.
2. The method of Claim 1, wherein the mRNA encodes TNFSF-2.
3. The method of Claim 2, wherein stimulating leukocytes in the first sample comprises intermixing heat-aggregated human IgG with the first sample.
4. The method of Claim 2, wherein at least one of the first and second samples comprises whole blood.
5. The method of Claim 2, wherein the control stimulus is phosphate-buffered saline.
6. The method of Claim 2, wherein the therapy comprises administration of an agent selected from the group consisting of infliximab and etanercept.
7. A method of determining whether a patient having rheumatoid arthritis is likely to respond to a therapy that inhibits anti-FcR-mediated expression of an mRNA encoding a tumor necrosis factor superfamily (“TNFSF”) protein, comprising:
 - stimulating the Fc receptors of leukocytes *in vitro* in a first sample from said patient;
 - measuring the amount of an mRNA encoding a tumor necrosis factor superfamily (“TNFSF”) protein in the first sample after the stimulation;
 - exposing leukocytes *in vitro* in a second sample to a control stimulus;
 - measuring the amount of said mRNA in the second sample;
 - determining a ratio of the amount of mRNA in the first sample to the amount of mRNA in the second sample; and
 - determining that the patient is likely to respond to the therapy if the ratio is about 2:1 or greater.

8. The method of Claim 7, wherein the mRNA encodes TNFSF-2.
9. The method of Claim 8, wherein stimulating leukocytes in the first sample comprises intermixing heat-aggregated human IgG with the first sample.
10. The method of Claim 8, wherein at least one of the first and second samples comprises whole blood.
11. The method of Claim 8, wherein the control stimulus is phosphate-buffered saline.
12. A method of identifying a putative agent for treating rheumatoid arthritis, comprising:

obtaining first, second, third, and fourth samples comprising leukocytes from a human whose leukocytes demonstrate at least a 1.5-fold increase in the transcription of an mRNA encoding a tumor necrosis factor superfamily ("TNFSF") protein when exposed to heat-aggregated human IgG;

stimulating the Fc receptors of leukocytes *in vitro* in the first sample;

exposing leukocytes *in vitro* in the second sample to a control stimulus;

measuring the amount of mRNA in the first and second samples after stimulation;

exposing the third and fourth samples to the agent;

stimulating the Fc receptors of leukocytes *in vitro* in the third sample after the exposure;

exposing leukocytes *in vitro* in the fourth sample to the control stimulus;

measuring the level of mRNA in the third and fourth samples after stimulation;

and

comparing the data obtained from the first sample to the data obtained from the third sample and comparing the data obtained from the second sample to the data obtained from the fourth sample, wherein a significant difference between the comparative data is indicative of a putative agent.

13. The method of Claim 12, wherein:

after measuring the amount of mRNA in the first and second samples, a first ratio of the amount of mRNA in the first sample to the amount of mRNA in the second sample is calculated;

after measuring the level of mRNA in the third and fourth samples, a second ratio of the amount of mRNA in the third sample to the amount of mRNA in the fourth sample is calculated; and

the first and second ratios are compared, wherein a significant difference in the ratios is indicative of a putative agent.

14. The method of Claim 13, wherein the TNFSF protein is TNFSF-2, TNFSF-8, or TNFSF-15.
15. The method of Claim 14, wherein stimulating leukocytes in the first and third samples comprises intermixing heat-aggregated human IgG with the samples.
16. The method of Claim 14, wherein the control stimulus is phosphate-buffered saline.
17. The method of Claim 14, wherein at least one of the first, second, third, and fourth samples comprises whole blood.
18. The method of Claim 14, wherein the significant difference in the ratios is that the first ratio is greater than the second ratio.
19. The method of Claim 14, wherein the mRNA encodes TNFSF-2.
20. The method of Claim 12, wherein the TNFSF protein is TNFSF-2, TNFSF-8, or TNFSF-15.
21. The method of Claim 20, wherein stimulating leukocytes in the first and third samples comprises intermixing heat-aggregated human IgG with the samples.
22. The method of Claim 20, wherein the control stimulus is phosphate-buffered saline.
23. The method of Claim 20, wherein at least one of the first, second, third, and fourth samples comprises whole blood.
24. The method of Claim 20, wherein the mRNA encodes TNFSF-2.
25. A method of evaluating the state of rheumatoid arthritis in a patient, comprising:
 - stimulating the Fc receptors of leukocytes *in vitro* in a first sample that comprises leukocytes and is obtained at a first time from the patient;
 - measuring the amount of an mRNA encoding a tumor necrosis factor superfamily ("TNFSF") protein in the first sample after stimulation;
 - exposing leukocytes *in vitro* to a control stimulus in a second sample comprising leukocytes obtained from the patient at the first time;
 - measuring the amount of mRNA in the second sample after stimulation;
 - determining a first ratio of the amount of mRNA in the first sample to the amount of mRNA in the second sample;
 - stimulating the Fc receptors of leukocytes *in vitro* in a third sample that comprises leukocytes and is obtained from the patient at a second time that is subsequent to the first time;
 - measuring the amount of mRNA in the third sample after the stimulation;

exposing leukocytes *in vitro* to a control stimulus in a fourth sample comprising leukocytes obtained from the patient at the second time;
measuring the amount of mRNA in the fourth sample after stimulation;
determining a second ratio of the amount of mRNA in the third sample to the amount of mRNA in the fourth sample; and
comparing the first and second ratios, wherein a significant difference in the first and second ratios is indicative of a change in the disease state.

26. The method of Claim 25, wherein the TNFSF protein is TNFSF-2, TNFSF-8, or TNFSF-15.

27. The method of Claim 26, wherein stimulating leukocytes in the first and third samples comprises intermixing human heat-aggregated IgG with the sample.

28. The method of Claim 26, wherein the control stimulus is phosphate-buffered saline.

29. The method of Claim 26, wherein at least one of the first, second, third and fourth samples comprises whole blood.

30. The method of Claim 26, wherein the significant difference in the ratios is that the second ratio is greater than the first ratio, and the change in disease state is a progression of the disease.

31. The method of Claim 26, wherein the significant difference in the ratios is that the first ratio is greater than the second ratio, and the change in disease state is a regression of the disease.

32. The method of Claim 26, wherein the mRNA encodes TNFSF-2.

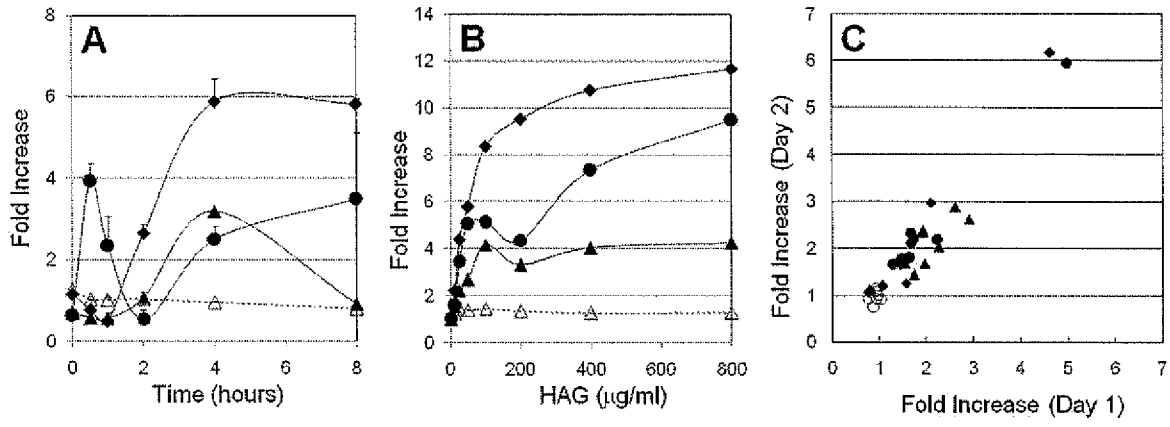


FIGURE 1

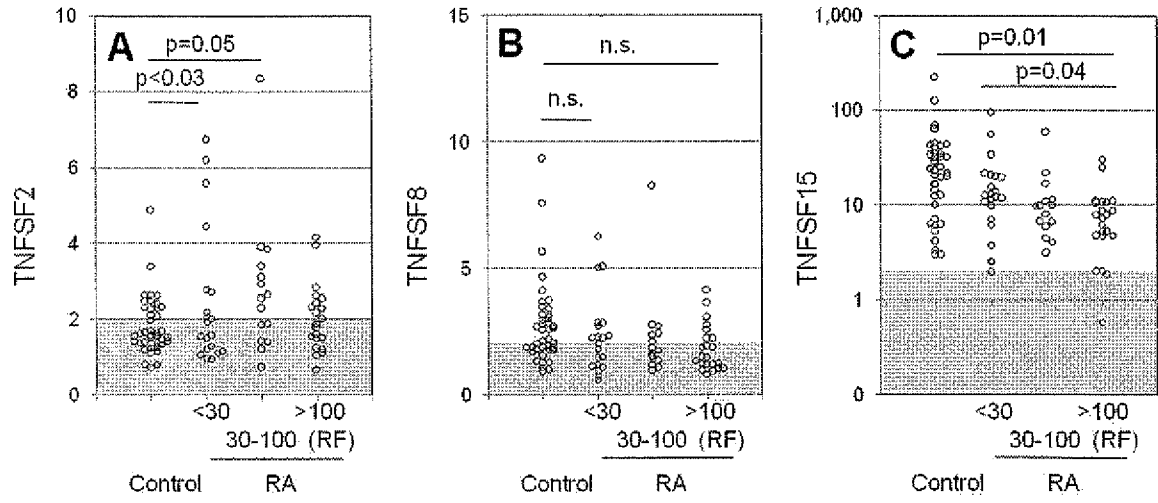


FIGURE 2

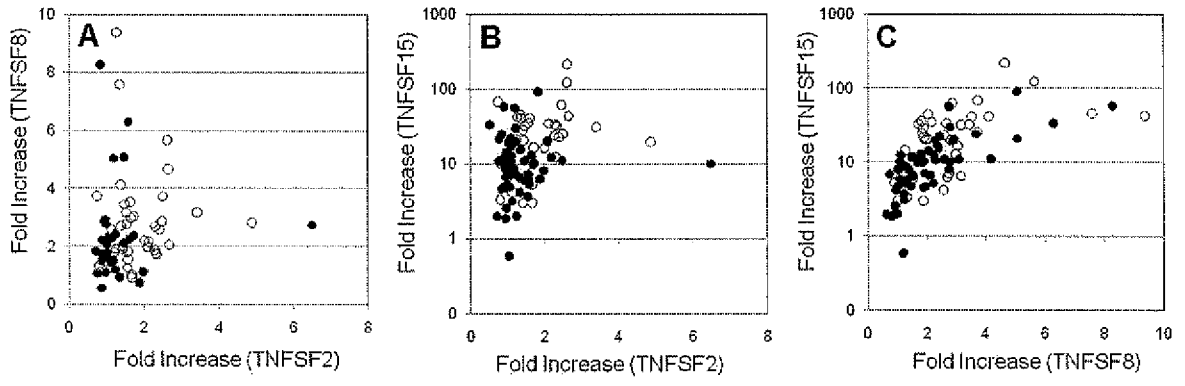


FIGURE 3