

Concurrent gene therapy strategies effectively destroy synoviocytes of patients with rheumatoid arthritis

E. Terzioglu^{1,2*}, A. Bisgin^{1,3*}, A. D. Sanlioglu^{1,3}, M. Ulker¹, V. Yazisiz^{1,2}, S. Tuzuner^{1,4}
and S. Sanlioglu^{1,3}

Objectives. Rheumatoid arthritis (RA) is characterized by the chronic inflammation of the synovial joints resulting from the hyperplasia of synovial cells and the infiltration of lymphocytes, macrophages and plasma cells. Currently, the aetiology of RA is not known, and new treatment modalities are needed to prevent the disease progression. Apoptosis induction of synovial cells through the use of death ligands has been explored as a treatment modality for RA. Thus, the primary objective of this study was the testing of the efficacy of adenovirus delivery of human TRAIL (Ad5hTRAIL) for the treatment of patients with RA.

Methods. Primary synovial cell cultures were established from eight patients with RA. Adenovirus permissiveness of synovial cells was determined by the infection of synoviocytes with adenovirus vector encoding green fluorescent protein (AdEGFP). TRAIL sensitivity of synoviocytes was assessed through the infection with Ad5hTRAIL vector using Live/Death Cellular Viability/Toxicity kit from Molecular Probe. TRAIL receptor profiles of synoviocytes were revealed by real-time RT-PCR assays followed by flow cytometric analyses.

Results. While the presence of TRAIL death receptors were necessary for the induction of cell death, high levels of TRAIL-R4 decoy receptor expression on surface were correlated with TRAIL resistance. A DcR2 siRNA approach in combination with Ad5hTRAIL infection eliminated apoptosis-resistant RA synovial fibroblasts.

Conclusion. Because a DcR2 siRNA approach in combination with Ad5hTRAIL infection exterminated RA synoviocytes to a greater extent than Ad5hTRAIL alone, the modulation of TRAIL receptor expression might be a new gene therapy strategy to sensitize RA synoviocytes to TRAIL.

KEY WORDS: Rheumatoid arthritis, Adenovirus, TRAIL, siRNA.

Rheumatoid arthritis (RA) is a disease in which synovial cells are phenotypically transformed to proliferate abnormally invading both bone and cartilage [1]. In RA, apoptosis-resistant synovial cells provoke tissue degradation through the production of elevated levels of pro-inflammatory cytokines and metalloproteinases [2]. Therefore, abnormal synoviocyte proliferation and lymphocyte–macrophage activation are the two major pathogenic hallmarks of RA [3]. Several strategies including gene therapy were explored for the treatment of RA in order to overcome current drug delivery problems to articular tissues [4]. With this perspective, prospected goals were to inhibit the production of pro-inflammatory cytokines [1] and matrix degrading enzymes [5] to prevent the activation of specific signal transduction pathways such as NF- κ B [6] and finally to promote apoptosis in synoviocytes and immune cells [7].

Induction of apoptosis is a common property of death ligands, which belong to tumour necrosis factor (TNF) family. TNF-related apoptosis inducing ligand (TRAIL) is a type II membrane protein of the TNF super family [8]. Five different receptors were identified to interact with TRAIL and these are TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and osteopontin [9, 10]. While the existence of multiple TRAIL receptors indicates that TRAIL is involved in multiple processes in the cell, the precise role of TRAIL in health and disease is unclear at this moment [11]. While TRAIL-R1 and TRAIL-R2 function as authentic death receptors inducing

apoptosis, TRAIL-R3 and TRAIL-R4 are unable to induce such signalling but can serve as decoy receptors [12]. Compared with the other members of TNF family such as FasL and TNF, TRAIL has some distinct apoptosis-inducing properties. For example, unlike TNF- α , which initiated and exacerbated autoimmune diseases, TRAIL was reported to down-regulate immune responses. This was demonstrated in TRAIL null mice, which were more susceptible to collagen-induced arthritis (CIA) due to the failure to properly silence activated T cells [13].

Even though, TRAIL and TRAIL receptors are expressed constitutively in various tissues, TRAIL does not induce apoptosis of most non-transformed cells [11, 14, 15]. Even today, no single mechanism is thought to be responsible for the TRAIL resistance in normal cells. There are at least two different hypotheses claimed for TRAIL resistance. The first hypothesis supports that normal cells carry decoy receptors (TRAIL-R3, TRAIL-R4), which compete with apoptosis-inducing TRAIL receptors (TRAIL-R1, TRAIL-R2) for binding to TRAIL [14, 15]. Here it is thought that decoy receptors either serve to dilute out TRAIL ligands (like TRAIL-R3) or provide anti-apoptotic signals (like TRAIL-R4) to cells. For instance, TRAIL-R4 binding activated anti-apoptotic NF- κ B signalling pathway, which led to the obstruction of TRAIL-mediated apoptosis [16]. More interestingly, activation of death receptors such as TRAIL-R1 and TRAIL-R2 also activated NF- κ B pathway [17, 18]. Under these circumstances, how cells undergo apoptosis despite the induction of both apoptotic and anti-apoptotic pathways is not known. The second hypothesis proposes the presence of apoptosis inhibitory substances. Such a molecule, cFLIP (FLICE inhibitory protein), a caspase 8 homologue, was shown to obstruct death ligand-induced apoptosis before [19, 20]. Despite these hypotheses, no clear evidence to account for TRAIL resistance has been asserted yet.

As gene delivery vehicles, vectors derived from retrovirus [21], adenovirus [2] and herpes simplex virus [22] as well as cationic liposomes and naked plasmid DNA were all tested to deliver

¹Human Gene Therapy Unit, ²Department of Rheumatology—Allergy and Immunology, ³Department of Medical Biology and Genetics and ⁴Department of Orthopedic Surgery, Akdeniz University Faculty of Medicine, Antalya 07070, Turkey.

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Correspondence to: S. Sanlioglu, Director of the Human Gene Therapy Unit, Akdeniz University, Faculty of Medicine, B-Block, 1st floor, Campus, Antalya 07070, Turkey. E-mail: sanlioglu@akdeniz.edu.tr

*The first two authors E.T. and A.B. contributed equally to this article.

therapeutic genes into the synovial lining of rabbit joints. Among the ones tested, adenovirus was found to be the most effective gene delivery vehicle [23]. Thus, in this study, adenovirus was chosen as gene delivery vector to transfer hTRAIL (Ad5hTRAIL) into synovial cells as a treatment modality for patients with RA. Furthermore, TRAIL receptor compositions of synoviocytes established from RA patients were analysed using real-time RT-PCR assays and flow cytometric analyses. DcR2 siRNA strategy was employed in order to alter the TRAIL receptor composition of RA synoviocytes. A putative connection between TRAIL sensitivity and TRAIL receptor profiles of RA synoviocytes was explored in this study.

Patients and methods

Synovial cell isolation and culturing from patients with RA

Synoviocyte cultures were established from eight patients diagnosed with RA based on the American Rheumatism Association 1987 revised criteria for the classification of RA [24]. RA synovial tissues were obtained at the time of knee/hip arthroplasty or arthroscopy in the Department of Orthopedic Surgery of Akdeniz University. The disease activity scores in 28 joints (DAS28) of eight patients were in the range of 3.8–6.5 (average 4.6). This study was approved by the Akdeniz University Hospital Committee on Ethics. In addition, patients' written consents based on Helsinki Declaration were obtained prior to the operation. Single-cell suspensions were prepared from the isolated human synovial tissues digested with 0.2% collagenase P as described earlier [25, 26].

Amplification of the first generation recombinant adenovirus vectors to deliver transgenes into RA synoviocytes

First generation recombinant adenovirus vectors used in the infections such as Ad5hTRAIL [27], AdEGFP [28] and AdCMVLacZ [29] were amplified as described previously [30]. Adenoviral vectors were stored at -80°C in 10 mM Tris containing 20% glycerol following CsCl banding and vector purification. The Ad5hTRAIL construct was used to overexpress hTRAIL in RA synoviocytes. Adenovirus vectors expressing β -galactosidase gene (AdCMVLacZ) was used as a negative control in the assays. The particle titres of adenoviral stocks, which were in the range of 10^{13} DNA particles/ml for each prep were determined by A260 measurements. Plaque titration on 293 cells and expression assays for encoded proteins were performed to obtain functional titres. In our purifications, the particle/plaque forming unit ratio was generally equal to 50.

The efficacy of adenovirus transduction of human synoviocytes

Briefly, synovial cells were incubated in RPMI 1640 medium supplemented with 10% FBS, 2.2 g/l sodium bicarbonate, 1 mM L-glutamine and 1% penicillin–streptomycin–amphotericin mixture (PSA) in Thermo SteriCult incubators. Adenovirus vectors expressing enhanced green fluorescent protein (EGFP) reporter gene (AdEGFP) were infected into synoviocytes at an increasing multiplicity of infection (MOI) and synoviocytes were incubated at 37°C in RPMI 1640 without FBS for 2 h. After that, an equal volume of RPMI 1640 supplemented with 20% FBS was added to increase the serum concentration in the media to 10%. The percentage of EGFP (+) cells was assessed under a fluorescent microscopy and subsequently by flow cytometry 48 h after the infection. Cell viability was monitored using propidium iodide exclusion technique.

Live/dead cellular viability and toxicity assays

Live/Dead Cellular Viability/Cytotoxicity Kit from Molecular Probes (Eugene, OR, USA) was used to discriminate live cells from dead cells. Calcein AM and ethidium homodimer-1 (EthD-1) are the two molecular reporters in the kit. The intracellular calcein esterase activity was assessed using calcein AM, a fluorogenic substrate. In this assay, active esterases present only in live cells with intact membranes, convert Calcein AM to a green fluorescent compound (calcein), which serves as a marker for viable cells. On the other hand, EthD-1 is a red fluorescent nucleic acid stain only taken up by cells with damaged membrane. Here unharmed cells with intact membranes do not allow EthD-1 to enter inside the cell; therefore, live cells do not get stained with EthD-1. However, harmed cells with damaged membrane up-take the dye and stain positive. Cellular viability assays were conducted 35 h following the infections.

Quantitative real-time RT-PCR assays for synovial cells established from RA patients

SDS 7500 ABI Prism instrument was used to perform relative quantities of TRAIL receptor mRNAs. Total RNA was extracted from RA synoviocytes using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). A $2\ \mu\text{g}$ of total RNA was included in each reverse transcription reaction using TaqMan Reverse Transcription Reagents (Applied Biosystems Cat. N8080234). Primer and probe sequences of TRAIL death receptors [31] and TRAIL decoy receptors [32, 33] used for the real-time RT-PCR assays were recently reported. In addition, ribosomal RNA (rRNA) primers and probes, which serve as an internal control in the same reaction, were acquired from PE Applied Biosystems (Cat. 4308329). $\Delta\Delta\text{Ct}$ method is employed to obtain the relative quantities of TRAIL receptors. The TaqMan PCR assays were performed as suggested by the manufacturer (Applied Biosystems Cat. N8080228). Reverse transcription step included a $50\ \mu\text{l}$ reaction mixture prepared in reverse transcription buffer with 2.25 mM MgCl_2 , $50\ \mu\text{M}$ from each dNTP, $2.5\ \mu\text{M}$ Random Hexamer, $0.6\ \text{U}/\mu\text{l}$ RNase inhibitor and $1.25\ \text{U}/\mu\text{l}$ reverse transcriptase. The thermal cycling conditions for the reverse transcription reactions were 10 min at 25°C , followed by 60 min at 48°C . TaqMan PCR assay was executed using TaqMan Universal Master Mix with 50 pmol of primer and probe mixture and 250 ng of cDNA. For the real-time RT-PCR step; the thermal cycling conditions are as follows: 2 min at 50°C , 10 min at 95°C , followed by 40 cycles of 15 s at 95°C and 1 min at 60°C . Here each reaction produced a specific threshold cycle (Ct). Then, ΔCt values were estimated for each receptor by taking the difference between the Ct values of the TRAIL receptors and that of rRNA internal controls. Moreover, a standard curve was generated using serial dilutions of rRNA. The comparative $\Delta\Delta\text{Ct}$ calculation was computed then by finding the difference between each receptor's ΔCt value and that of 25 pg of rRNA. Final relative expression levels were computed by the formula $2^{-\Delta\Delta\text{Ct}}$. Thus, relative expression levels of TRAIL receptors were normalized to that of 25 pg of rRNA.

Flow cytometric analyses of TRAIL receptor protein expression profiles in RA synoviocytes

Anti-TRAIL receptor flow cytometry kit (Cat. ALX-850-273-KI01) was used to detect the surface TRAIL receptor expression profiles of synoviocytes. $100\ \mu\text{g}$ of MAbs to TRAIL-R1 (clone HS101, Cat. 804-297A), TRAIL-R2 (clone HS201, Cat. 804-298A), TRAIL-R3 (clone HS301, Cat. 804-344A) and TRAIL-R4 (clone HS402, Cat. 804-299A) were included in each kit. Primary antibodies were used at $5\ \mu\text{g}/\text{ml}$ concentration. Since the secondary antibody was the biotinylated goat anti-mouse IgG1 (Cat. ALX-211-202), the streptavidin-PE conjugate

(Cat. ANC-253-050) was added before the flow cytometric analysis. All the flow assays were performed in duplicates and repeated at least twice to confirm the observation ($n=4$). Newly acquired Beckman Coulter EPICS ALTRA (HyPerSort™) instrument of the Human Gene Therapy Unit of Akdeniz University Hospitals was used to perform the flow cytometric analyses. The marker distribution (immune staining) based on flow cytometric quantification of TRAIL and its receptors was analysed similar to the one described recently [11]. The marker distribution was scored as 0 (–, negative), <10%; 1 (+, weak), between 10% and 40%; 2 (++, moderate), between 40% and 70% and 3 (+++, strong), >70% of the synoviocytes stained. Surface TRAIL expression was analysed using a monoclonal antibody to TRAIL (human) (ALX-804-296-C100) followed by a polyclonal antibody to mouse IgG1 (R-PE) (ALX-211-201-CO50).

Down-regulation of TRAIL-R4 expression by a DcR2 siRNA approach

A DcR2 siRNA kit (sc-35185), siRNA transfection medium (sc-36868) and siRNA transfection reagent (sc-29528) were used to perform DcR2 siRNA experiments as described by the manufacturer (Santa Cruz Biotechnology). Gene Bank Access Number: NM_003840. A fluorescein conjugated control siRNA (sc-36869) was used to determine the transfection efficiency under the fluorescent microscope prior to analysis. More than 97% of the cells were transfected by this procedure. As a negative control, control siRNA-A (sc-37007) containing scrambled sequences which did not lead to the specific degradation of any cellular RNA was used in siRNA experiments. Transfections were performed 35 h prior to the infections. The efficacy of the siRNA approach was measured 30 h after the transfection using real-time RT-PCR assay and flow cytometry.

Results

Adenovirus transduction of RA synovial cells

Recombinant adenovirus vectors carrying AdEGFP were infected into synovial cells established from patients with RA. The percentage of GFP (+) cells was determined 48 h after the infection by both fluorescent microscopy (Fig. 1A) and flow cytometry (Fig. 1B). Flow cytometric analyses of synoviocytes isolated from eight patients suggested that >90% of the cells (± 7) were transduced at an MOI of 5000 DNA particles/cell of AdEGFP vectors. Almost all synoviocytes were GFP-positive ($97 \pm 2\%$) when the MOI of AdEGFP virus was increased to the MOI of 10000 DNA particles/cell as shown in Fig. 1, Panel B. Since no patient's synoviocytes exhibited resistance to adenovirus infection, it was concluded that synoviocytes isolated from RA patients were readily transducible by recombinant adenovirus vectors.

TRAIL receptor compositions of RA synovial cells

In order to reveal TRAIL receptor profiles in patients with RA; real-time RT-PCR assays and flow cytometric analyses (Table 1) were employed. TRAIL-R1 death receptor mRNA expression was detectable in the synoviocytes of six out of eight RA patients, but only two of these synovial cultures displayed TRAIL-R1 death receptor protein expression on the surface. On the other hand, TRAIL-R2 death receptor mRNA expression was noticeable in all RA patients' synoviocytes and all synoviocytes displayed TRAIL-R2 death receptor protein to varying degrees on their cell surface. Furthermore, while TRAIL-R3 decoy receptor mRNA expression was evident in all patients to some degrees, low levels of TRAIL-R3 surface protein expression were measurable only in two out of eight patients. RA synoviocytes isolated from three out of eight

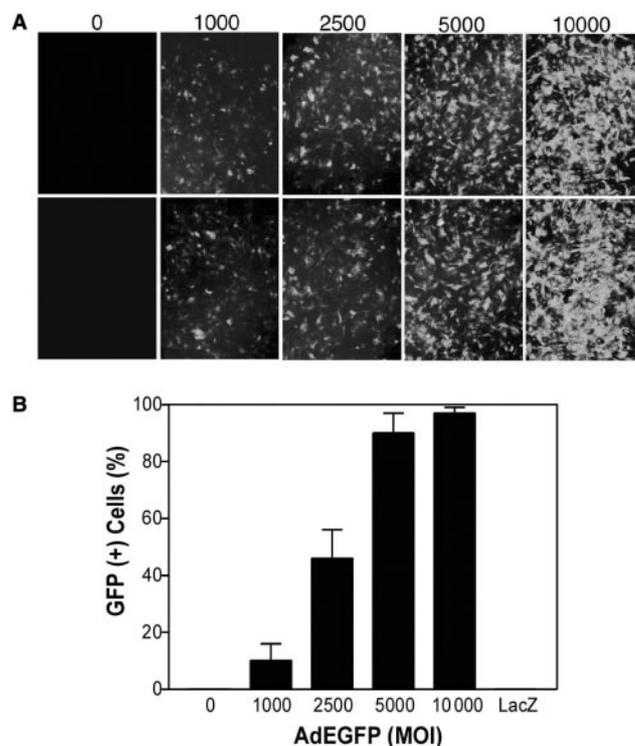


FIG. 1. Adenovirus transduction of RA synoviocytes. Panel A indicates fluorescent micrographs of RA synoviocytes infected with various MOIs of AdEGFP vectors. MOI values used in the infection are provided above each panel. Fluorescent micrograph images are provided in duplicates from only one patient out of eight. Quantitative results of the flow cytometric analyses of eight patients are given in Panel B. Values represent the mean (\pm S.E.M.) of 32 independent data points ($n=32$). The percentage GFP (+) cells is illustrated on y-axis. Viral doses applied as MOI values in DNA particles/cell are shown below x-axis. AdCMVLacZ infections are carried out at an MOI of 10000 DNA particles/cell.

RA patients showed evidence of an expression of TRAIL-R4 decoy mRNA in the cell and these cells were also positive for TRAIL-R4 protein expression on the cell surface. As strong TRAIL-R2 death receptor mRNA expression was correlated with the prominent protein expression on the surface of RA synoviocytes in some patients, even low TRAIL-R2 mRNA expression inside the cell still generated detectable levels of the surface protein expression in other patients. Similarly, as shown in Figs 4 and 5, strong TRAIL-R4 decoy receptor mRNA expression inside the cell was linked to high levels of surface TRAIL-R4 decoy receptor expression. While synoviocytes of three out of eight patients exhibited all four TRAIL receptors on mRNA levels (Figs 3–5), synoviocytes isolated from none of these RA patients demonstrated both the death and the decoy receptors as a complete set on the surface.

TRAIL sensitivity profiles of RA synoviocytes

Molecular Probes Live/Dead Cellular Viability/Toxicity kit was used to determine the cellular viability of RA synoviocytes following the infection with Ad5hTRAIL vector. Those synovial cells with strong death receptor expression especially TRAIL-R2 on the surface either without the decoy receptor expression (Fig. 2) or low levels of TRAIL-R4 decoy receptor expression (Fig. 3), showed the highest degree of TRAIL sensitivity upon infection. One patient displayed very low levels of TRAIL-R2 death receptor on the surface which were resistant to the cytotoxic effects of Ad5hTRAIL. But in this case, TRAIL resistance can be attributed to the insufficient amounts of death receptor expression on the cell surface but not to the presence of decoy receptors.

TABLE 1. TRAIL receptor expression profile of synoviocytes isolated from patients with RA.

Patient number	TRAIL receptor			
	TR1	TR2	TR3	TR4
1	-	+++	-	-
2	++	+++	-	-
3	+	+++	-	-
4	-	+	-	-
5	-	++	+	-
6	-	+	++	+++
7	-	+++	-	+++
8	-	+++	-	+

Flow cytometric analysis and quantifications were performed as described in 'Patients and methods' section. Numbers on the left refer to the patient number (1–8). TRAIL receptor subtype (TR-1–TR-4) is given at the top of each column

The patient who displayed low levels of both TRAIL-R2 death receptor and TRAIL-R3 decoy receptor expression on synoviocytes were still partly sensitive to Ad5hTRAIL infection. In this case TRAIL-R2 expression was slightly higher than TRAIL-R3 despite the fact that both were low. This suggested that low levels of TRAIL-R3 did not protect cells from TRAIL cytotoxicity. However, the TRAIL resistance was restored in synoviocytes with elevated TRAIL-R4 decoy receptor expression (but low levels of TRAIL-R2 death receptor expression) on surface (Fig. 4). Interestingly, a DcR2 siRNA approach employed for the patient displayed on Fig. 5, lowered TRAIL-R4 decoy receptor expression on the surface (Fig. 6, Panel A, label 4 to the left) and this sensitized RA synoviocytes to Ad5hTRAIL as shown in Fig. 6, Panel B. RA synoviocytes of this particular patient exhibited $82 \pm 6\%$ decrease in surface TRAIL-R4 expression and $89 \pm 5\%$ reduction in TRAIL-R4 transcripts following DcR2 siRNA approach as demonstrated by flow cytometry and real-time RT-PCR assays, respectively. By deploying the DcR2 siRNA approach, TRAIL resistance of synovial cells was reduced from $73 \pm 5\%$ to $20 \pm 6\%$. Neither a reduction in TRAIL-R4 expression (Fig. 6, Panel A, label 4 to the right) nor a sensitization was noticeable when siRNA-A was used in the transfection instead of DcR2 siRNA.

Discussion

Neither TRAIL receptor expression profiles nor the mechanism of TRAIL resistance in RA synoviocytes are clearly known yet. In one recent study, the lack of TRAIL receptor gene expression was blamed for the failure to induce TRAIL-mediated apoptosis on cultured RA synovial fibroblasts [34]. In another study, the presence of high levels of TRAIL-R2 death receptor expression was reported in synovial fibroblasts of RA patients using flow cytometry [35]. As the presence of all four TRAIL receptors in synovial fibroblasts were also confirmed by western blotting, increased AKT activity was pointed out as an inhibitor of TRAIL-mediated cytotoxicity [36]. Although both the intra-articular adenovirus delivery of TRAIL and/or recombinant TRAIL administration induced apoptosis and reduced inflammation in a rabbit model of arthritis [37, 38], TRAIL was reported to cause cell death only in a small fraction of RA synovial fibroblasts (30%); conversely, the remaining apoptosis resistant synoviocytes were stimulated to proliferate by TRAIL [39]. Thus, TRAIL resistance manifested by synovial cells remains as a major challenge for any adenovirus-mediated TRAIL gene therapy approach. Nevertheless, a histone deacetylase inhibitor, Trichostatin A, was reported to sensitize RA synoviocytes to TRAIL [40]. Therefore, in terms of the mechanisms, both the TRAIL sensitivity status and the TRAIL receptor composition of RA synoviocytes deserve an extensive investigation.

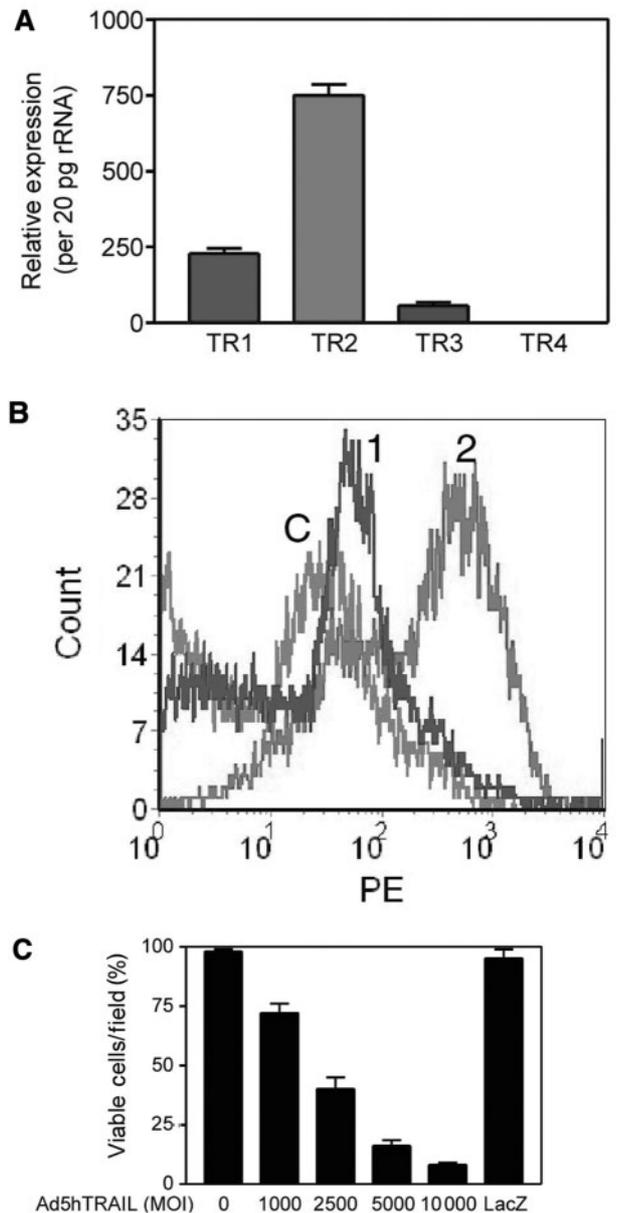


FIG. 2. Death receptor gene expression on surface is the main modulator of TRAIL sensitivity. While intracellular mRNA composition of TRAIL receptors is given in Panel A, extra-cellular protein composition is provided in Panel B. As shown in Panel B, C represents isotype control staining and the number above the each peak displays particular TRAIL receptor. Each histogram represents 10^4 gated cells. Cell survival analysis following infection with Ad5hTRAIL is provided in Panel C. AdCMVLacZ infections (LacZ) are performed at an MOI of 10000 DNA particles/cell. Values represent the mean (\pm S.E.M.) of six independent data points. These data (Panels A–C) belong to one patient only.

For this purpose, real-time RT-PCR and conventional flow cytometric analyses were employed in order to determine TRAIL receptor profiles of patients with RA. There were mainly two purposes for performing these two assays in order. First, mRNA levels do not necessarily correlate with the protein expression on the cell surface, which is a more relevant issue for TRAIL sensitivity. Second, an mRNA for a gene might still be expressed in the cell even if the protein expression is undetectable on the cell surface using flow cytometry. For example, while all TRAIL receptors are expressed inside the cell displayed for the patients depicted in Figs 3–5, surface TRAIL receptor expression is present

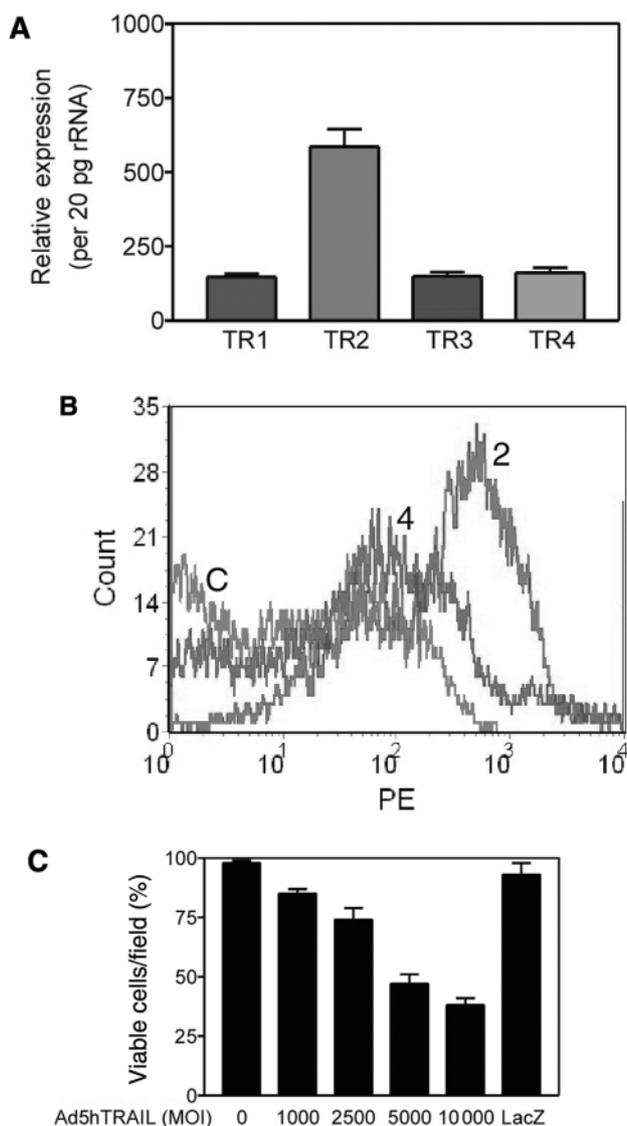


FIG. 3. Low levels of TRAIL-R4 expression on the surface cannot protect cells from TRAIL induced cell death. While real-time RT-PCR analysis is given in Panel A, Panel B shows a flow cytometric assessment of synovial cells. Isotype staining is shown as C, numbers above the each peak indicate each TRAIL receptor subtype in Panel B. 10^4 cells are gated for each histogram. The assay is repeated twice to confirm the observation. Ad5hTRAIL infection results in cell death of synovial cells (Panel C). As a control, an adenovirus expressing β -galactosidase gene (AdCMVLacZ) is infected at an MOI of 10 000 DNA particles/cell into synoviocytes. Data represent the mean (\pm s.e.m.) of three independent assays. Panels A–C represent a single patient only.

as a complete set (1–4) in none of these patients. Thus, our result proposes that intracellular TRAIL receptor compositions do not necessarily match to the surface TRAIL receptor expression profiles. Furthermore, Ad5hTRAIL infection mainly killed RA synoviocytes with high levels of TRAIL-R2 death receptor expression on the surface (Figs 2 and 3). The fact that synovial fluid fibroblast express functionally active TRAIL-R2 death receptor is also confirmed by a recent report [41]. On the other hand, synoviocytes with considerable levels of TRAIL-R4 decoy receptor expression (Figs 4 and 5) are resistant to TRAIL-mediated cell killing. It is intriguing to note that the level of TRAIL resistance is correlated with the amount of TRAIL-R4 decoy receptor expression on surface. This result also supports

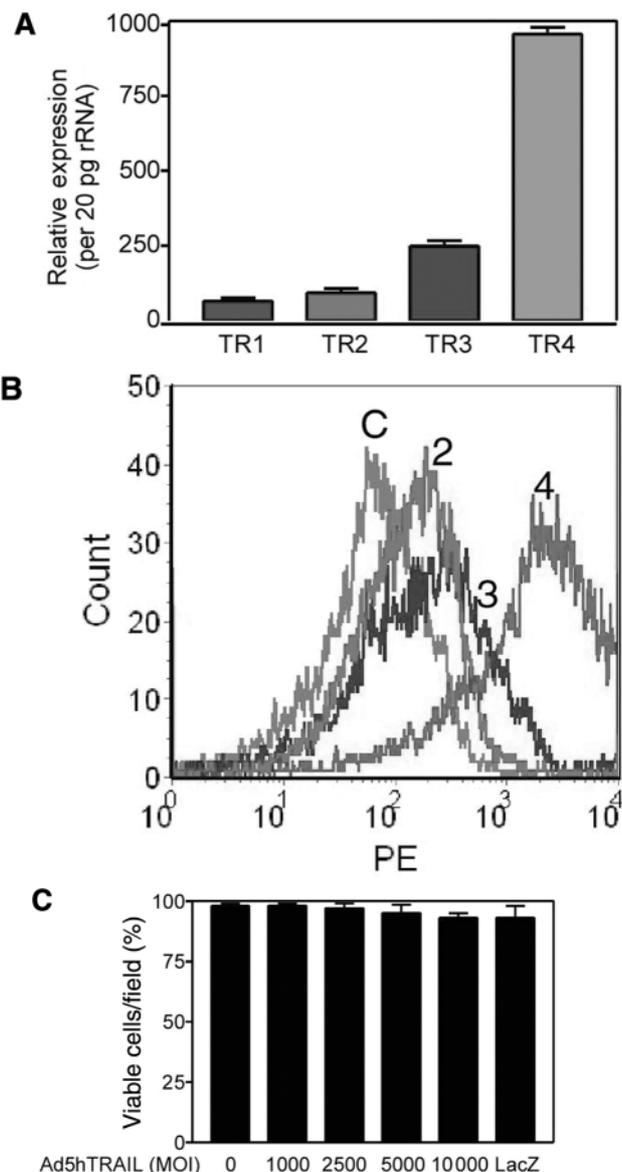


FIG. 4. Synovial cells expressing high levels of TRAIL-R4 decoy receptor is resistant to the cytotoxic effects of hTRAIL. Panel A shows an intracellular TRAIL receptor mRNA profile while Panel B indicates surface TRAIL receptor expression pattern of synovial cells. 10^4 cells are gated for the flow cytometric analyses. Live/Dead cellular viability and cytotoxicity assay is provided in Panel C. AdCMVLacZ (LacZ) infections are performed at an MOI of 10 000 DNA particles/cell. The assay is repeated three times to confirm the experimental findings. These panels (A–C) are derived from a single patient.

previous reports on surface TRAIL-R4 decoy receptor expression pattern and its connection to TRAIL resistance as demonstrated in prostate and breast cancer cells [32, 33]. A Dcr2 siRNA strategy but not an siRNA-A transfection in combination with Ad5hTRAIL infection were necessary to destroy TRAIL-resistant RA synoviocytes. Intriguingly, endogenous TRAIL death ligand expression could not be detected on RA synoviocytes isolated from the patients using flow cytometry (data not shown). This is in accordance with that recently reported by Morel *et al.* [39].

Due to its antigenic properties and transient gene expression, adenovirus is the most commonly used viral vector in the clinical trials of cancer gene therapy [42]. In addition, delivering adenovirus into each cell appears to be quite easy in a laboratory

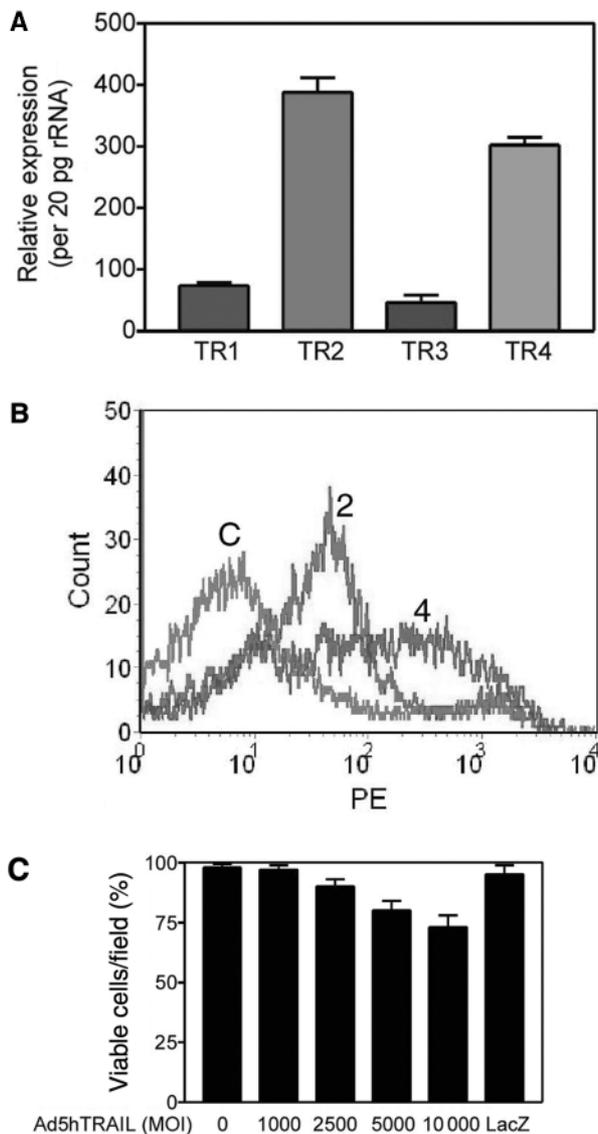


FIG. 5. High levels of TRAIL-R4 decoy receptor expression confer some degree of TRAIL resistance in synoviocytes despite the expression of TRAIL-R2 death receptor on the surface. Intracellular mRNA composition is provided in Panel A and the surface TRAIL receptor expression profile is given in Panel B. Each histogram represents 10^4 gated cells. Treatment conditions are shown above the each peak. The assay is repeated twice but only one is given for clarity. TRAIL sensitivity profile of synovial cells expressing both the decoy receptor (TRAIL-R4) and the death receptor (TRAIL-R2) on surface is given in Panel C. LacZ implies AdCMVLacZ infections performed at an MOI of 10000 DNA particles/cell. Only one out of the three independent assays is shown. Panels A–C are composed of data from a single patient.

setting. On the other hand, despite its high transduction properties, targeting each cell is a challenging task to accomplish under a clinical setting. Furthermore, the effect of the siRNA approach is transient. Thus, both of these concurrent gene therapy modalities have a short period of time to exert their action. If TRAIL resistant cells are not completely eliminated along the process, these cells may overtake the synovium. Then, a different serotype of adenovirus carrying hTRAIL has to be administered in combination with DcR2 siRNA due to the presence of neutralizing antibodies in the patient's sera. Since RA is characterized by the constant proliferation of synoviocytes, these cells but not the chondrocytes have to be targeted for the apoptosis inducing

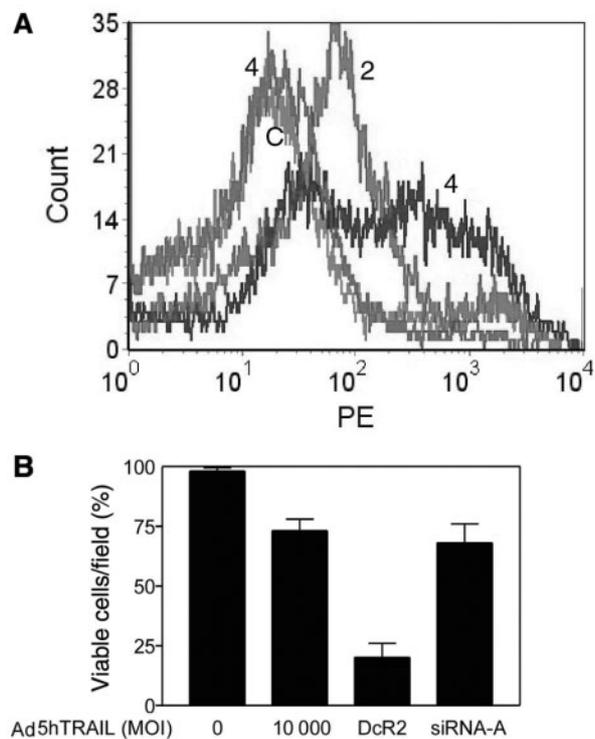


FIG. 6. Down-regulation of TRAIL-R4 expression further sensitized RA synoviocytes to Ad5hTRAIL. Panel A is a flow cytometric analysis of TRAIL receptor composition of synoviocytes following a siRNA approach. C represents isotype control staining. Numbers above the each peak represent TRAIL receptor subtype. Peak labelled as 4 (far right) received siRNA-A while the rest of the samples received DcR2 siRNA. 10^4 cells are gated for each histogram. Panel B is the cell viability assay following either DcR2 siRNA or siRNA-A approach. Both DcR2 and siRNA treated samples are also infected with an Ad5hTRAIL virus at an MOI of 10000 DNA particles/cell. Panels A and B are composed of data from a single patient.

therapeutic approaches. In order to confirm our findings, experiments have to be performed on a larger group of patients with a wide spectrum of disease progression. In addition, while TRAIL-R2 was the predominant receptor type expressed in RA synoviocytes, other TRAIL receptors exhibited a heterogeneous expression profile for a given patient (Table 1). This might raise a concern in the efficacy of Ad5hTRAIL application as a single treatment modality since only a certain fraction of RA synoviocytes was TRAIL sensitive, and therefore TRAIL resistance cells might prevail in the long run due to cellular proliferation stimulated by the TRAIL as reported previously [39]. Although, our study demonstrated that the DcR2 siRNA approach in combination with Ad5hTRAIL delivery was effective in reducing the number of TRAIL-resistant cells, whether such an approach is useful in eliminating TRAIL resistant cells in the long term remains to be seen.

Rheumatology key messages

- Synoviocytes of RA patients express various TRAIL receptors on the surface and these surface TRAIL receptor profiles mainly influence whether RA synoviocytes are sensitive or resistant to TRAIL.
- A DcR2 siRNA approach in combination with Ad5hTRAIL infection eliminated apoptosis-resistant RA synoviocytes to a greater degree than Ad5hTRAIL alone.

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The authors have declared no conflicts of interest.

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