Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) Induces Rheumatoid Arthritis Synovial Fibroblast Proliferation through Mitogen-activated Protein Kinases and Phosphatidylinositol 3-Kinase/Akt*

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Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of joints leading to progressive and irreversible joint destruction. The aggressive front of synovial tissue, called pannus, invades and destroys local articular structure. The pannus is characterized by a synovial hyperplasia that is mainly composed of fibroblast-like synoviocytes (FLSs) combined with a massive infiltration of lymphocytes and macrophages. Both increased proliferation and/or insufficient apoptosis might contribute to the expansion of RA FLSs, and several reports suggest inducing apoptosis of RA FLSs as a therapeutic approach.

Intraarticular expression of proinflammatory cytokines, in particular tumor necrosis factor (TNF-α) and IL-1β, plays a crucial role in the pathogenesis of RA (1). An anti-inflammatory role has been suggested for the TNF family member TRAIL (TNF-related apoptosis-inducing ligand) following the observations that the blockade of TRAIL in mice exacerbated autoimmune arthritis and that intraarticular TRAIL gene transfer relieved the symptoms (2). In addition, one report described an increased sensitivity of TRAIL-deficient mice to collagen-induced arthritis (3).

TRAIL can interact with five different receptors, namely two agonistic receptors called DR4/TRAIL-R1 and DR5/TRAIL-R2 (hereafter referred to as TRAIL-R1 and -R2, respectively), two membrane-anchored decoy receptors designated DcR1/TRAIL-R3 and DcR2/TRAIL-R4 (hereafter referred to as TRAIL-R3, and -R4, respectively), and a soluble decoy receptor known as osteoprotegerin (OPG) (4). The physiological relevance of OPG as a receptor for TRAIL is unclear, because the affinity for this ligand at physiological temperatures has been described to be very low (5). Notably, the affinities of TRAIL-R3 and -R4 for TRAIL at physiological temperatures are ~50–100-fold lower than those between TRAIL and its cognate receptors TRAIL-R1 and -R2 (5).

The receptors TRAIL-R1 and -R2 contain a cytoplasmic death domain and can induce apoptosis through the activation of caspases upon ligation with a TRAIL ligand (reviewed in Ref. 6). Nevertheless, the binding of TRAIL to these TRAIL receptors can also activate the transcription factor NFκB, which is known to control cell proliferation (7). Indeed, although TRAIL is known to induce apoptosis in tumor cells (reviewed in Ref. 6), TRAIL has also been shown to promote endothelial cell proliferation (8). Thus, depending on the cellular system, TRAIL is capable of initiating apoptosis or cell survival.

The effect of TRAIL on the RA FLS appears to be controversial. Ichikawa et al. described TRAIL-R1 and -R2 expression on primary isolated FLSs from RA patients and reported that an agonistic anti-TRAIL-R2 antibody, as well as cross-linked TRAIL, induces apoptosis on these FLSs (9). Similar observations have been reported by analyzing synovial fluid fibroblasts of RA patients (10). The conclusion that TRAIL induces apo-
TRAIL Induces RA Synovial Fibroblast Proliferation

Preparation of Fibroblast-like Synoviocytes of Rheumatoid Arthritis Patients—Fibroblasts were isolated from synovium samples obtained from patients meeting the American College of Rheumatology criteria for RA (revised 1987) who had undergone surgery for synovectomy or total joint replacement surgery (12). Fresh synovial tissues were minced and digested in a solution of dispase (Roche Applied Science), collagenase (Sigma), and DNase (Calbiochem). Synovial fibroblasts were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were used at passage 4–10, at which time they were a homogenous population of fibroblasts. Upon reaching confluence, the cells were passaged by brief trypsinization as described previously (13). For enzyme-linked immunosorbent assay, Western blot, and apoptosis evaluation (FACS), cells were seeded in 6-well plates at 2 × 10^4 cells/well or in 6 cm-dishes at 4 × 10^5 cells/well or in 6 cm-dishes at 4 × 10^5 cells. Fetal calf serum content of the media was progressively decreased from 10 to 1% with final starvation for 12 h in RPMI and 1% FCS. Cells were stimulated with TNF-α (2 nM), IL-1β (1 nM), or TRAIL (at indicated concentrations).

Flow Cytometric Analysis—RA FLSs were harvested with Versene (Invitrogen) according to the manufacturer’s instructions and suspended in PBS supplemented with 0.1% bovine serum albumin and 0.01% NaN3. Fifty to one hundred thousand cells were incubated for 20 min on ice in with 0.5 µg of goat anti-TRAIL-R1 antibody, anti-TRAIL-R2 antibody, anti-TRAIL-R4 antibody, or mouse anti-TRAIL antibody. As a negative control, cells were incubated with an isotype control antibody. Cells were then washed and resuspended in 1% paraformaldehyde for 20 min. Cells were then fixed in PBS with 4% paraformaldehyde for 20 min. Cells were then analyzed on a FACSCalibur (BD Biosciences). 

Immunofluorescence Staining—To determine the nuclear translocation of NF-κB, RA FLSs were seeded at 2 × 10^4 cells/well in 8-well Lab-Tek chamber slides (Falcon). Cells were stimulated with TRAIL at 0.5 nM or with IL-1 at 1 nM for 30–120 min, washed with cold PBS, and then fixed in PBS with 0.5% paraformaldehyde for 20 min. Cells were permeabilized with PBS and 0.5% Triton X-100 for 10 min. Unspecific binding was prevented with blocking buffer containing 5% goat serum diluted in PBS. Cells were incubated with the rabbit polyclonal anti-NF-κB p65 antibody (Santa Cruz Biotechnology) at 5 µg/ml or an isotype control for 60 min at room temperature. Cyan3-conjugated goat anti-rabbit antibody was used at 20 µg/ml for 60 min at room temperature. Coverslips were mounted with mounting medium, and the subcellular localization of NF-κB was visualized using a fluorescence microscope (Leica Microsystems, Heidelberg, Germany).

Statistical Analysis—Results are expressed as the mean ± S.E. Statistical studies were performed with a non-parametric Wilcoxon test (Instat software), p values of <0.05 were considered statistically significant.

RESULTS

TRAIL Induces Initially Apoptosis of RA FLS, but Proliferation of Surviving Cells—To test the effect of TRAIL on RA FLSs, cells were co-cultured for 3 days with 0.5 nM TRAIL. The first 24 h of co-culturing with TRAIL resulted in apoptosis of ~30% of the cells as determined by annexin V staining/TO-PRO-3 uptake (Fig. 1). This technique allows us to distinguish early apoptotic (annexin V+/TO-PRO-3−) from late apoptotic cells (annexin V+/TO-PRO-3+) (15). As shown in Fig. 1A, the highest percentage of early apoptotic cells was observed upon 4 h of TRAIL treatment. At later time points the cells shifted from the early apoptotic gate toward the late apoptotic cell gate. Apoptosis of RA FLS was also obvious in an analysis of forward angle light scatter (FSC; data not shown), because a decrease of FSC is characteristic of apoptotic cell death (15). After 24 h of co-culturing, ~70% of RA FLSs were still viable. Similar results were obtained for RA FLSs derived from different patients (data not shown). Apoptotic cell numbers remained unchanged in unstimulated cells throughout the 72-h observation period.
After 72 h of TRAIL exposure, proliferation of RA FLS was determined by [3H]thymidine incorporation (Fig. 2A). Significant increases in DNA synthesis were observed for all of the concentrations of TRAIL tested, with a maximum proliferation already detectable for 0.25 nM. TRAIL induced a 5-fold increase in RA FLS proliferation similar to that induced by the proinflammatory cytokine IL-1β (Fig. 2B). Simultaneous addition of TRAIL and IL-1β enhanced proliferation, demonstrating the additive effect of these two cytokines on RA FLS cells (Fig. 2B).

To confirm the specificity of TRAIL-induced proliferation, we evaluated the inhibitory effect of a neutralizing anti-TRAIL antibody. This led to an almost complete inhibition of TRAIL-mediated RA FLS proliferation, whereas the isotype control antibody had no effect (Fig. 2C). Treatment with either the anti-TRAIL antibody alone or the control antibody alone did not affect proliferation (data not shown).

**RA FLSs Express TRAIL mRNA and TRAIL-R1 and -R2 Proteins**—We next analyzed the expression of TRAIL and its receptors in RA FLS. Semi-quantitative RT-PCR revealed the up-regulation of TRAIL mRNA expression by TNF-α and IL-1β by ~2 and 5-fold, respectively, versus unstimulated cells (Fig. 3A). The TRAIL protein could not be detected either in soluble form in conditioned media or on the RA FLS cell surface (data not shown). This finding might be due to the limited sensitivity of the antibodies employed or a low translation efficiency. The expression of different TRAIL receptors on RA FLS was evaluated by flow cytometry. TRAIL-R1 and -R2 on RA FLSs were constitutively expressed, whereas TRAIL-R4 was not detectable (Fig. 3C). Expression of the receptors was unaltered when RA FLSs were treated with IL-1β, TNF-α, or TRAIL (data not shown).

**TRAIL-R2/DR5 Receptor Mediates TRAIL-induced Proliferation**—Treatment of RA FLS with an agonistic anti-TRAIL-R2 antibody induced a ~5-fold increase in RA FLS proliferation in comparison with untreated cells (Fig. 4). This increase corresponds to the maximal induction observed upon the addition of recombinant TRAIL (Fig. 4), suggesting that TRAIL-R2 mediates TRAIL-induced proliferation to a large extent.

**TRAIL Activates the Kinases p38, ERK1/2, and Akt but Not NFκB**—To determine the intracellular mechanisms involved in TRAIL-induced cell proliferation, we tested the effect of TRAIL on signaling pathways that control cell proliferation, specifically those containing the MAP kinases p38 and ERK1/2 and PI3 kinase/Akt (16). RA FLSs were treated with 0.5 nM TRAIL and subsequently analyzed by Western blotting. Activating
phosphorylation of p38 MAP kinase was rapidly induced by TRAIL and persisted up to 60 min, although with a lower intensity in comparison with that of TNF-α (Fig. 5, A and B).

Treatment with TRAIL also induced activation of the phosphorylation of ERK1/2 and Akt in a time-dependent manner, with maximum activation occurring between 5 and 10 min (Figs. 5,
The kinetics and intensities of ERK and Akt phosphorylation were comparable with those induced by TNF-α/H9251.

The transcription factor NFκB plays a major role in cell survival and can be activated by MAP kinases and Akt (17, 18). Upon activation, NFκB translocates from the cytoplasm to the nucleus, which can be visualized using indirect immunofluorescence (19). We therefore tested whether nuclear accumulation of NFκB could be detected in the RA FLS following TRAIL stimulation. Very little NFκB was in the nucleus in non-stimulated RA FLSs (4.3 ± 0.9%). IL-1β strongly induced its nuclear translocation (97.9 ± 1.1%). In contrast, only 6.7% (±1.2) of NFκB was nuclear upon exposure to TRAIL (Fig. 7, A and B).

Western blotting confirmed these observations when NFκB was detectable in the nuclear fraction only upon TNF-α/H9251 stimulation (Fig. 7 C). Thus, the exposure of RA FLS to TRAIL activates the kinases p38 and ERK1/2 and PI3 but not the transcription factor NFκB.

Akt, p38, and ERK Control TRAIL-mediated RA FLS Proliferation—To confirm that MAP kinases and PI3 kinase/Akt play a role in TRAIL-induced proliferation, we used the specific inhibitors LY294002 (PI3 kinase), PD98059 (ERK1/2), and SB203580 (p38). RA FLSs were pretreated with the specific inhibitors at different concentrations or with solvent (Me2SO) alone for 1 h and then stimulated with 0.5 nM TRAIL. Under these experimental conditions, cell viability was ≥90% (14). Proliferation was assessed by [3H]thymidine incorporation. ERK 1/2 inhibition (Fig. 8A) and p38 inhibition (Fig. 8B) significantly diminished TRAIL-induced proliferation in a dose-dependent manner. Strikingly, the PI3 kinase inhibitor almost completely blocked the proliferation induced by TRAIL, underlining the crucial role played by this pathway (Fig. 8C).
DISCUSSION

The TRAIL ligand/receptor system has raised much interest for cancer therapy, because it appears to kill tumor cells but not normal ones (20). Fibroblast-like synoviocytes play a crucial role in the physiopathology of rheumatoid arthritis, as these cells are involved in inflammation and joint destruction. The pseudo-tumoral proliferation of RA FLS is considered to be the major mechanism for the hyperplastic growth of the RA syno-

**Fig. 5.** TRAIL activates the MAP kinases p38 and ERK1/2. RA FLSs were stimulated with TRAIL (0.5 nM) or TNF-α (2 nM) at the indicated times. Cell lysates were analyzed by Western blotting for phospho-p38 MAP kinase (A and C) and phospho-ERK1/2 kinases (B and D). Each blot was stripped and reprobed with a mouse anti-human β-actin antibody. Blots were scanned and quantified with Kodak 1D image analysis software. Band intensities for phospho-p38 MAP kinase and phospho-ERK1/2 kinases were normalized to the corresponding band intensities for β-actin. Data from five RA donors were averaged and are represented as mean ± S.E. in the panels C and D. Asterisks (*), p values of <0.05; NS, non-stimulated.
vium and can be mimicked by in vitro culturing, because ex vivo RA FLS cells grow in normal medium without requiring additional stimulation.

RA FLSs exhibit certain tumor-like features, leading us to investigate the effect of TRAIL on RA FLS survival. Treatment of RA FLSs with TRAIL induced apoptosis within the first 24 h in ~30% of the cells as detected by TO-PRO-3-uptake, annexin V staining, and decreased forward angle light scatter. Unexpectedly, TRAIL induced RA FLS proliferation in a dose-dependent manner at later culture times, i.e. 72 h. This was inhibited by neutralizing anti-TRAIL antibodies, confirming that cell proliferation is specifically induced by this cytokine. Moreover, combined stimulation with TRAIL and IL-1β had an additive effect on RA FLS proliferation, suggesting two independent mechanisms of stimulation. Further studies are required to analyze whether endogenous TRAIL participates in the pathogenesis of RA patients. The increased levels of TRAIL transcripts detected in RA FLS treated with proinflammatory cytokines argue for fratricide activation, but a contribution of TRAIL derived from synovial T cells is also possible.

Our results suggest that TRAIL has two different effects on RA FLS, namely an initial rapid induction of apoptosis that is followed by an increased proliferation of the surviving population. These observations differ from those in the collagen-induced mouse model of arthritis that describes an inhibitory role for TRAIL by blocking the proliferation of synovial cells (2, 3). This discrepancy could reflect the different pathogenic mechanisms between RA in the joints of patients and the respective mouse model. Furthermore, only one membrane-anchored TRAIL receptor and two soluble decoy receptors have been identified in the mouse (21), indicating differences in the organization of TRAIL signaling between man and mouse.

The name TRAIL derives from its capacity to induce apoptosis (22). However, depending on the cellular system, TRAIL can exert different functions that also include survival, proliferation, and maturation. For example, TRAIL has been shown to promote cell survival and proliferation on endothelial and vascular smooth muscle cells (8, 23) and to regulate erythroid and monocytic maturation (24, 25). Here we describe for the first time that TRAIL is able to induce proliferation of RA FLS. A previous study analyzed the effect of TRAIL on RA FLSs for shorter culturing periods, i.e. up to 24 h, and reported the induction of apoptosis (9). Varying levels of apoptosis were induced by TRAIL on the different RA FLS cultures tested in which a portion of cells survived (9). These RA FLSs strongly express TRAIL-R2 and were highly susceptible to an agonistic anti-TRAIL-R2 antibody, identifying TRAIL-R2 as the receptor mediating TRAIL-induced apoptosis (9). In agreement with these observations, Miranda-Caruso et al. analyzed (10) fibroblasts of 50 RA synovial fluid samples and found that about half of them express functionally active TRAIL-R2. These cells underwent apoptosis when treated in vitro with an agonistic anti TRAIL-R2 antibody (10). Although these reports suggested the specific targeting of TRAIL-R2 on RA FLSs as a potential therapeutic approach, Perlman et al. drew the opposite conclusion because they could not detect the expression of TRAIL-R1 or TRAIL-R2 or the susceptibility to TRAIL in RA FLSs (11). A possible explanation for these contradictory observations might be the use of different protocols to isolate and/or culture RA fibroblasts.

The results described here suggest two distinct types of modulation of RA fibroblasts by TRAIL. TRAIL induced rapid cytotoxicity in a subset of RA FLSs that was followed by an induction of proliferation in the surviving cells. This proliferative effect appears to be mainly mediated by TRAIL-R2, because an agonistic anti-TRAIL-R2 antibody induced proliferation of RA FLS in a similar manner as that of recombinant TRAIL. Therefore, it emerges that TRAIL can promote both proliferation as well as apoptosis, as has been established for other members of the TNF family (26). For example, Fas ligand/receptor interaction stimulates T cells during the early activation phase, whereas it mediates apoptosis at later phases of T cell activation (27).

We show here that TRAIL promotes cell proliferation by activation of the MAP kinases ERK1/2 and p38 and the serine/threonine kinase Akt. While this manuscript was under preparation, Myashita et al. reported that TRAIL activates ERK1/2 and Akt in RA FLSs, preventing these cells from undergoing...
apoptosis (28). The ERK1/2 and PI3 kinase/Akt pathways have been reported to activate NFkB, and, in certain cell types, TRAIL has been reported to activate NFkB via ligation of TRAIL-R1 (29). We could, however, detect no activation of NFkB in RA FLSs by TRAIL. Notably, very similar observations were made in a different cellular system (8) where TRAIL promoted the proliferation of primary human vascular endothelial cells (HUVECs) by activating the ERK1/2 and Akt but not the NFkB pathways. These data suggest that TRAIL promotes cell growth through an NFkB-independent mechanism.

**Fig. 7.** TRAIL enhances nuclear translocation of NFkB in RA FLSs. A, NFkB localization in the RA FLS, untreated (NS) or stimulated with TRAIL (0.5 nM) or IL-1β (2 nM), is visualized in red; the nucleus, stained with Hoechst, is blue. Nuclear localization of NFkB is confirmed by superposition of both signals (right column). B, percentages represent cells positive for nuclear localization of NFkB. The graph represent means ± S.E.; NS, non-stimulated. C, nuclear protein were extracted from untreated (NS), TRAIL- (0.5 nM), or TNF-α-stimulated (2 nM) RA FLSs and subsequently analyzed for NFkB levels by Western blotting.
The inhibition of MAP kinases abrogated TRAIL-mediated RA FLS proliferation partially, and the inhibition of PI3 kinase abrogated it completely, confirming that both pathways are essential for RA FLS proliferation.

Different regulatory pathways have been proposed to control the sensitivity of RA FLSs toward apoptotic signals. These include altered expression levels of apoptosis inhibitory proteins such as FLIP and Bcl-2 (30, 31) and the over-activation of MAP and PI3 kinases (18, 32). These kinases have been shown to be activated by pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-18, which are present at elevated levels in the joints of arthritis patients (17, 32, 33). The present study sug-

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**Fig. 8.** Inhibition of TRAIL-induced FLS proliferation with specific inhibitors of MAP and PI3 kinases. Serum-starved cells were pre-treated with inhibitors of ERK 1/2 (PD98059) (A), p38 MAPK (SB203580) (B), PI3 kinase (LY294002) (C), or Me2SO control for 1 h and then stimulated with TRAIL (0.5 nM) for 72 h. Cell proliferation was assessed by [³H]thymidine incorporation. The proliferation rate represents the percentage of proliferation of TRAIL-stimulated RA FLSs treated with the respective specific inhibitor. Results are presented as means ± S.E; n = number of patients samples; NS, non-stimulated.
gests that TRAIL stimulation may contribute to survival/proliferation by inducing Akt and ERK1/2. In accordance with this idea, RA FLS proliferation is enhanced by the combined addition of IL-1β and TRAIL.

There is some precedent for TRAIL having both apoptotic and proliferative functions. Treatment of the promyelocytic human HL-60 cell line with TRAIL resulted in rapid cytoxicity within 6–24 h, followed by progressive maturation of the surviving cells along the monocytic lineage (25). The capacity of TRAIL to sequentially induce death and growth signals in one cell type might therefore be a common mode of function. Consequently, the stimulatory effect of TRAIL observed on RA FLS complicates the proposed strategy to use TRAIL for targeting RA FLS. On the other hand, our results suggest Akt is a potential target to block joint destruction in RA.

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