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Rheumatoid Arthritis Synoviocyte Survival Is Dependent on Stat3

Anja Krause,* Nicholas Scalaletta,* Jong-Dae Ji,* and Lionel B. Ivashkiv2*†

Rheumatoid arthritis (RA) synovial fibroblasts (SFs) are relatively resistant to apoptosis and exhibit dysregulated growth secondary to production of autocrine-acting growth factors and the accumulation of cell-autonomous defects. Many of the cytokines and growth factors expressed during RA synovitis, including IL-6, epidermal growth factor (EGF), and platelet-derived growth factor, activate the transcription factor Stat3 that has been implicated in promoting cell growth and survival. We analyzed the role of Stat3 in mediating the abnormal growth and survival properties of RA synoviocytes using retroviral-mediated gene transfer of a dominant negative mutant of Stat3, termed Stat3-YF. Approximately 3- to 5-fold overexpression of Stat3-YF effectively blocked endogenous Stat3 activation and Stat3-dependent gene expression, including expression of the sos3 and myc genes. Stat3-YF-transduced RA synoviocytes failed to grow in culture, exhibited markedly diminished [3H]thymidine incorporation (>90% decreased), and died spontaneously. Cell death occurred by apoptosis, as confirmed by annexin V staining, propidium iodide exclusion, and identification of cells with subdiploid levels of DNA. In marked contrast to control cells, EGF accelerated death of Stat3-YF-transduced SFs, such that >90% of cells were dead within 24–48 h of transduction. These results indicate that ablation of Stat3 function converts EGF from a growth/survival factor for RA synoviocytes to a death factor. Stat3-YF also induced apoptosis in osteoarthritis synoviocytes, and levels of apoptosis were increased by exogenous EGF. Apoptosis in Stat3-YF-transduced osteoarthritis synoviocytes was suppressed when Stat1 activity was blocked using a dominant negative Stat1 mutant. Our results identify Stat3 as an important molecule for RA SF survival, and suggest that Stat3 may represent a good target for gene therapy. The Journal of Immunology, 2002, 169: 6610–6616.

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2 Address correspondence and reprint requests to Dr. Lionel B. Ivashkiv, Department of Medicine, Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021. E-mail address: IvashkivL@HSS.edu
3 Abbreviations used in this paper: RA, rheumatoid arthritis; SF, synovial fibroblast; cGFP, enhanced green fluorescence protein; SOCS, suppressor of cytokine signaling; PI, propidium iodide; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; Jak, Janus kinase; OsM, oncostatin M; OA, osteoarthritis; IRES, internal ribosomal entry site.
tyrosine phosphorylation and activation of latent cytoplasmic transcrip-
tion factors termed STATs. STATs are rapidly tyrosine-phos-
phorylated after stimulation with cytokines, and subsequently
dimerize and translocate to the nucleus, where they can activate
transcription. Of seven known STAT proteins, Stat3 has been
shown to be active in synovial lining cells in adjuvant arthritis and
RA, and in freshly isolated RA SFs (16, 17). Stat3 is activated by
a number of cytokines and growth factors expressed in RA syno-
vitis, including IL-6, oncostatin M (OsM), EGF, and PDGF. Stat3
has been strongly implicated in promoting cell survival and growth
in many cell types, and contributes to cellular transformation (18).
The underlying mechanisms described so far include conferring
resistance to apoptosis, transcriptional activation of genes impor-
tant in cell cycle progression such as cyclins, and activation of
expression of protooncogenes such as myc or pim-1 (19–22). We
have examined the role of Stat3 in RA SF growth and survival
using retroviral-mediated transduction of dominant negative Stat3.

Materials and Methods

Cells and retroviral transduction

Synovial tissues were obtained perioperatively from patients who
fulfilled the revised American College of Rheumatology criteria for
definite RA or osteoarthritis (OA) and were undergoing total joint replacement; the pro-
tocol was approved by the Institutional Review Board of the Hospital for
Special Surgery. Synovial cells were obtained by finely mincing freshly
isolated synovial tissue followed by treatment with collagenase A (1 mg/
ml; Boehringer Mannheim, Indianapolis, IN) for 2 h at 37°C. Fibroblasts
were obtained by allowing cells to adhere to tissue culture plates, followed
by removal of nonadherent cells. Similar to previous reports, most of the
experiments were performed using SFs after the third passage in tissue
culture. At this time, there were <2% contaminating lymphocytes, NK
cells, or macrophages, as assessed by flow cytometry and staining with Abs
against CD3, CD14, CD16, and CD19 as previously described (23). SFs
were cultured in DMEM supplemented with 10% FBS, and cells were
routinely split and replated the day before an experiment. Experiments
were performed using third passage SFs derived from 11 different patients.

Wild-type Stat3 and Stat3-YF and Stat1-YF dominant negative mutants
containing a C-terminal FLAG tag (20) were subcloned into the SFG ret-
roviral vector (24) as a translational fusion with the initiator methionine of
the viral envelope protein. Recombinant retroviral particles pseudotyped
to express the gibbon-ape leukemia virus envelope were produced using tran-
sient transfection of SFG plasmids into the 293-GPG2 (H29) packaging
cell line (25), followed by cross-infection of the PG13 cells (26) to obtain
stable producer cell lines. PG13 producer cell lines were subcloned to
identify cells that produced titers of 1–2 × 10^6 retroviral particles/ml. RA
SFs were transduced by continuous exposure to retroviral particle-contain-
ing supernatants over the course of 3 days in the presence of 8 μg/ml
polybrene, as described (27).

Immunoblotting

Cell lysates were fractionated on 7.5% SDS-polyacrylamide gels, trans-
ferred to polyvinylidene difluoride membranes, and incubated with phos-
pho-Tyr-specific Stat1 and Stat3 Abs (NEB, Beverly, MA) or monoclonal
FLAG and Stat1 Abs (Transduction Laboratories, Lexington, KY). ECL
was used for detection.

Analysis of mRNA levels

Total cellular RNA was isolated using TRIzol (Life Technologies, Gaith-
ersburg, MD) according to the instructions of the manufacturer. RNA was
treated with RNase-free DNase, and cDNA was obtained using Moloney
murine leukemia virus reverse transcriptase (Life Technologies). A total of
2.5% of each cDNA was subjected to 22 cycles of PCR using condi-
tions that resulted in a single specific amplification product of the correct size,
as previously described (23, 28): 30 s denaturation at 94°C, 1 min anneal-
ing at 55°C, and 30 s extension at 72°C in a GeneAmp 9600 thermal cycler
(PerkinElmer, Norwalk, CT). dNTPs were used at 100 μM, and 1 μCi of
[^32P]dATP was added to each reaction. No amplification products were
obtained when reverse transcriptase was omitted, indicating the absence of
contaminating genomic DNA. Amplification was empirically determined
to be in the linear range.

Apoptosis and proliferation analysis

Apoptotic cells were detected using annexin V staining, propidium iodide
(P1) staining, and analysis of cellular DNA content using flow cytometry,
as previously described (23, 28), and cell counts were performed in duplica-
tion using trypan blue exclusion to identify viable cells. For proliferation
assays, 5 × 10^4 cells in 100 μl of medium were seeded in triplicate in
96-well tissue culture plates and cells were pulsed for the final 16 h of
culture with 25 μCi/ml of [3H]thymidine and harvested using an automated
cell harvester (Harvester 96; Tomtec, Orange, CT), and [3H]thymidine in-
corporation was quantitated using a Wallac Microbeta TriLux scintillation
counter (Wallac, Gaithersburg, MD).

Results

Transduction of RA SFs

RA SFs after the third passage were transduced with retroviruses
encoding enhanced green fluorescence protein (eGFP) or wild-type
Stat3 (controls) or Stat3-YF (dominant negative mutant) containing
a C-terminal FLAG tag. When Stat3-encoding retroviral particles
were used, infection rates of 30–50% were obtained (Fig. 1A, left panel; 1 representative experiment of 11 performed is
shown). Although eGFP expression was relatively low, secondary
to expression from the 3' position in a bicistronic transcript, the
GFP signal was sufficient to monitor transduction efficiency and to
purify cells by FACS sorting. Indeed, nearly pure transduced cells
were obtained after sorting for cells that expressed GFP (Fig. 1A, right panel). These synoviocytes expressed exogenous Stat3 en-
coded by the transgene, as assessed by immunoblotting with Abs
against the FLAG tag (Fig. 1B, left panel) and Stat3 (Fig. 1B, right panel). Approximately 3- to 5-fold overexpression of STAT pro-
teins was typically achieved. These results demonstrate successful
and relatively high efficiency gene transfer into RA SFs.

Dominant negative effect of Stat3-YF in RA SFs

The capacity of Stat3-YF to act as a dominant negative mutation in
our system was tested by analyzing the effects of Stat3-YF on
activation of endogenous Stat3 and on the activation of Stat3-in-
ducible genes. Transduced synoviocytes were purified by FACS

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** Transduction of primary SFs with Stat3. Third passage RA
synoviocytes were transduced with gibbon-ape leukemia virus-
pseudotyped recombinant retroviral particles as described in Materials and
Methods. Transduced synoviocytes were replated and then analyzed 1 day
later using flow cytometry; a fraction of cells was used to make cell ex-
tracts. A, Retroviruses expressing a bicistronic transcript with FLAG-Stat3
in the 5' position and eGFP 3' to the IRES sequence were used. A total of
41% of RA synoviocytes were successfully transduced (left panel); and cell
sorting was used to obtain nearly pure transduced cells (right panel). B, RA
synoviocytes transduced with WT or mutant (YF) Stat3 were analyzed for
expression of the FLAG-Stat3 transgene using immunoblotting.
Dominant negative effect of Stat3-YF. Sorted transduced synoviocytes were treated with IL-6 (50 ng/ml) + soluble IL-6Ra chain (100 ng/ml), OsM (100 ng/ml), or IFN-γ (100 U/ml). A, Cell extracts were prepared 10 min after cytokine stimulation and analyzed by immunoblotting. B, RNA was prepared 3 h after cytokine stimulation and analyzed using semiquantitative RT-PCR. C, RNA was prepared from unstimulated cells 24 h after transduction and analyzed using semiquantitative RT-PCR.

When transduced cells were grown in culture, it became readily apparent just from visual inspection that Stat3-YF-transduced cells did not expand, and cell numbers actually decreased over time (Fig. 3; a representative experiment of eight experiments performed is shown). Therefore, levels of apoptotic cell death and proliferation were analyzed. Apoptosis was assessed directly using flow cytometry and staining with annexin V (which detects early apoptotic cells) (Fig. 4A) and exclusion of PI (a marker for late apoptotic or necrotic cells) (Fig. 4B). Higher levels of apoptosis and cell death were apparent in Stat3-YF-transduced cells relative to control eGFP-transduced cells already after 1 day of culture (Fig. 4, A and B). Fig. 4 shows one representative experiment of seven where apoptosis of Stat3-YF-transduced cells was analyzed using annexin V and PI staining. Statistical analysis of the differences in levels of apoptosis in all seven experiments using ANOVA revealed that the increased apoptosis in Stat3-YF-transduced cells was significant (eGFP vs Stat3-YF, p = 0.05 for annexin V staining and p = 0.007 for PI staining). In these experiments, wild-type Stat3-transduced cells could not be used as an additional control, as these cells assumed an aberrant, enlarged morphology; additional controls using Stat1-YF were similar to the eGFP-transduced cells (data not shown). [³H]thymidine incorporation assays were used to assess the effects of Stat3-YF on cell proliferation. [³H]thymidine incorporation in Stat3-YF-transduced
cells was appreciably lower than in control cells after 2 days of culture, and the difference was dramatic after 5 days of culture (Fig. 4C; one representative experiment of seven is shown). Because decreased \(^{3}H\)thymidine incorporation in this system was likely secondary to increased cell death, and possibly inhibition of proliferation as well, the effects of Stat3-YF on progression of RA SFs through the cell cycle were determined. Expression of Stat3-YF for 3 days of culture resulted in a decrease in the fraction of cells in the S and G2/M phases of the cell cycle from 26 to 16%, and in an increase in numbers of apoptotic cells that contain subdiploid levels of genomic DNA, from 8 to 50% (Fig. 4D). These results further support the conclusion that Stat3-YF induces apoptosis, and suggest that Stat3-YF may induce growth arrest as well. The increased levels of cell death in Stat3-YF-transduced cells had a striking effect on recovery of viable cells from these cultures (Fig. 4E). In contrast to control cells that expanded in culture, the number of viable Stat3-YF-transduced cells did not increase, and actually decreased, such that there were very few viable cells after 4 days of culture (Fig. 4E). IL-6 suppressed expansion of control cells (Fig. 4E) similar to previous reports (29, 31). The basis for this effect is not understood, but may be secondary to strong activation of Stat1 at the dose of IL-6 used (see Discussion). IL-6 did not reverse induction of apoptosis by Stat3-YF-transduced cells (Fig. 4E). A suppressive effect of Stat3-YF on cell expansion has been observed in eight of eight experiments with RA SFs performed to date, suggesting that Stat3-YF blocks an important constitutive growth/survival pathway.

**Stat3-YF induces a switch in EGF from a growth factor to a death factor**

EGF is a growth factor for many cell types, but has pleiotropic effects and, in certain cells, may contribute to growth arrest or apoptosis by a mechanism that is dependent on Stat1 (32). EGF is a major growth and survival factor for RA synoviocytes, and acts, at least in part, via the ErbB2 receptor that is overexpressed on these cells (10). Control and Stat3-YF-transduced RA synoviocytes were cultured for 1–6 days with or without exogenous EGF, and cell growth and survival were evaluated. Consistent with the results in Fig. 4, after 2 days of culture fewer viable cells were recovered from wells containing cells transduced with Stat3-YF (Fig. 5A), even in the absence of any added factors. Addition of exogenous EGF to control internal ribosomal entry site (IRES)-eGFP-transduced RA synoviocytes resulted in an increased number of viable cells (Fig. 5A). In marked contrast, EGF treatment had the opposite effect on Stat3-YF-transduced cells, namely, a greater decrease in viable cells (Fig. 5A; one representative experiment of five performed is shown). Consistent with the cell counts, EGF-treated Stat3-YF-transduced cells exhibited markedly lower \(^{3}H\)thymidine incorporation than EGF-treated control cells (Fig. 5B). EGF suppressed background apoptosis levels to very low levels in control IRES-eGFP-transduced cells (Fig. 5C, left panel), but did not suppress, and actually increased apoptosis levels in Stat3-YF-transduced cells (Fig. 5C, right panel, and data not shown). Induction of increased cell death by EGF was consistently detected in five of five experiments in which EGF was used and the differences were statistically significant (–EGF vs +EGF, \(p = 0.003\) for annexin V staining and \(p = 0.05\) for PI staining). These results indicate that ablation of Stat3 function converts EGF from a growth/survival factor for RA synoviocytes to a death factor. The most plausible basis for this effect is that endogenous wild-type Stat3 opposes proapoptotic signals from the EGFR/ErbB2, and these signals are unmasked and act unopposed when Stat3 is not functional. The acceleration of cell death by exogenous EGF suggests that the increased apoptosis observed in Stat3-YF-transduced cells in the absence of exogenous factors (Fig. 4) may be secondary to the unbalanced action of autocrine growth factors, PDGF and EGF, that are produced by these cells.

We wished to determine whether Stat3 is also required for survival of OA SFs. OA SFs from three different patients were transduced to express eGFP or dominant negative Stat3-YF, and cells were analyzed 1 day later by annexin V and PI staining. Similar to RA SFs, Stat3-YF induced increased apoptosis in OA SFs (Fig. 6;
two experiments using OA SFs are shown). The increased apoptosis induced by Stat3-YF was statistically significant (eGFP vs Stat3, \( p = 0.03 \) for annexin V staining and \( p = 0.009 \) for PI staining). Addition of EGF to cells transduced to express Stat3-YF resulted in a higher level of apoptosis (EGF vs Stat3-YF, \( p = 0.05 \) for annexin V staining and \( p = 0.04 \) for PI staining). One potential mechanism for increased apoptosis in cells expressing Stat3-YF is unopposed activation of Stat1, which has proapoptotic properties, by synoviocyte-derived factors or exogenous EGF. The role of Stat1 in synoviocyte apoptosis was tested by first transducing SFs with dominant negative Stat1-YF, sorting to obtain transduced cells, followed by a second transduction with Stat3-YF. In the absence of EGF, expression of Stat1-YF resulted in a modest but significant decrease in PI positive Stat3-YF-transduced cells ( \( p = 0.03 \); annexin V positive cells also decreased, but to a variable extent and the difference was not statistically significant ( \( p = 0.2 \)). When exogenous EGF was added, Stat1-YF effectively blocked apoptosis in Stat3-YF-transduced cells (–EGF vs + EGF, \( p = 0.03 \) for annexin V staining and \( p = 0.03 \) for PI staining). These results suggest that one mechanism by which Stat3 promotes survival of SFs is by opposing the action of Stat1. When we examined the role of Stat1 in apoptosis of RA SFs transduced with Stat3-YF, a pilot experiment showed a weak and partial effect (data not shown). Additional experiments will be required to assess whether differences between OA and RA SFs are significant, and whether any differences may be related to higher EGF receptor expression in RA SFs.

**Discussion**

In this report, we present data that Stat3 is important for the survival and expansion of growth factor-dependent SFs that are important in the pathogenesis of RA. Importantly, ablation of Stat3 function led to accelerated cell death in response to growth factors such as EGF. These results extend our knowledge of the role of Stat3 in growth regulation to a cell type that is important in arthritis and manifests certain aspects of cellular transformation. Dominant negative Stat3-YF also induced apoptosis in OA SFs, thus identifying primary SFs as a cell type that is dependent upon Stat3 for survival. Interestingly, the partial transformation of synoviocytes that occurs during RA and makes them resistant to induction of apoptosis by other pathways did not abrogate their dependence upon Stat3 for survival. The major emphasis in RA cytokine research focuses on targeting pathways activated by cytokines related to TNF and IL-1, and the downstream signaling pathways mediated by NF-κB and AP-1 transcription factors (33). The role of the Jak-STAT pathway in inflammatory arthritis has not been extensively investigated. Our results, together with data that Stat3 is active during inflammatory arthritis (16, 17), suggest that Stat3 represents a promising novel therapeutic target in RA. It may also be useful to target ligands for receptors that activate Stat3, or other components of the Jak-STAT signaling pathway.

**FIGURE 6.** Stat3-YF induces apoptosis in OA SFs, and induction of apoptosis is blocked by dominant negative Stat1-YF. OA SFs were transduced with Stat1-YF, and subsequently control or Stat1-YF-expressing cells were transduced with eGFP, Stat3-YF, or Stat1-YF. After overnight culture without or with EGF (100 ng/ml), SFs were analyzed using annexin V and PI staining and flow cytometry.
The notion that Stat3 and related Jak-STAT signaling proteins may play an important role in the pathogenesis of several inflammatory diseases is supported by recent reports showing that overexpression of wild-type SOCS3 (also known as CIS3), an inhibitor of Jak-STAT signaling and Stat3 activation, suppressed experimental arthritis, and a dominant negative SOCS3/CIS3 mutant potentiated inflammatory bowel disease in murine models (17, 34).

A substantial amount of evidence has accumulated supporting a role for Stat3 in cellular transformation and oncogenesis. Stat3 can be activated by oncogenic kinases such as v-Src, and in several tumor models or transformed cell lines, a causal role for Stat3 in driving abnormal growth has been proposed (18, 35–37). The mechanisms by which Stat3 contributes to oncogenesis include increasing expression of antiapoptotic Bcl family proteins, proteins important for the cell cycle, protooncogenes such as myc and pim-1 (22), and suppressing cell surface Fas expression (38). In primary cells where oncogenic pathways are not activated, the role of Stat3 is more complex, as Stat3 promotes survival of T cells, but suppresses the proliferation of myeloid precursors, promotes the apoptosis of mammary epithelial cells, and in keratinocytes, has a more prominent role in regulating cell migration than cell survival or growth. We have shown that in primary SFs that have abnormal growth properties but remain growth factor-dependent, Stat3 is an important survival factor. The mechanisms by which Stat3 contributes to survival in RA SFs have not yet been fully elucidated, but the data suggest that Stat3 works, at least in part, by maintaining myc expression (Fig. 2), and by opposing a proapoptotic signal delivered by EGF, and possibly other factors. In OA SFs cultured in the presence of EGF, an important mechanism by which Stat3 promotes cell survival is by opposing the proapoptotic effects of Stat1, as Stat3-YF-induced apoptosis was almost completely abrogated when Stat1 function was suppressed (Fig. 6). This scenario presents an interesting analogy to signaling by cytokines such as TNF, where activation of NF-κB serves to oppose simultaneously activated death pathways (39). Antagonism of Stat1 proapoptotic effects by Stat3 have recently been convincingly demonstrated (40), and unbalanced activation of Stat1 by EGF that leads to apoptosis is consistent with the emerging data demonstrating that Stat1 and Stat3 can serve as, respectively, a tumor suppressor and a tumor promoter (18, 41). A pilot experiment indicated that unopposed Stat1 activity may play less of a role in Stat3-YF-induced apoptosis in RA than in OA SFs, suggesting that Stat3 suppresses additional proapoptotic pathways in RA cells. One possibility is that the partial transformation of RA SFs may make them more dependent upon Stat3-inducible genes, such as myc, and this will be resolved in future experiments.

One important goal in the therapy of RA is suppression of inflammation. However, suppression of inflammation does not necessarily result in proportionate suppression of tissue destruction (42, 43). Thus, there has been a strong emphasis on developing therapeutic strategies to induce apoptosis of joint macrophages and fibroblasts, thereby ablating the inflammatory pannus. Induction of apoptosis of these cells requires an understanding of the pathways and molecules that promote their survival, and NF-κB, Akt, and senin have been previously reported to be important for the survival of RA synoviocytes (44–46). These molecules serve as targets for novel therapeutic approaches, many of which use gene therapy approaches in murine models of arthritis, and results inhibiting NF-κB have been promising (46). The work presented herein adds Stat3 to the list of therapeutic targets in RA. Stat3 is an attractive therapeutic target, as loss of Stat3 function led to exceptionally effective induction of apoptosis in RA synoviocytes. Another potential advantage of targeting Stat3 is that this approach converts endogenously produced growth factors, such as EGF, into death factors, and thus uses endogenous factors produced during synovitis to more effectively eliminate pathogenic cells.

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