# Differential Survival of Leukocyte Subsets Mediated by Synovial, Bone Marrow, and Skin Fibroblasts

## Site-Specific Versus Activation-Dependent Survival of T Cells and Neutrophils

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*Objective*. Synovial fibroblasts share a number of phenotype markers with fibroblasts derived from bone marrow. In this study we investigated the role of matched fibroblasts obtained from 3 different sources (bone marrow, synovium, and skin) to test the hypothesis that synovial fibroblasts share similarities with bone marrow–derived fibroblasts in terms of their ability to support survival of T cells and neutrophils.

*Methods*. Matched synovial, bone marrow, and skin fibroblasts were established from 8 different patients with rheumatoid arthritis who were undergoing knee or hip surgery. Resting or activated fibroblasts were cocultured with either CD4 T cells or neutrophils, and the degree of leukocyte survival, apoptosis, and proliferation were measured.

*Results*. Fibroblasts derived from all 3 sites supported increased survival of CD4 T cells, mediated principally by interferon-$\beta$. However, synovial and bone marrow fibroblasts shared an enhanced site-specific ability to maintain CD4 T cell survival in the absence of proliferation, an effect that was independent of fibroblast activation or proliferation but required direct T cell–fibroblast cell contact. In contrast, fibroblast-mediated neutrophil survival was less efficient, being independent of the site of origin of the fibroblast but dependent on prior fibroblast activation, and mediated solely by soluble factors, principally granulocyte–macrophage colony-stimulating factor.

*Conclusion*. These results suggest an important functional role for fibroblasts in the differential accumulation of leukocyte subsets in a variety of tissue microenvironments. The findings also provide a potential explanation for site-specific differences in the pattern of T cell and neutrophil accumulation observed in chronic inflammatory diseases.

Fibroblast-like synoviocytes (FLS) interact with a wide variety of leukocyte subtypes within the synovium. In particular, direct interactions between T cells and FLS have been suggested to contribute to the persistence of synovial inflammation (1). Activated T cells modify fibroblast proliferative capacity and matrix production (2,3), while resting T cells activate FLS in vitro to produce stromelysin, interleukin-6 (IL-6), IL-8, and prostaglandin E$_2$ (4). A recent study demonstrated that surface-bound IL-15 and intercellular adhesion molecule 1 (ICAM-1) expressed by rheumatoid FLS in vitro induce T cell activation and production of tumor necrosis factor $\alpha$ (TNF$\alpha$), interferon-$\gamma$ (IFN$\gamma$), and IL-17, which, in turn, feed back on FLS to induce IL-6, IL-8, and further IL-15 and ICAM-1 expression (5). A complex paracrine, proinflammatory loop is therefore gen-

erated and perpetuated by a mixture of soluble and contact-mediated mechanisms.

FLS have also been shown to rescue T cells from apoptosis in vitro (6,7). Part of this effect can be accounted for by the generic property of stromal cell–derived type I IFNs in keeping T cells alive (8). Similarly, interactions between FLS and B cells result in increased B cell survival, mediated by vascular cell adhesion molecule 1 (VCAM-1)–very late activation antigen 4 and CXCL12–CXCR4 binding (9,10). More recently, BAFF secretion by cytokine-activated mesenchymal cells from the synovium has also been suggested to rescue B cells from apoptosis in vitro (11). However, it remains unclear whether the ability to support lymphocyte survival is a common, generic property of fibroblasts at all sites or whether FLS are particularly good at keeping lymphocytes alive.

The role of neutrophils in maintaining synovial inflammation has been poorly studied, despite their presence at the synovial cartilage–pannus junction and their well-documented numbers in inflamed synovial fluid (12). The relative lack of neutrophil accumulation within synovial tissue compared with synovial fluid is thought to result from a lack of appropriate integrin receptors for matrix components, such as fibronectin and collagen, but why neutrophils continue to survive within synovial fluid remains unclear. Neutrophil survival is exquisitely sensitive to signals from the inflammatory microenvironment, some of which may be provided by stromal cell subpopulations. For example, bronchial epithelial cells and human umbilical vein endothelial cells (HUVECs) have been shown to release soluble factors that inhibit neutrophil apoptosis in response to TNF$\alpha$ (13,14). Furthermore, vascular smooth muscle cells inhibit neutrophil apoptosis in response to IL-1$\beta$ stimulation, by means of granulocyte–macrophage colony-stimulating factor (GM-CSF) and G-CSF secretion (15). Whether FLS also play a direct role in neutrophil survival in rheumatoid arthritis (RA) has not yet been explored.

Recent study results have suggested that fibroblasts are a diverse cell type that display topographic differentiation and positional memory. It has been proposed that these site-specific differences account for the ability of different stromal microenvironments to support the differential accumulation of leukocyte subsets (16–18). FLS derived from the rheumatoid synovium are heterogeneous, express a range of markers more commonly associated with bone marrow stromal cells, and, similar to bone marrow–derived mesenchymal progenitor cells, have the capacity to differentiate into a variety of stromal cell types (19,20). The systemic nature of RA and the finding of bone marrow abnormalities in patients with RA have therefore led some investigators to propose that FLS might share similar developmental origins with bone marrow mesenchymal cells (21,22). However, it remains unclear whether such lineage similarities translate into functional similarities that might explain the ability of both the rheumatoid synovium and bone marrow microenvironments to support sustained levels of leukocyte accumulation.

In this study we set out to investigate whether fibroblasts derived from the synovium and bone marrow were equally able to support the survival of leukocyte subsets involved in both acquired (T cell) and innate (neutrophil) immune responses. We used matched fibroblasts from synovium, bone marrow, and skin. Sampling fibroblasts from 3 different sites in the same patient eliminated potential confounding effects of prior treatment and permitted us to determine whether site-specific differences within the same patient really exist.

Our findings confirmed that some of the similarities in phenotype between synovial and bone marrow fibroblasts are reflected at a functional level. Moreover, we were able to illustrate that different leukocyte subpopulations have quite different molecular requirements for survival induced by stromal cells such as fibroblasts.

## PATIENTS AND METHODS

**Media, antibodies, and cytokines.** All tissue culture reagents were purchased from Sigma (St. Louis, MO) unless stated otherwise. Recombinant IL-2 was purchased from Chiron (Middlesex, UK). The following antibodies were used for fluorescence microscopy and were purchased from Dako (Cambs, UK), unless stated otherwise: CD68 (M0718), cytokeratin (M3515), prolyl-4-hydroxylase (M0977), VCAM-1 (IG11), von Willebrand factor (M0616), CD31 (MA3105; Pierce, Rockford, IL), and antifibronectin (rabbit IgG; Sigma). Negative controls were IgG1 and IgG2b (X0931 and X0944, respectively) and rabbit immunoglobulin fraction (X0903). Secondary antibodies used were fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse pan IgG (1031-02; Southern Biotechnology, Birmingham, AL). The following neutralizing antibodies, each used at 10 $\mu$g/ml unless stated otherwise, were purchased from R&D Systems (Abingdon, UK): anti–IL-6 (MAB206, 20 $\mu$g/ml), anti–ICAM-1 (BBA4), anti–IL-15 (MAB247), anti–CD40 ligand (MK13A4; Alexis Biochemicals, Notts, UK), and anti–GM-CSF (MAB215). Sheep antisera capable of neutralizing IFN$\beta$ activity were a kind gift from Dr. Tony Meager (National Institute for Biological Standards and Control, Herts, UK). Recombinant TNF$\alpha$ and IL-17 (R&D Systems) were used at 10 ng/ml, IFN$\gamma$ (Biogen, Cam-

bridge, MA) at 10 ng/ml, and IL-1$\beta$ at 1 ng/ml. GM-CSF (PeproTech, London, UK) was used at various concentrations.

**Fibroblast and leukocyte cell cultures.** Tissue samples were collected from 8 patients who fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for RA (23). All patients exhibited erosions on radiographs of the hands and feet; 6 of 8 patients were rheumatoid factor positive. Synovial, bone marrow, and overlying skin tissues were obtained from the hip or knee joint of each patient at the time of joint replacement. Bone marrow tissue was removed from femoral bone at the time of deep drilling for prosthesis fixation. Tissue came from within the femoral neck or the distal femur in patients undergoing hip replacement or knee replacement, respectively. This study was reviewed and approved by the South Birmingham local ethics committee (LREC 5735).

Tissue samples were enzymatically dissociated and cultured in RPMI 1640 medium (Life Technologies, Paisley, UK), supplemented with 20% heat-inactivated fetal calf serum (Life Technologies), 1% penicillin/streptomycin, 1% L-glutamine, 1% sodium pyruvate, and 1% nonessential amino acids (Sigma) as previously described (7,24,25). Fibroblast phenotype was confirmed by morphology and immunofluorescence microscopy. All cells expressed fibronectin and prolyl-4-hydroxylase, while fewer than 0.5% of cells stained positive for CD68, von Willebrand factor, CD31, or cytokeratin.

Despite the use of cells at varying passage numbers (passages 3, 4, or 5 only), all comparisons for cytokine secretion and functional assays, within a matched group of fibroblast strains, were made on fibroblasts at an identical passage number. Similarly, the time in culture for each of the matched sets of fibroblasts was the same, since cells were brought out of liquid nitrogen from passage 2 onward, in groups of 3 (synovium, bone marrow, and skin), and discarded beyond passage 5. Activated CD4 T cell lines were prepared from peripheral blood T cells and expanded by regular restimulation in the presence of phytohemagglutinin- and Epstein-Barr virus–transformed B cells as described previously (7,26). CD4 T cells were always used at the identical passage number (passage 5) and day of culture after stimulation (day 7). Neutrophils were freshly isolated from peripheral blood and cultured as previously described (27).

**Flow cytometry.** Analysis of absolute cell numbers by fixed volume counting, determination of the percentage of apoptotic cells, and assessment of T cell proliferation using labeling with 5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) were performed using an EPICS XL flow cytometer (Beckman Coulter, High Wycombe, UK), as previously described (8). Cytometer calibration was standardized using Flow-set fluorospheres (Beckman Coulter).

**Enzyme-linked immunosorbent assays (ELISAs).** Twenty-four–well plates were seeded with 20,000 fibroblasts per well and incubated with or without TNF$\alpha$ (10 ng/ml) for 24 hours in fibroblast culture medium. Tissue culture supernatants were collected at 24 hours and analyzed for IL-6 and CCL2 (monocyte chemoattractant protein 1 [MCP-1]) using OptEIA ELISA kits (PharMingen, San Diego, CA), according to the manufacturer's instructions. For analysis of GM-CSF, a DuoSet ELISA kit (Genzyme, Cambridge, MA) was used according to the manufacturer's instructions. The lower detec-

tion limits for the ELISA kits used were 15 pg/ml for IL-6, 2 pg/ml for MCP-1, and 1 pg/ml for GM-CSF.

**Cell staining and fluorescence microscopy.** Fibroblasts (5,000) were seeded into 8-well chamber slides (Falcon; Becton Dickinson, San Diego, CA) and grown for 3 days. The cells were fixed using acetone and washed in phosphate buffered saline (PBS), and primary antibodies were added in a 2% (weight/volume) solution of bovine serum albumin in PBS for 30 minutes at room temperature. After washing, FITC-conjugated secondary antibody was added in a similar way. The cells were then washed and a solution of 4′,6-diamidino-2-phenylindole (40 ng/ml; Sigma) was added for 2 minutes at room temperature to stain cell nuclei. The cells were then washed and mounted in 1% (w/v) $p$-phenylene diamine–90% (volume/volume) glycerol. Fibroblasts were imaged by epifluorescence microscopy using a Zeiss Axiovert 200 microscope (Zeiss, Wetzlar, Germany). Live, unstained cells were visualized using differential interference contrast. Images were captured and merged using a Hamamatsu C4742-95 camera and Simple PCI software (Digital Pixel, Brighton, UK).

**Survival assays.** Fibroblasts were seeded into 96-well or 24-well plates at densities of 3,000/well or 20,000/well, respectively, and cultured for 2 days in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin, 1% L-glutamine, 1% sodium pyruvate, and 1% nonessential amino acids. Fibroblasts were washed and treated with cytokines or fresh medium for 24 hours before being washed again. Either cytokine-deprived T cells or neutrophils were then added at a density of $10^5$ cells per well in the presence or absence of conditioned media and/or inhibitors with which the appropriate cells had been preincubated for 90 minutes. In some 24-well plate wells, 0.4-$\mu$m–pore size transwell inserts (Falcon; Becton Dickinson) were interposed between fibroblasts and T cells. Absolute numbers of input cells were assessed at the time of leukocyte addition by fixed volume counting, as described previously (7,8). Neutrophil survival was assessed similarly after 24 hours. The percentage of cells surviving was calculated as the number of live cells at the end of the experiment compared with the total number of cells added at the beginning of the experiment.

**Apoptosis assays.** Apoptosis of T cells was assessed simultaneously in parallel triplicate wells with survival assays using an antibody to active caspase 3 (559565; PharMingen) or control rabbit immunoglobulin (X0903; Dako) for 30 minutes at room temperature. Neutrophil apoptosis was measured using 3,3′-dihexyloxacarbocyanine iodide (DiOC6; Sigma) to measure mitochondrial membrane integrity (27). Rates of apoptosis indicated by DiOC6 labeling were confirmed episodically by use of cytospins and Diff-Quik staining and examination of nuclear morphologic features.

**Proliferation assays.** In order to assess T cell proliferation, some CD4 T cells were labeled with CFSE at the time of cytokine deprivation. To assess fibroblast proliferation, parallel wells in survival assays were seeded with fibroblasts, and fibroblast numbers were counted by flow cytometry on the days of fibroblast seeding, leukocyte addition, and leukocyte harvesting, in order to directly assess proliferation during the experiment.

**Cytokine depletion.** Depleting antibodies (MAB215 for GM-CSF or MAB271 for CCL4 [macrophage inflammatory protein 1$\beta$ (MIP-1$\beta$)] [both from R&D]) and isotype-
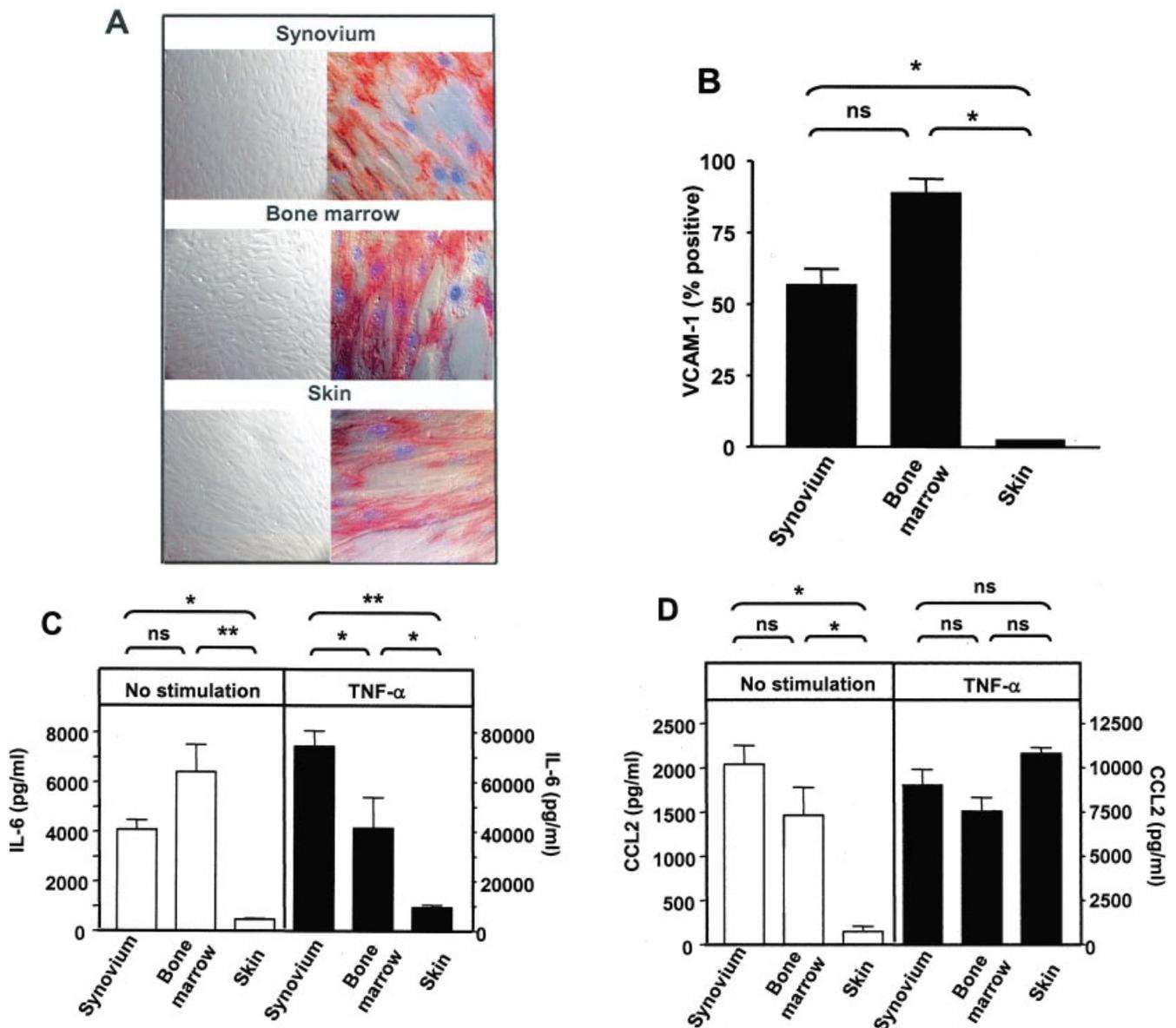
**Figure 1.** Structural and cytokine expression profiles of synovial- and bone marrow–derived fibroblasts as compared with dermal fibroblasts. **A,** Morphologic findings (at confluence) in a matched set of resting synovial, bone marrow, and skin fibroblasts. Cells were grown at passage 5 in glass-bottom chamber slides and then visualized by differential interference contrast (DIC). The DIC images of cells overlaid with fluorescence images (right panels) show staining for fibronectin (red) and nuclear staining with 4′,6-diamidino-2-phenylindole (blue) (original magnification × 20 in left panels; × 32 in right panels). **B,** Surface vascular cell adhesion molecule 1 (VCAM-1; CD106) expression of resting fibroblasts was quantified by immunofluorescence microscopy. Results are the combined mean and SD number of positively staining cells per field for all 8 matched samples. **C** and **D,** Fibroblasts (from 3 patients) were cultured in the absence or presence of tumor necrosis factor α (TNFα) for 24 hours. Supernatants were collected after a further 24 hours and production of interleukin-6 (IL-6) **(C)** and CCL2 **(D)** was analyzed quantitatively by enzyme-linked immunosorbent assay. Results are the mean and SD of duplicate assays of combined fibroblasts from 3 patients. ∗ = $P < 0.05$; ∗∗ = $P < 0.01$. ns = not significant.

matched irrelevant control antibodies ($\alpha_9\beta_1$, MAB2078Z; Chemicon International, Temecula, CA) were first bound to agarose gel beads (Upstate Biotechnology, Lake Placid, NY) at a concentration calculated to yield 2 μg antibody per depletion step. Depletion was confirmed by measuring cytokine levels by ELISA before and after depletion.

**Statistical analysis.** A non-Gaussian distribution was assumed for all assays. Statistical comparisons of data on fibroblast expression, neutrophil and T cell survival, and apoptosis induced by matched fibroblast groups were performed using Kruskal-Wallis one-way analysis of variance and Dunn's post hoc test. For the difference between paired samples (on depletions and blocking experiments) and results with or without transwells, significance was assessed using the Wilcoxon signed rank test with 2-tailed $P$ values. Results are expressed as the mean ± SD or mean ± SEM as appropriate.

## RESULTS

**Characteristic morphologic features, patterns of cytokine production, and expression of VCAM-1 (CD106) and fibronectin in matched synovial, bone marrow, and skin fibroblasts.** Previous studies have indicated that synovial- and skin-derived fibroblasts, obtained from different donors, exhibit distinct cytokine and chemokine profiles (24,25,28). We used matched sets of fibroblasts, each obtained from the same patient, in order to eliminate the confounders frequently present in studies of fibroblast lines obtained from different subjects whose age, disease subtype, and prior therapy can vary widely.

As previously observed (25,29), rheumatoid synovial fibroblasts exhibited a heterogeneous, stellate morphologic appearance as compared with the elongated, spindle-shaped fibroblasts from skin, which formed flowing patterns in culture (Figure 1A). Bone marrow fibroblasts were larger than synovial fibroblasts but exhibited similar stellate morphologic features, frequently forming clusters of cells separated by relatively uncolonized areas. Consistent with our previous findings (25), the expression of fibronectin, as measured by immunohistochemistry in the fibroblasts from different sites, was similar quantitatively (all of the fibroblasts produced fibronectin) yet qualitatively different.

Site-specific expression of VCAM-1 (CD106) by matched fibroblasts confirmed previous findings (10,21,30), in that bone marrow fibroblasts were generally positive for VCAM-1, while ~50% of synovial fibroblasts expressed the antigen; skin fibroblasts were all VCAM-1 negative (Figure 1B). Production of IL-6 and CCL2 demonstrated similar patterns between synovial and bone marrow fibroblasts, but not skin fibroblasts. Unstimulated bone marrow fibroblasts secreted the highest amounts of IL-6, with both synovial and bone marrow fibroblasts secreting significantly greater quantities compared with dermal fibroblasts. On stimulation with TNFα, IL-6 production increased ~10-fold

for all fibroblasts. However, bone marrow and synovial fibroblasts still secreted the highest levels of IL-6, whereas dermal fibroblasts produced significantly lower levels (Figure 1C). Production of CCL2 showed a different pattern. Synovial and bone marrow fibroblasts secreted higher basal levels, whereas TNFα stimulation raised secretion of CCL2 to similar levels in all fibroblasts (Figure 1D). In general, therefore, resting synovial and bone marrow fibroblasts share a characteristically high basal cytokine production profile that is quite distinct from that of matched skin-derived fibroblasts.

**Site-specific survival of CD4 T cells supported by synovial and bone marrow fibroblasts.** Previous studies using coculture systems have shown that fibroblasts are able to maintain the survival of cytokine-deprived CD4 T cells in coculture, and that soluble survival factors, of which type I IFNs are the most important, partially mediate this effect (7,8). To determine whether fibroblasts from different sites would support differing levels of survival of CD4 T cells, we cocultured synovial, bone marrow, and skin fibroblasts with cytokine-deprived CD45RO+,CD4+ T cell lines. These T cell lines are highly differentiated (CD45RO^{bright},CD45RB[dull]) and have a phenotype very similar to that of T cells found in synovial fluid (31). All fibroblasts induced increased survival of cytokine-deprived CD4 T cells, compared with that in control cultures (Figure 2A), but synovial and bone marrow fibroblasts induced a consistently greater degree of survival. A similar site-specific pattern was observed with all 8 matched sets of fibroblasts, and differential survival was maintained for up to 11 days in culture (results not shown).

Increases in absolute survival due to fibroblast coculture were reflected in decreased apoptosis, as measured by caspase 3 activation in parallel experiments (Figure 2B). These reciprocal findings suggested that fibroblasts induce the survival of T cells by inhibiting apoptosis without any effect on T cell proliferation (32). We confirmed the absence of proliferation by labeling CD4 T cells with CFSE and measuring CFSE dilution after coculture with fibroblasts (Figure 2C).

In order to determine whether the enhanced ability of bone marrow and synovial fibroblasts to keep CD4 T cells alive might reflect differences in their rates of proliferation compared with skin fibroblasts, we correlated fibroblast proliferation rates with their ability to maintain CD4 T cell survival for 3 different sets of matched fibroblast cell strains. CD4 T cell survival was independent of fibroblast proliferation over a wide range of proliferation rates (Figure 2D). This suggested to us that the ability of synovial and bone marrow
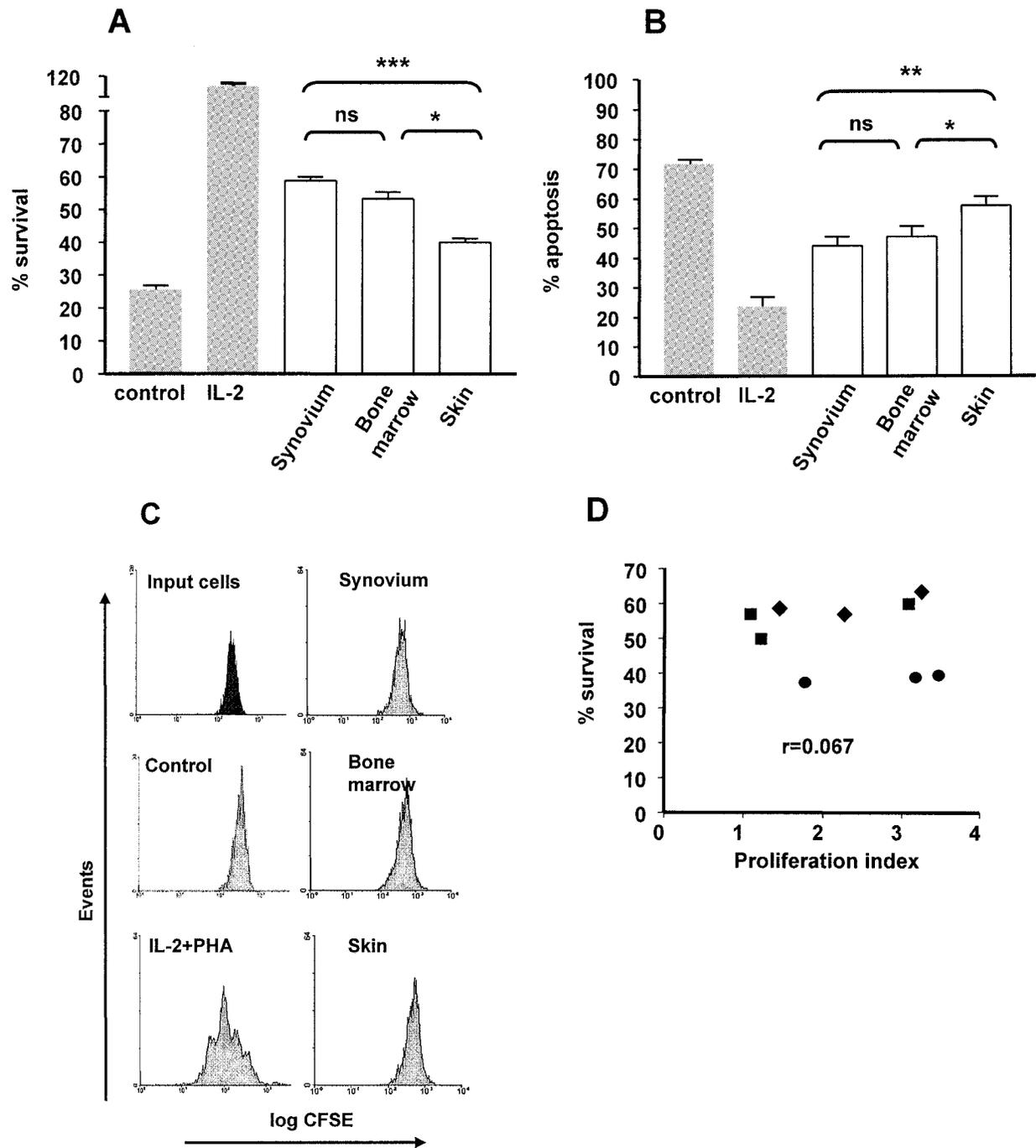
**Figure 2.** Support of site-specific survival of CD4 T cells by resting fibroblasts in the absence of T cell proliferation. **A,** Increased survival of CD4 T cell lines cocultured for 3 days with synovial, bone marrow, and dermal fibroblasts as compared with that in cultures without fibroblasts (control) or with interleukin-2 (IL-2) (as a positive control). **B,** Association of T cell survival with the decreased rates of apoptosis, as measured by caspase 3 activation. Results in **A** and **B** are the mean and SEM from 8 individuals. At least 2 separate experiments with triplicate assays were performed using matched synovial, bone marrow, and skin fibroblasts at identical passage. $* = P < 0.05$; $** = P < 0.01$; $*** = P < 0.001$. ns = not significant. **C,** Labeling of T cells with 5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) demonstrating the absence of proliferation on cocultures with fibroblasts as compared with positive control T cells treated with IL-2 plus phytohemagglutinin (PHA). **D,** Lack of association between T cell survival and fibroblast proliferation. Results are depicted as a scatter plot of T cell survival against the proliferation index (ratio of mean triplicate fibroblast cell counts at day 6 compared with day 0). Each point represents an individual cell strain (squares = bone marrow; triangles = synovium; circles = skin). The r value is the Spearman's correlation coefficient.
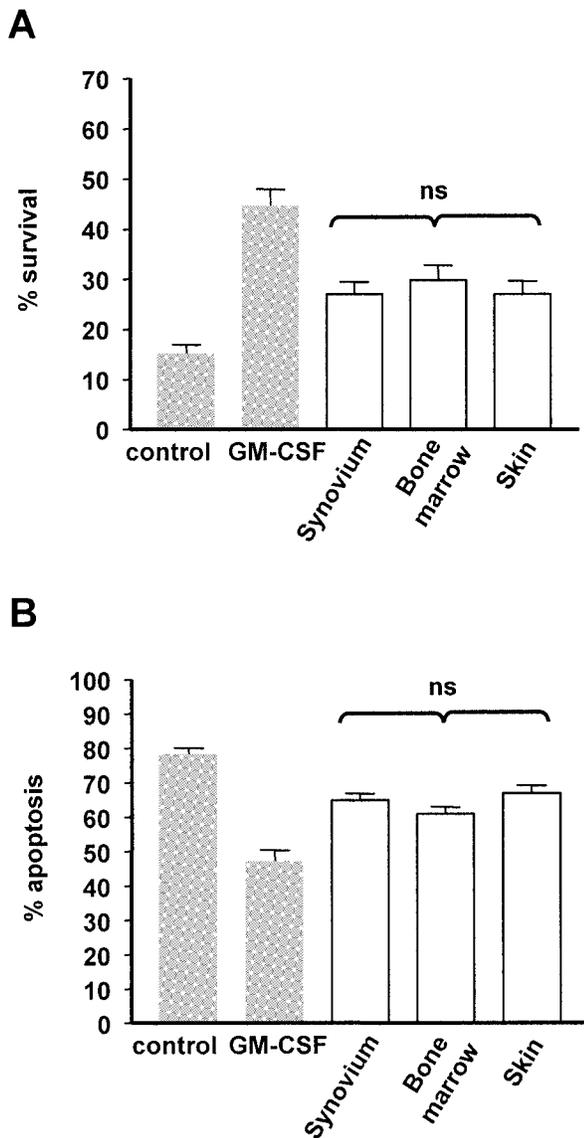
**A**



**B**



**Figure 3.** Support of neutrophil survival by resting fibroblasts, with no site specificity. **A,** Neutrophil survival is modestly increased (as compared with control cultures) on cocultures with all fibroblasts ($P <$ 0.01), but no difference between sites of origin of the fibroblasts is evident. Granulocyte–macrophage colony-stimulating factor (GM-CSF) was included as a positive control. **B,** Increased neutrophil survival is mirrored by decreased apoptosis as measured by mitochondrial 3,3′-dihexyloxacarbocyanine iodide retention ($P < 0.05$). Results are the mean and SEM from 8 individuals. At least 2 separate experiments with triplicate assays were performed using matched synovial, bone marrow, and skin fibroblasts at identical passage. ns = not significant.

fibroblasts to maintain CD4 T cell survival was an intrinsic property of fibroblasts from these sites and not in any way related to their rates of proliferation.

**Lack of site specificity for neutrophil survival.** We next determined whether the matched fibroblast strains could also support neutrophil survival, indicating a generic leukocyte survival mechanism, or whether site-specific differences were a particular feature unique to CD4 T lymphocytes. In contrast to the pattern of T cell survival, all 8 matched sets of resting synovial, bone marrow, and dermal fibroblasts supported only a modest degree of additional survival compared with that of
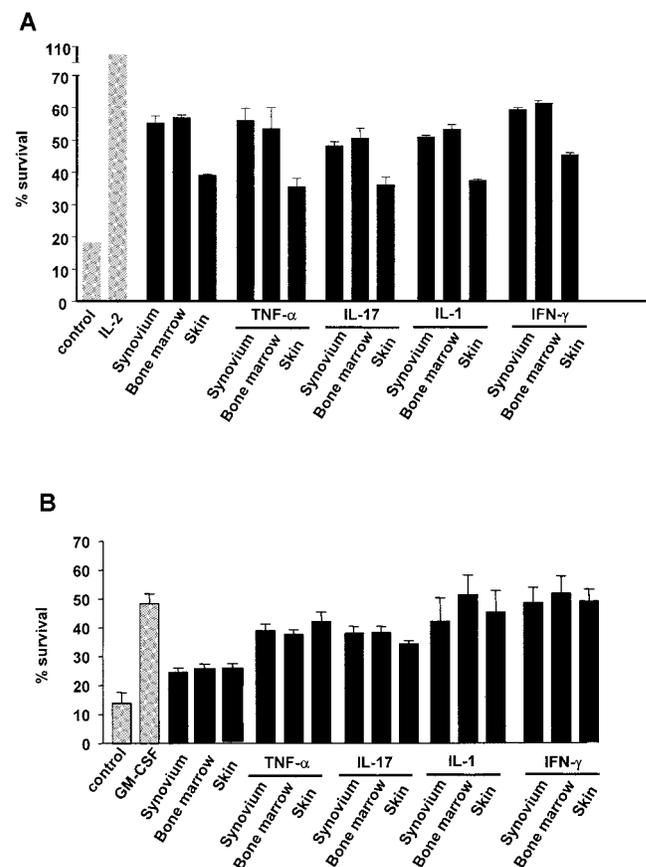
**A**



**B**



**Figure 4.** Effect of prior fibroblast activation with proinflammatory cytokines on leukocyte survival. **A,** Matched synovial, bone marrow, and skin fibroblasts were preactivated with tumor necrosis factor $\alpha$ (TNF$\alpha$), interleukin-17 (IL-17), interferon-$\gamma$ (IFN$\gamma$) (each at 10 ng/ml), and 1 ng/ml of IL-1$\beta$, and then cocultured with CD4 T cells. No significant effect of preactivation on CD4 T cell survival can be seen. IL-2 was included as a positive control. Results are the mean and SEM from 3 independent experiments. **B,** Following preactivation of matched fibroblasts with TNF$\alpha$, IL-17, IFN$\gamma$, and IL-1$\beta$ at the same concentrations as in **A** and coculture with neutrophils, enhanced survival of neutrophils can be seen with all 3 types of fibroblasts. Granulocyte–macrophage colony-stimulating factor (GM-CSF) was included as a positive control. Results are the mean and SEM from 8 individuals in at least 2 separate experiments for each.

neutrophils cultured alone. No site-specific differences in the survival of neutrophils were seen (Figure 3A). Increased survival was accounted for by reduced apoptosis (Figure 3B).

**Enhanced survival of neutrophils, but not CD4 T cells, supported by cytokine-activated fibroblasts.** To determine whether the differences between survival of T cells and survival of neutrophils mediated by the 3 types of fibroblasts were due to intrinsic differences or whether they could be modified by extrinsic factors, we assessed the effect on leukocyte survival of prior activation of fibroblasts with a range of proinflammatory cytokines, including TNFα, IL-17, IL-1, and IFNγ. Preactivation of fibroblasts with any of these cytokines made no difference to the survival of CD4 T cells (Figure 4A), suggesting that rescue from apoptosis is an inherent property of synovium and bone marrow fibroblasts compared with skin fibroblasts. In complete contrast, when neutrophils were cocultured with cytokine-activated fibroblasts, their survival was significantly increased, as observed with all 4 stimuli tested (Figure 4B). The increase in neutrophil survival was similar with all 3 types of fibroblasts, with no site-specific differences observed.

**Mediation of site-specific CD4 T cell survival by soluble factors, with prerequisite of T cell–fibroblast interactions.** To explore whether differential CD4 cell survival was mediated by soluble factors, we interposed transwells between fibroblast monolayers and T cells, which allowed passage of soluble mediators only. The presence of transwells decreased CD4 T cell survival to the same extent in cultures mediated by all 3 fibroblasts, but did not abolish the generic ability of all 3 fibroblasts to support higher levels of T cell survival compared with culture in the absence of fibroblasts. This suggested to us that direct cell–cell contact (or very close proximity between T cells and fibroblasts) was necessary for the majority of site-specific survival (Figure 5A), but that a soluble factor was responsible for the generic ability of fibroblasts from all 3 sites to support CD4 T cell survival.

Cell contact between T cells and fibroblasts has been shown to be a requirement for increased expression of potential survival factors such as VCAM-1 and ICAM-1 (33), and has recently been shown to be important in surface IL-15– and ICAM-1–mediated T cell–fibroblast crosstalk (5). Furthermore, CXCL12 (stromal cell–derived factor 1) expressed by synovial fibroblasts has been shown to regulate the retention of T cells (26,34) and has been proposed as a T cell survival factor (35). Interaction of CD40 and CD40 ligand has also been implicated in fibroblast–T cell crosstalk (36). We
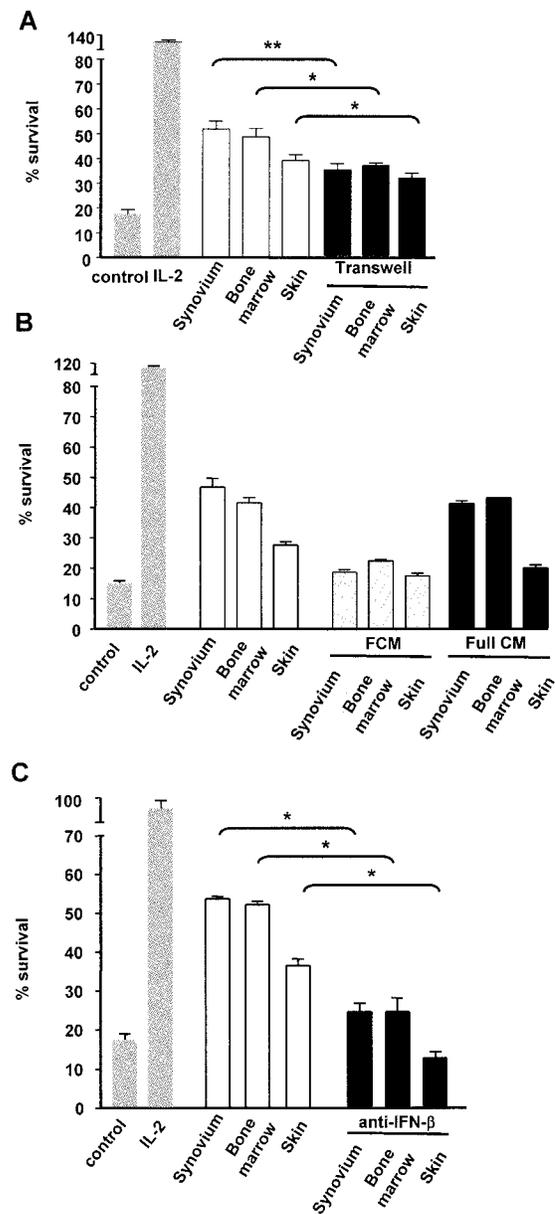


**Figure 5.** Prerequisite of initial cell–cell contact for enhanced T cell survival, and partial mediation by interferon-β (IFNβ). **A,** CD4 T cells were cocultured with matched fibroblasts from synovium, bone marrow, and skin in the presence or absence of transwells. Interleukin-2 (IL-2) was included as a positive control. **B,** CD4 T cells were incubated with fibroblasts, fibroblast-conditioned medium (FCM), or medium obtained from whole coculture (full CM). No effect of FCM on T cell survival can be seen, whereas full CM is sufficient to reconstitute site-specific survival. Results are the mean and SEM of 4 independent experiments in **A** and 3 independent experiments in **B. C,** CD4 T cells were incubated with full CM in the presence or absence of specific antisera capable of blocking IFNβ activity. IFNβ-specific antisera blocked survival to a similar degree regardless of site of origin. Results are the mean and SEM of 4 independent experiments. $* = P < 0.05$; $** = P < 0.01$.

therefore used specific inhibitors targeted to a variety of candidates to further investigate the molecular basis for site-specific T cell survival. Blocking antibodies to ICAM-1 and CD40 ligand had no effect on T cell survival. Furthermore, neither culture with AMD3100, a specific small-molecule CXCR4 antagonist, nor culture with specific $\alpha_4\beta_1$ blocking peptides had an effect (results not shown).

To further explore the molecular basis of site-specific survival, we tested whether fibroblast-conditioned medium could support T cell survival. Conditioned media obtained directly from fibroblasts cultured in the absence of T cells (fibroblast-conditioned medium) yielded only a marginally increased CD4 T cell survival and displayed no site specificity. In contrast, media obtained from T cell–fibroblast cocultures (full conditioned medium) was sufficient to reproduce the site-specific differences in T cell survival (Figure 5B). Furthermore, fixation of fibroblasts prior to coculture led to a complete inhibition of additional survival, indicating that live fibroblasts (with appropriate bidirectional signals) are required to support T cell survival (results not shown).

We analyzed the full conditioned medium (from T cell–fibroblast cocultures) for differential expression of a range of cytokines (IL-1$\beta$, IL-2, IL-4, IL-7, IL-8, IL-10, IL-15, GM-CSF, IFN$\gamma$, inflammatory protein 10, MIP-1$\alpha$, MCP-1, RANTES, TNF$\alpha$, endothelial growth factor, fibroblast growth factor 2, platelet-derived growth factor, and vascular endothelial growth factor [VEGF]) to assess whether any of these might have been overexpressed in full (coculture) conditioned medium as compared with fibroblast-conditioned medium. The results demonstrated significant up-regulation of potential survival factors, including IL-6, VEGF, GM-CSF, and CCL2 (MCP-1). However, none of these soluble factors, either alone or in combination, were able to reconstitute T cell survival (results not shown). There was no measurable IL-2 or IL-15 in the full (coculture) conditioned medium, consistent with our findings that fibroblast-mediated rescue did not lead to T cell proliferation (Figure 2C). Blocking antibodies to the candidate soluble survival factors, including IL-6, GM-CSF, and IL-15, were also unable to inhibit T cell survival when added to cocultures (results not shown).

**Role of IFN$\beta$ production in the generic ability of fibroblasts to maintain CD4 T cell survival.** We and others have demonstrated that IFN$\beta$ is a crucial element in the rescue of activated CD4 T cells by a wide variety of stromal cells, and is present in fibroblast-conditioned medium (8,37). We therefore used antisera specific for IFN$\beta$ to investigate the contribution of type I IFNs to
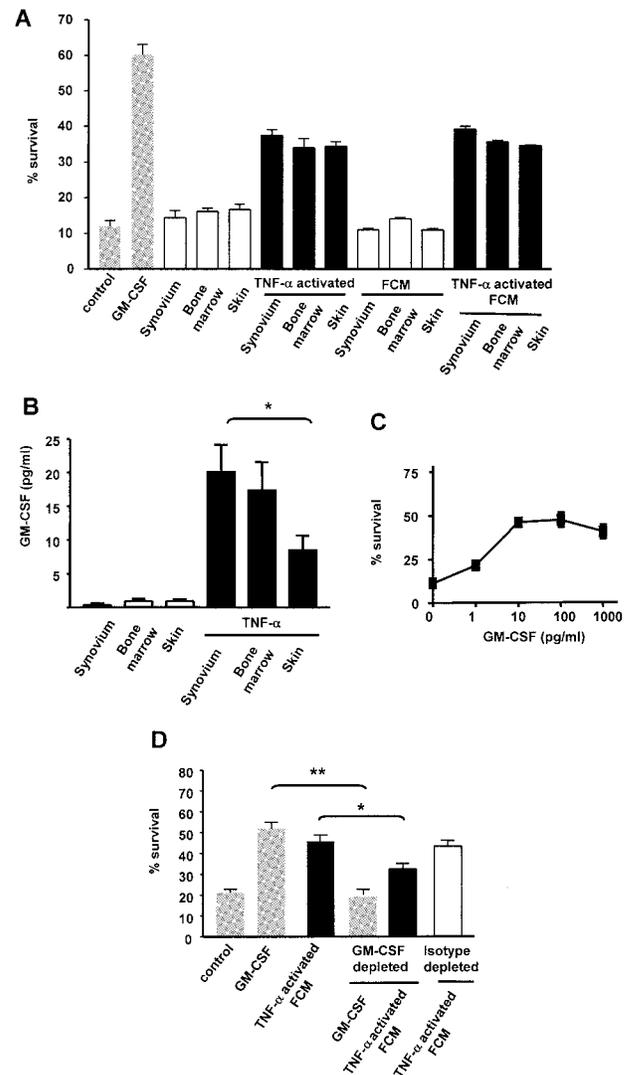


**Figure 6.** No requirement for initial cell–cell contact in enhanced neutrophil survival, and partial mediation by granulocyte–macrophage colony-stimulating factor (GM-CSF). **A,** Peripheral blood neutrophils were incubated with either resting or tumor necrosis factor $\alpha$ (TNF$\alpha$)–stimulated fibroblasts, or with fibroblast-conditioned medium (FCM) or TNF$\alpha$-activated FCM prepared from the same fibroblasts. Neutrophil survival induced by TNF$\alpha$-activated FCM was identical to that induced by direct coculture with resting or stimulated fibroblasts. **B,** Matched fibroblasts from synovium, bone marrow, and skin were cultured in the absence or presence of TNF$\alpha$ for 24 hours. Supernatants were collected after a further 24 hours and GM-CSF was measured by enzyme-linked immunosorbent assay. $* = P < 0.05$. **C,** Peripheral blood neutrophils were incubated with varying amounts of GM-CSF, and survival of neutrophils was determined. **D,** Peripheral blood neutrophils were incubated with medium alone, GM-CSF, or TNF$\alpha$-activated FCM, some of which had been depleted using antibodies against GM-CSF or isotype-matched irrelevant antibodies. Depletion using antibodies to GM-CSF resulted in a mean inhibition of enhanced neutrophil survival of ~54% ($P = 0.029$). As a positive control, GM-CSF was added to neutrophils either before or after depletion with anti–GM-CSF antibodies. $* = P < 0.05$; $** = P < 0.01$. Results are the mean and SEM of 3 independent experiments.

CD4 T cell survival induced by the 3 types of fibroblasts in this study. Antisera to IFN$\beta$ significantly reduced T cell survival induced by full (coculture) conditioned medium (Figure 5C), but, consistent with the findings from other studies using transwells and coculture-conditioned media, anti-IFN$\beta$ did not affect the site-specific differences observed between synovium and bone marrow fibroblasts compared with skin fibroblasts. T cell survival was decreased to a similar degree regardless of fibroblast origin, consistent with the constitutive basal production of IFN$\beta$ by all fibroblasts (8).

**Mediation of enhanced neutrophil survival by soluble factors, but without requirement for fibroblast–neutrophil interactions.** To determine the factors responsible for enhanced neutrophil survival, we used a similar approach as that described above, by comparing the results in conditioned medium from resting fibroblasts with those in conditioned medium from TNF$\alpha$-stimulated fibroblasts. TNF$\alpha$-activated fibroblast-conditioned medium had an effect on neutrophil survival that closely mirrored that induced by direct neutrophil–fibroblast coculture (Figure 6A). This suggested that soluble factors are responsible for increased neutrophil survival, but that no prior cell–cell interactions between neutrophils and fibroblasts are required in order to elicit increased survival.

**Role of GM-CSF as the major factor secreted by TNF$\alpha$-stimulated fibroblasts in enhanced neutrophil survival.** The observation that the factor responsible for neutrophil survival was secreted by activated fibroblasts without any requirement for prior contact with neutrophils suggested to us that GM-CSF might be a possible candidate (38,39). Furthermore, GM-CSF was only produced at significant levels by fibroblasts after TNF$\alpha$ stimulation (Figure 6B). Although synovial fibroblasts produced significantly higher levels of GM-CSF, the levels of production ($\sim$10–20 pg/ml) by bone marrow and skin fibroblasts were functionally relevant, since neutrophils could be kept alive by as little as 10 pg/ml of GM-CSF when recombinant GM-CSF was titrated to neutrophil survival (Figure 6C). Moreover, depletion of GM-CSF from TNF$\alpha$-activated synovial fibroblast supernatants resulted in a significant reduction in neutrophil survival compared with that in nondepleted coculture medium or an isotype control depletion. Functional depletion using the anti–GM-CSF antibody was confirmed using recombinant GM-CSF, which, when depleted, was unable to promote neutrophil survival (Figure 6D).

In an attempt to identify other factors in addition to GM-CSF that might play a role in neutrophil survival, we performed ELISAs for additional candidates. The results indicated that G-CSF was not produced by activated fibroblasts in adequate quantities to explain the increased neutrophil survival. Furthermore, depletion of CCL4 (MIP-1$\beta$), a recently identified soluble survival factor released by fibroblasts under activating conditions including hypoxia (40), did not affect neutrophil survival (results not shown). These results suggested to us that the majority of the survival factor produced by TNF$\alpha$-activated fibroblasts could be attributed to GM-CSF.

## DISCUSSION

In this study we tested whether the site of origin and state of activation of fibroblasts can affect fibroblast-induced survival of CD4 T cells and neutrophils. We used matched sets of fibroblasts obtained from synovium, bone marrow, and skin to avoid the potential confounding effect of fibroblasts obtained from different individuals with varying disease phenotypes and prior drug therapy.

We first confirmed the phenotype of the 3 types of fibroblast strains by demonstrating that synovial, and not skin, fibroblasts express VCAM-1 (CD106), fully consistent with previous findings (26,41). Bone marrow fibroblasts expressed high levels of VCAM-1, as expected from existing data (42). Autonomous IL-6 production by synovial fibroblasts is well documented (24), and a similar production of IL-6 from bone marrow–derived fibroblasts was confirmed here. In the context of the bone marrow, IL-6 secretion is necessary as a supporting factor for hemopoietic cell populations, including B cell and myeloid lineage growth and differentiation (42).

We found that T cell survival was enhanced, to some extent, by coculture with fibroblasts from all sites. This observation and the lack of CD4 T cell proliferation are consistent with our previous findings (7). A clear hierarchy of T cell survival was observed, with synovial and bone marrow fibroblasts being much more efficient at keeping T cells alive compared with skin fibroblasts. In some autologous coculture systems, increased T cell survival has been suggested to be the result of proliferation of T cells (6,43). However, the majority of data, including our current results, do not support a role for T cell proliferation when T cells are cocultured with fibroblasts. This is fully consistent with the production of IFN$\beta$ by fibroblasts, which induces T cells to enter the Go phase of the cell cycle (6,8,44).

Site-specific CD4 T cell survival was independent of the rate of fibroblast proliferation (Figure 2D) and prior activation with a range of activating cytokines

(Figure 4A). However, it was dependent on cell–cell interactions between live fibroblasts and T cells. Enhanced survival of T cells was paralleled by increased secretion of VEGF, GM-CSF, IL-6, and CCL2 in coculture supernatants, confirming earlier observations (4,5,33,45).

Since direct cell contact appeared to be important, a number of candidate molecules that could potentially be involved in such an interaction were examined. The interaction of CD40 with CD40 ligand has been shown to play a part in T cell–fibroblast interactions (46), particularly by increasing induction of CXCL12 in synovial fibroblasts (47), expression of which has been shown to be important in the retention of synovial T cells in the inflamed synovium (26,34). Cell contact has also been shown to be important in the induction of ICAM-1 and VCAM-1 on fibroblasts (33), and ICAM-1–lymphocyte function–associated antigen 1 interactions have been implicated in T cell survival induced by bone marrow stromal cells (48). Blockade of CD40/CD40 ligand, IL-15/IL-15 receptor, CXCL12/CXCR4, ICAM-1, and $\alpha_4\beta_1$/VCAM-1 pathways had no effect on the pattern of survival seen, indicating that these pathways are unlikely to contribute significantly. These findings with T cells are reminiscent of findings with B cell fibroblast cocultures, in which it appears that no one factor is responsible for B cell survival, and rather, a combination of factors, including the CXCL12/CXCR4 and $\alpha_4\beta_1$/VCAM-1 pathways, are required (9–11).

In complete contrast to the findings with T cells, neutrophil survival induced by fibroblasts showed no dependence on the site of origin of the fibroblast, but was critically dependent on prior fibroblast activation (Figure 4B) and was mediated principally by GM-CSF. Such survival effects have also been demonstrated for other stromal cell types, including bronchial epithelial cells (13,14) and HUVECs, which similarly elaborate GM-CSF in response to cytokine stimulation (14). Vascular smooth muscle cells also inhibit neutrophil apoptosis in response to cytokine activation by means of GM-CSF and G-CSF secretion. Under these conditions, cyclooxygenase 2 (COX-2) inhibitors prevented G-CSF secretion, but augmented GM-CSF secretion, suggesting that COX-2 activity may differentially regulate neutrophil survival under inflammatory conditions (15).

Hypoxia may be an important factor in neutrophil survival within synovial microenvironments. Walmsley and colleagues recently demonstrated that hypoxia induced autocrine secretion of CCL4 (MIP-1$\beta$), which delayed neutrophil apoptosis (40). However, we were not able to demonstrate that CCL4 played any role in fibroblast-induced neutrophil survival.

Stromal cells such as fibroblasts clearly make an important contribution to neutrophil survival by secreting soluble survival factors in response to proinflammatory cytokines, regardless of the organ involved (27,38,39). The abundance of synovial fibroblasts in pannus tissue may therefore lead to significantly enhanced survival, due to soluble factors such as GM-CSF, which is found at high levels in the synovium. The effect of synovial fluid (as a mimic of the synovial microenvironment) on neutrophil apoptosis remains highly controversial, particularly with respect to the pro- and antiapoptotic effects of synovial fluid and the possible role of short-lived mediators such as adenosine (49). In our study, a defined coculture system was used, and our findings suggest that the contribution of soluble factors derived from synovial fibroblasts is primarily antiapoptotic in nature.

The bone marrow niche plays a critical role in the early development of all hemopoietic populations, but also acts as an active reservoir for terminally differentiated leukocyte subpopulations, including CD4 and CD8 T cells and neutrophils. The bone marrow stromal microenvironment therefore maintains not only production of immature cells, but, in some cases, the recruitment of their mature counterparts. The functional similarity that we have observed between synovial and bone marrow fibroblasts may help to explain the persistent accumulation of both CD4 T cells and neutrophils within the synovium.

Our findings emphasize that fibroblasts play an important role in regulating the survival of different leukocyte subpopulations within tissues. Furthermore, it is apparent that the requirements of different leukocyte subpopulations for fibroblast-derived survival factors may not only be complex, but may also differ considerably at different sites, and at different stages of the inflammatory response.

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