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Fibroblasts are a dominant cell type in most human solid tumors. The possibility that fibroblasts have the capacity to interact with and modulate the function of tumor-associated T lymphocytes makes them a potential therapeutic target. To address this question, primary cultures of fibroblasts derived from human lung tumors were established and cultured with T cells derived from the same tumor. The tumor fibroblasts significantly enhance the production of IFN-γ and IL-17A by the tumor-associated T cells following a CD3/CD28-induced activation of the T cells. This enhancement was fibroblast cell dose-dependent and did not require direct contact between the two cell types. Tumor-associated fibroblast-conditioned media similarly enhanced both IFN-γ and IL-17A in activated T cells, and this enhancement was significantly reduced by Abs to IL-6. Conditioned media derived from activated lymphocyte cultures significantly enhanced IL-6 production by tumor fibroblasts. A similar enhancement of IFN-γ and IL-17A was observed when activated T cells from a normal donor were cultured with skin fibroblasts derived from the same donor. These results establish that fibroblasts and autologous lymphocytes, whether derived from the tumor microenvironment or from nonmalignant tissues, have the capacity to reciprocally interact and modulate function. In contrast to other reports, fibroblasts are shown to have an immunostimulatory effect upon activated T lymphocytes. The ability of fibroblasts to enhance two T cell cytokines known to have an impact upon tumor progression suggests that fibroblasts play an important role in tumor pathogenesis that could be exploited therapeutically.

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extensively studied for their ability to suppress T lymphocyte proliferation and IFN-γ production (reviewed in Ref. 12). Although fibroblasts make many immunosuppressive molecules, they also produce immunostimulatory factors, and this aspect has been an intense area of research for fibroblast-like synoviocytes found in rheumatoid arthritis (13, 14). Few studies have examined which of these opposing roles dominates for tumor-associated fibroblasts (TAFs) in cancer. Knowing how fibroblasts are capable of influencing T lymphocytes may prove valuable for manipulating the tumor microenvironment to favor the activation of T effector cells for an anti-tumor immune response for cancer eradication.

The immunosuppressive nature of the tumor microenvironment represents the sum total of complex interactions between many different cell types. In this paper, we begin to tease apart this puzzle by examining how autologous human tumor-associated T cells directly impact the function of TAFs and vice versa. We use primary cells isolated from human tissue from patients undergoing surgical resection for non-small cell lung carcinoma as our primary model. We find that TAFs have the ability to simultaneously promote both proliferation and IFN-γ production (reviewed in Ref. 12). Although described (16), IL-6, IL-17A, and TGF-β1 ELISA kits were ordered from eBioscience (San Diego, CA) and used according to the manufacturer’s protocol. Multiplex assays for IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, and TNF-α were performed utilizing BD Flex sets for the cytokometric bead array according to the manufacturer’s protocol (BD Pharmingen, San Diego, CA). To avoid cytokine degradation due to freezing and thawing, supernatant samples were stored at 4°C and assayed within a week of being collected.

**Assessment of cytokine production**

Culture supernatants were assayed by sandwich ELISA for IFN-γ using Endogen Abs (Thermo Fisher Scientific, Rockford, IL), as previously described (16). IL-6, IL-17A, and TGF-β1 ELISA kits were ordered from eBioscience (San Diego, CA) and used according to the manufacturer’s protocol. Multiplex assays for IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, and TNF-α were performed utilizing BD Flex sets for the cytokometric bead array according to the manufacturer’s protocol (BD Pharmingen, San Diego, CA). To avoid cytokine degradation due to freezing and thawing, supernatant samples were stored at 4°C and assayed within a week of being collected.

**TAF-conditioned media**

To make TAF-conditioned AIM-V media (TAF-conditioned media), fibroblasts were seeded at 3.5–4 × 10⁴ live cells per 25 cm² flasks in DMEM/F12/10% FCS. After overnight incubation, media were replaced with 12 ml serum-free AIM-V (Invitrogen) media. Every 5 d, media were removed and replaced. TAF-conditioned media were centrifuged to remove cells and stored at 4°C prior to use with PBLs.

**PBL-conditioned media**

Normal donor monocyte-depleted PBLs were brought to 50,000 cells/ml AIM-V media, activated with 5 μl CD3/CD28 Dynabeads and incubated in 24-well plates (2 ml/well) for 1–3 d. Cells and Dynabeads were pelleted by centrifugation and media were transferred to new tubes and stored at 4°C prior to use with fibroblasts.

**Cytokine neutralization**

Neutralizing Abs to IL-6 (AB-206-NA), TGF-β1 (MAB240 and AF-101-NA), and TNF-α (MAB 210) were purchased from R&D Systems (Minneapolis, MN) and used at 5 μg/ml unless specified otherwise.

**Proliferation analysis**

Cells were stained in Dulbecco’s phosphate buffered saline, using 5 μM CFSE (CellTrace Kit; Invitrogen) for 15 min at 37°C. Cells were then washed with 1 ml volume of media and rested for at least 30 min in complete media to allow for stabilization of the probe prior to being washed and used in proliferation experiments. Cells were incubated in 24-well plates with 1 μg/ml CD3/CD28 Dynabeads and media at a concentration of 5 × 10⁴ cells/ml. After 7 days, cells were washed 2 times with 2 ml AIM-V in Dulbecco’s phosphate buffered saline, fixed in 2% formaldehyde, and analyzed on the FACS Calibur (BD Biosciences, San Jose, CA).

**Statistical analysis**

Data are expressed as the mean ± SEM. Data were analyzed using a two-tailed Student t test calculated with SigmaPlot 8.0 software (Systat Software, San Jose, CA). A p value <0.05 was considered significant.

**Results**

**TAFs enhance IFN-γ and IL-17A production by activated tumor immune cell infiltrates**

To determine the effect of TAFs upon the function of CD3/CD28 activated tumor-associated T lymphocytes, CD45⁺ cells were enriched from single-cell suspensions of human lung tumor biopsies. Adherent autologous TAF cultures were established and CD45⁺ enriched cells were applied to wells containing the TAFs or empty wells and activated with CD3/CD28 Ab immobilized on synthetic beads. To monitor the T cell response to CD3/CD28 activation, IFN-γ and IL-17A cytokine levels were quantified in the culture supernatants by ELISA over a 7-d period. These cytokines were detectable only when anti-CD3/CD28-activating beads were present in the CD45⁺ enriched population. When tumor-associated T lymphocytes were activated in the presence of fibroblasts, levels of both...
IFN-γ and IL-17A were increased significantly \((p < 0.05)\) over conditions that were not enriched with fibroblasts (Fig. 1). The levels of IFN-γ detected were greater than those for IL-17A. Statistically significant fibroblast-mediated enhancement of IFN-γ in the autologous activated tumor-associated T cell/fibroblast cocultures was evident within the first 24 h, whereas IL-17A was not significantly enhanced until day 5. No IFN-γ or IL-17A was detectable in the wells containing fibroblasts alone. We conclude from these studies that fibroblasts derived from the microenvironment of human lung tumors have the capacity to enhance cytokine production by activated T cells derived from the same tumor microenvironment.

**TAFs enhance IFN-γ production by purified autologous activated patient T lymphocytes**

In initial experiments, the TAFs were cultured with a single-cell suspension derived from solid tumors that included, in addition to T cells, a mixture of B cells, monocytes, endothelial cells, stromal fibroblasts, and tumor cells. To determine if TAFs were acting directly on T lymphocytes and not through one of the other cell types, CD3+ T cells were purified from the tumor cell suspension of a lung cancer patient. Coculture of the anti-CD3/CD28 Dynabead-activated purified patient CD3+ tumor-associated T lymphocytes with autologous TAFs led to significantly increased levels of IFN-γ over that of the activated purified CD3+ T cells alone at days 1, 3, and 5 postactivation (Fig. 2), as was seen with the unpurified CD45+ cell enriched suspension. IL-17A was detectable at days 3 and 5 and was found at increased levels when the T cells were cultured with the autologous TAFs. Although found at much lower levels, IL-4 was also increased when tumor-associated T cells were cultured with the TAFs. At days 1, 3, and 5, significant increased levels of IL-2 were found in the autologous tumor-associated T cell and fibroblast cocultures. IL-6 was produced by both the activated T cells alone and the fibroblasts alone. When cultured together, IL-6 increased to levels greater than addition of the IL-6 levels of the two cell types cultured alone. Amplified IL-6 levels were apparent at day 1 of the culture and persisted for the 5-d duration. IL-1β was found at enhanced levels in the coculture of T cells and fibroblasts at days 3 and 5. IL-10, although initially enhanced in the T cell and fibroblast cocultures, was found at a reduced level in the coculture compared with the T cells alone by day 5. These results establish that the enhancing effect of TAFs is mediated by a direct interaction of the fibroblast or fibroblast-secreted products upon the T lymphocytes.

**TAFs enhance IFN-γ and IL-17A production by purified autologous activated patient peripheral blood T lymphocytes**

To determine if the enhanced levels of IFN-γ and IL-17A observed in cocultures of TAFs and activated tumor-associated T cells was a phenomenon unique to T cells derived from the tumor microenvironment, cocultures utilizing patient peripheral blood T cells and TAFs were also performed. Coculture of the anti-CD3/CD28 Dynabead-activated purified patient CD3+ T lymphocytes with autologous TAFs led to significantly increased levels of IFN-γ over that of the activated purified CD3+ T cells alone at days 1 and 3 postactivation (Fig. 3). IL-17A was detected at day 3 and was enhanced in a statistically significant manner in the autologous patient peripheral blood T cell and TAF cocultures by day 5. The effect of coculture of patient peripheral blood T cells and TAFs on IL-4 and IL-2 was not consistent between patients. Fig. 3 shows an example in which coculture had no effect on IL-4 levels or early IL-2 and TNF-α levels. As seen with the tumor-associated T cells, IL-6 was produced by both the activated peripheral blood T cells and TAFs, and these levels were greatly magnified when the two cell types were cultured together. IL-10 was significantly enhanced at days 1, 3, and 5 in the cocultures of autologous patient peripheral blood T cells and lung TAFs. These results establish that the enhancing effect of TAFs is not restricted to tumor-associated T lymphocytes.

**TAFs augment early T lymphocyte cytokine production in a cell dose-dependent fashion**

To further explore the effect of TAF numbers on the activation of T lymphocytes, a constant number of monocyte-depleted normal donor PBMCs were activated with anti-CD3/CD28 Dynabeads in the presence of increasing numbers of TAFs. To ensure adequate numbers of T cells to complete these studies, normal donor PBLs were used as a source of T cells. Unstimulated coculture wells were included with each experiment to rule out cytokine production due to a response to alloantigens. IL-17A and IFN-γ were detected at levels that were not increased in the cocultures at days 1 and 3 for all cell doses, the enhancement was lost at later time points (Fig. 4C). The more fibroblasts that were present, the more quickly the levels of IFN-γ and IL-17A fell to below that of the PBLs alone. This reversal occurred for IFN-γ before it did for IL-17A. These data show that when an early time point is used (day 1), fibroblasts over a range
of different cell numbers all augment the IFN-γ and IL-17A production by T lymphocytes in dose-dependent fashion. However, at later time points such as day 3, the dose-response of fibroblasts on T cell cytokine production begins to level out. At late time points (day 5), less IFN-γ is present in the fibroblast and lymphocyte cocultures, compared with the lymphocytes alone. The effect of fibroblasts on IL-17A levels at day 5 of the coculture depends upon the starting number of fibroblasts. These studies used allogeneic PBL which includes other cell types besides T cells, which may be contributing to the observed effects. However, a net enhancement in IFN-γ and IL-17A is still initially observed, as was seen with the autologous cocultures of purified T cells and TAFs. Therefore, we conclude that the kinetics and cell number must be taken into account when interpreting data regarding the effect of fibroblasts upon T lymphocyte activation.

Enhancement of IFN-γ and IL-17A is mediated by a soluble factor produced by fibroblasts

To characterize the fibroblast-associated factor that enhanced activated T lymphocyte production of IFN-γ and IL-17A, experiments were designed to determine if a soluble factor was involved using Transwell plates (Corning). TAFs were separated from autologous tumor-associated T cells present in the CD45+ enriched mixed population of cells by a cell-impermeable polycarbonate membrane that permitted free exchange of soluble proteins through 0.4-μM pores. Under these culture conditions, a fibroblast-dependent significant enhancement of cytokine production by activated tumor-associated T cells was observed (data not shown). These results were consistent with the notion that the fibroblast enhancement of the T cell response did not require a direct contact between the cells, and was thus likely to be mediated by a soluble factor produced by the fibroblasts. However, because these experiments were conducted with T cells that were present in a mixed population of cells that may have included some fibroblasts—and because the culture media contained FBS, which includes cytokines to which human cells can respond (17)—additional experiments were designed to establish that the enhancement was indeed due to a soluble fibroblast-produced factor.

![FIGURE 2.](image-url) CD3+ purified tumor-associated T cell IFN-γ and IL-17A production is enhanced by autologous lung TAFs. Purified tumor-associated T cells (1 × 10⁶) were activated with anti-CD3/CD28 beads in the presence or absence of 2 × 10⁵ autologous lung TAFs in serum-free AIM-V media. Levels of IFN-γ, IL-17A, IL-4, IL-2, IL-6, IL-1β, TNF-α, and IL-10 were measured over a 5-d period by cytometric bead array. The p values compare triplicate wells of the coculture of T cells and fibroblasts (black bars) versus either cell type alone.
TAFs were cultured in serum-free AIM-V media for 5 d. These fibroblast-conditioned media were found to be free of IFN-γ and IL-17A. Monocyte-depleted normal donor PBLs were then activated with anti-CD3/CD28 Dynabeads in the presence or absence of varying concentrations of fibroblast-conditioned media. PBLs activated in the presence of TAF-conditioned media produced more IFN-γ (Fig. 5A) and IL-17A (Fig. 5B) than did PBLs activated without fibroblast-conditioned media. Both IFN-γ and IL-17A levels were directly correlated with the percentage of fibroblast-conditioned media in the well, with higher concentrations of fibroblast-conditioned media leading to higher levels of IFN-γ and IL-17A. These results confirm that a TAF-derived soluble factor is able to enhance IFN-γ and IL-17A cytokine production of anti-CD3/CD28 bead-activated T lymphocytes in a dose-dependent fashion.

TAF-produced IL-6 contributes to IFN-γ and IL-17A augmentation

Fibroblast cultures derived from human lung tumors constitutively produce a variety of different biologically active factors, including cytokines and chemokines (10). Among these biological factors produced by fibroblasts, we have focused on the effects of two cytokines, IL-6 and TGF-β1, which have been demonstrated to influence the generation of IL-17A-producing Th17 cells (18). To
test the possibility that IL-6 was the fibroblast-produced factor that was responsible for the observed enhancement of IL-17A. TAFs were cultured for 5 d in serum-free AIM-V to generate fibroblast-conditioned media. Monocyte-depleted PBLs were cultured in fibroblast-conditioned media diluted to 90, 45, or 11% in AIM-V media with or without 5 μg/ml of anti–IL-6 neutralizing Ab. The T lymphocytes in the monocyte-depleted PBL cultures with fibroblast-conditioned media were activated with anti-CD3/CD28 Dynabeads, and the cytokine levels were monitored 3 d after activation. The anti–IL-6 Ab significantly decreased the

FIGURE 4. Dose response of lymphocytes to TAFs. IFN-γ (A–C) and IL-17A (D–F) response of 7 × 10⁴ activated normal donor monocyte-depleted PBLs to increasing numbers of allogeneic lung TAFs on days 1 (A, D), 3 (B, E), and 5 (C, F). The p values represent statistical significance of fibroblast addition compared with the PBL alone of triplicate wells for one of three independent experiments.

FIGURE 5. Fibroblast-produced soluble factor responsible for enhancement of T lymphocyte activation. TAFs were allowed to condition serum-free AIM-V media for 5 d. Media were then serially diluted and added to anti-CD3/CD28 bead-activated 5 × 10⁵ monocyte-depleted normal donor PBLs, and the concentration of IFN-γ (A) and IL-17A (B) was measured by ELISA. No IFN-γ or IL-17A was detected in the fibroblast-conditioned media prior to addition to lymphocytes. Day 3 of culture shown. The p values represent statistical significance of conditioned media treatment compared with fresh AIM-V media of triplicate wells for one of three independent experiments.
enhanced production of IL-17A that was observed with the 90% TAF-conditioned media (Fig. 6B). A decreased enhancement was also observed for IFN-γ when IL-6 was neutralized in the fibroblast-conditioned media (Fig. 6A). The effect of anti–IL-6 on IFN-γ enhancement was observed at all three levels of TAF-conditioned media (90, 45, and 11%). Similar results were found for conditioned-media made by fibroblasts derived from three different patients paired with three different donors of PBL. These results establish that IL-6 produced by the fibroblasts contributes to the observed fibroblast enhancement of the T cell activation.

Neutralization of TGF-β1 does not decrease IFN-γ and IL-17A levels of T cells activated in TAF-conditioned media

TGF-β1, like IL-6, is a cytokine produced by fibroblasts that contributes to the polarization of Th17 subset. Therefore, it was possible that the fibroblast-conditioned media contained TGF-β1 that could be contributing to the development of Th17 cells and subsequent levels of IL-17A. CD3+ T cells were activated with anti-CD3/CD28 beads in TAF-conditioned media in the presence or absence of neutralizing anti–TGF-β1. Although the TAF-conditioned media enhanced the levels of IFN-γ and IL-17A made by the T cells over that of the cells activated in fresh AIM-V, neutralization with either of two TGF-β1 Abs (MAB240 or AF-101-NA) failed to reduce the levels of either IFN-γ (Fig. 6C) or IL-17A (Fig. 6D). Both Abs had no effect on the levels of IFN-γ (Fig. 6C). Contrary to what would be expected if TGF-β1 were contributing to Th17 development, MAB240 blockade of TGF-β1 actually increased the levels of IL-17A (Fig. 6D) in our cultures of T cells activated in TAF-conditioned media. The other neutralizing Ab, AF-101-NA, had no effect on IL-17A levels of the T cells activated in TAF-conditioned media (Fig. 6D). These results fail to support a role for TGF-β1 in enhancing the production of IFN-γ or IL-17A by the T cells activated in TAF-conditioned media.

TAF-derived factors augment T cell proliferation

Although neutralization of IL-6 reduced the fibroblast-conditioned media–induced enhancement of IFN-γ and IL-17A levels produced by activated T cells, it did not bring it completely down to the level of the T cells activated in fresh media. Thus, we hypothesized that other mechanisms may also be involved in the modulation of T cell activation. One such possibility would be that fibroblasts were influencing the proliferation of activated T cells. Th cells purified from normal donor monocyte-depleted PBLs...
were labeled with CFSE and activated with plate-bound CD3/CD28 Abs for 4 d. CFSE dye dilution was analyzed in the entire ungated population as an indicator of cellular proliferation. The T cells that were exposed to TAF-conditioned media (Fig. 6E) for the duration of the experiment contained a greater fraction of cells that had undergone CFSE dye dilution (i.e., proliferation) than the population of cells that were activated in fresh AIM-V (Fig. 6E). The results show that at this day 4 time point a greater percentage of cells have undergone division when activated in the presence of TAF-conditioned media. No proliferation was evident when T cells were cultured in TAF-conditioned media without CD3/CD28 Abs (data not shown). From these experiments, we conclude that it is possible that part of the increase in T cell-produced IFN-γ and IL-17A levels observed when these cells are activated in TAF-conditioned media may be due to an increase in cell numbers present.

**T lymphocytes produce soluble factors that enhance fibroblast production of IL-6**

Having established that the TAFs produced a factor responsible for the enhanced production of IFN-γ and IL-17A by activated T cells, we next examined whether the CD3/CD28-activated T cells could be producing a factor that would directly affect cytokine production by the TAFs. To address this question, monocyte-depleted peripheral blood T lymphocytes were activated with anti-CD3/CD28 Dynabeads in serum-free AIM-V media for 1 or 3 d. The supernatants from these cultures (activated T cell-conditioned media) were then added to cultures of TAFs, and their effect upon the fibroblast production of IL-6 was monitored after 3 d in culture. The day 1 T cell-conditioned media significantly enhanced the fibroblast production of IL-6 in a dose-dependent fashion (Fig. 7A). No IL-6 was detected in the day 1 T cell-conditioned media. A much greater increase in the TAF production of IL-6 was seen when the fibroblasts were cultured with day 3 activated T cell-conditioned media (Fig. 7B). We conclude from these results that T cells and fibroblasts reciprocally and positively interact to regulate each other’s activity by the production of soluble factors.

**Nonmalignant fibroblasts and T cells from autologous normal donor peripheral blood exhibit a reciprocal enhancing effect similar to that observed in cocultures of TAFs and tumor-associated T cells**

All of the data presented above use TAFs or conditioned media generated by TAFs. To determine whether the enhancement of T cell activation by fibroblasts was a property unique to TAFs, autologous cocultures of skin fibroblasts with PBMCs were performed. With the use of two different ratios of PBLs to fibroblasts (4:1 and 1:1), the presence of the nonmalignant fibroblasts was found to enhance T cell-produced IFN-γ (Fig. 8A). IL-17A was also significantly enhanced in the skin fibroblast/PBL cocultures (Fig. 8B). These data suggest that fibroblasts derived from both a malignant source (non-small cell lung carcinoma, Fig. 1) and a nonmalignant source (skin, Fig. 8) have the ability to augment T cell–fibroblast coculture IFN-γ and IL-17A cytokine levels. Whereas IFN-γ has been shown by flow cytometry to be produced in low quantities by a small percentage of fibroblasts (10), the cocultures of T lymphocytes and fibroblasts only yielded detectable amounts of IFN-γ by ELISA when T cell-activating anti-CD3/CD28 Dynabeads were used. IL-17A is believed to be a lymphocyte-specific cytokine. These data are consistent with the notion that nonmalignant fibroblasts, such as fibroblasts derived from the tumor microenvironment, have the capacity to act upon T cells to enhance their response to activation via CD3/CD28.

Because TAFs were able to modulate the cytokine production of T cells without the need for accessory cells, we next set out to verify that nonmalignant fibroblasts similarly do not require an accessory cell to cause enhancement of autologous T lymphocyte cytokine production. T cells purified from the blood of a normal donor were cultivated in the presence or absence of skin-derived fibroblasts (striped bars) and PBLs activated alone (white bars).
fibroblasts from the same normal donor, and the supernatants were assayed to quantify the expression of a panel of cytokines by cytometric bead array. As seen for the tumor-associated cocultures, cocultures of skin fibroblasts with autologous CD3+ T lymphocytes demonstrated significantly enhanced levels of IFN-γ and IL-17A (Fig. 9). Similar to what was observed with the TAFs, we found a comparatively low level of IL-6 produced by fibroblasts cultured alone, and IL-6 was significantly increased when these cells were cultivated with activated T cells (Fig. 9). Statistically significant increases were also observed for IL-2, IL-4, and IL-10 in normal donor fibroblast–T cell cocultures. We conclude from these studies that fibroblasts from nonmalignant sources are able to directly modulate the cytokine production of activated T cells in a fashion similar to TAFs.

Cytokine production by both activated CD4+ and CD8+ cells is enhanced by fibroblast-secreted products

Fibroblasts from both malignant and nonmalignant sources were able to augment the cytokine production of T cells derived from tumors, the peripheral blood of cancer patients, and the blood of normal donors. Next, we sought to determine if this fibroblast-mediated enhancement was restricted to certain types of T cells. Normal donor peripheral blood cells were sorted into CD4+ or CD8+ fractions. Fibroblasts from tumors, lung tissue, or skin were grown in AIM-V media for 5 d, and the resultant culture medium was collected. These conditioned fibroblast media were applied to the purified CD4+ or CD8+ cells activated with anti-CD3/CD28 beads. On day 3, the IFN-γ and IL-17A levels were assayed by ELISA. The CD4+ cells produced more IFN-γ (Fig. 10A) and IL-17A (Fig. 10B) than CD8+ cells.
elicit T lymphocyte production of IFN-γ without an additional activating stimulus, such as anti-CD3/CD28, even when allogeneic cells are used. Stimulation of T cells with anti-CD28 without anti-CD3 did not result in the production of IFN-γ or IL-17A even if the cells were cultivated with allogeneic fibroblasts. However, when a T cell-activating stimulus is present (such as anti-CD3 with anti-CD28), fibroblasts are able to modulate the T cell response and serve as an immunostimulatory cell.

Because we have observed that the immunoenhancing effect is lost—or even reversed—with increasing numbers of cells in the fibroblast–T cell coculture and with prolonged time in culture, we speculate that this apparent loss of immunostimulation could be the result of a depletion of nutrients and/or a severe drop in pH in the media (i.e., an in vitro culture artifact). It is also possible that this loss in immunostimulatory ability could be due to the consumption of cytokines by or death of either cell type. The activated T cells could be producing products such as granzyme B that may be toxic to the fibroblasts. Alternatively, the T cells may be succumbing to activation-induced cell death over time. In contrast to studies reported in this paper, where we titrate the number of cells and focus on early (3–4 d) time points and consistently see enhancement, previous studies showing either no effect or a suppressive effect of fibroblasts have used higher numbers of cells and looked at later time points (25–27).

Two of our early key findings were that the fibroblasts were acting directly upon the T cells to enhance their production of IFN-γ and IL-17A, and that this effect was due to a soluble factor produced by the fibroblasts. These early findings led us to discover that IL-6 in fibroblast-conditioned media was much more stimulatory than IL-17A produced by the fibroblasts. From these findings came the recognition that a positive stimulatory reciprocal loop exists between the two cell types. Because IL-6 is required for generating IL-17A–producing Th17 cells (17, 18, 28, 29) and IL-17A has been shown to stimulate the production of IL-6 by fibroblasts (30, 31), the mutual enhancement of these two cytokines in the coculture experiments was not unexpected. However, we did not immediately anticipate that IL-6 would also contribute to the significant increase in IFN-γ production by the T cells. One possible explanation may lie in the fact that IL-6 is known to inhibit Foxp3 (32, 33), the transcription factor associated with Tregs, which inhibit the activation of other T cell subsets. Therefore, the presence of IL-6 in T cell–fibroblast cocultures might reduce the number or function of Tregs and thus increase the activation potential and cytokine production of the rest of the T lymphocytes. Attempts to explore this possibility were confounded by the fact that human T lymphocytes transiently express Foxp3 upon activation (34, 35). Previous studies have demonstrated that IL-6 can act upon cells to reduce apoptosis (36). Therefore, an alternative possibility is that an increase in cell survival (and/or decrease in activation-induced cell death) may be contributing to the observed increase in both IFN-γ and IL-17A in the fibroblast–lymphocyte cocultures. It is of interest that although the depletion of IL-6 from the fibroblast-conditioned media significantly reduced the observed enhancement in the T cell cultures, it did not result in a complete elimination of the effect. Our CFSE dye dilution analysis suggests that fibroblast-derived soluble factors lead to an increased fraction of T cells that have undergone cell division after activation. Further studies are necessary to clarify whether this represents an increased proliferation rate and/or the result of an improved cell survival.

Given the recognized heterogeneity of fibroblasts and the rather distinct phenotype that is associated with TAFs (10, 11, 37), we
were expecting the immunoenhancing effects we observed with TAFs to be limited to cells from the tumor microenvironment. On the contrary, we established that fibroblasts derived from a nonmalignant tissue exhibited the same immunoenhancing effects when cultivated with activated lymphocytes derived from the peripheral blood of the same normal donor. We conclude that the reciprocal relationship of fibroblasts and T cells is not restricted to the cells in tumor microenvironments, but rather is a more general effect that is applicable to both cells derived from neoplastic tissues and cells derived from nonmalignant and noninflammatory microenvironments. Indeed, others have suggested that the phenotype of fibroblasts in tumor microenvironments does not differ greatly from that of resident fibroblasts in the surrounding tissue (38). This observation neither supports nor refutes the possibility that the observed immunostimulation is mediated by a phenotypically distinct subset of fibroblasts. In addition, our results do not gainsay the possibility that, in addition to an immunostimulatory subset of fibroblasts, other phenotypically distinct immunosuppressive fibroblasts may be present in normal and/or neoplastic tissues. With respect to the latter, we have previously shown the existence of TAFs that express the ligands for PD-1 (B7H1 and B7DC) (10), coregulatory molecules capable of binding to PD-1 present on T cells and suppressing their activity (39, 40).

In view of our demonstrated ability of fibroblasts to enhance T cell cytokine production and proliferation, and because many of these cells are present in most solid human tumors, these cells represent a potential target for therapeutic applications for cancer. The potential for developing novel cancer therapeutic strategies that are based upon modulation of fibroblasts has been suggested by others (41). Clinical trials have already been initiated that 1) attempt to decrease fibroblast recruitment by targeting chemotactic signals from tumor cells; 2) inhibit fibroblast-dependent mechanisms that initiate and promote tumor growth and that facilitate invasion and metastasis; and 3) deplete fibroblasts from the tumor to abolish all fibroblast activities.

Our results show that TAFs are able to directly alter T lymphocyte activation in terms of IFN-γ and IL-17A expression when a strong CD3/CD28 stimulus is provided without need for any other cell type, and autologous T lymphocytes from both peripheral blood and the tumor microenvironment can activate TAFs to produce elevated amounts of IL-6. However, this cellular crosstalk is not unique to the tumor microenvironment. Nonmalignant fibroblasts derived from skin and fibroblasts derived from healthy lung also enhance IFN-γ and IL-17A by stimulated T lymphocytes. Therefore, our findings may be applicable to other fibroblasts as well and have been shown for rheumatoid arthritis fibroblast-like synoviocytes (14). We also suspect that fibroblast-like mesenchymal stem cells cultured in a similar manner may show a similar augmentation of activated T cells. Fibroblasts are important for the repair of damaged tissues, and thus may express a universal repair program that includes the stimulation of inflammation through increases in trafficking of immune cells, angiogenesis of endothelial cells, and increases in the levels of inflammatory cytokines produced by T lymphocytes. Fibroblasts increase the activation potential of T lymphocytes, whereas T lymphocytes are also able to enhance the activation of fibroblasts. This stimulatory cell loop likely occurs between the fibroblasts and T cells present in the tumor microenvironment in which chronic inflammation is perpetuated until T lymphocytes become exhausted and are rendered unresponsive to tumor Ag. However, it is important to recognize that the CD3/CD28 stimulus provided in these experiments is of differing strength from the physiologic stimulation provided by microenvironments such as the tumor where T cells may be actively suppressed. Given the very complex, and as yet poorly defined, potential of fibroblasts to modulate lymphocyte function, as well as the ability of these cells to augment a cytokine IFN-γ that is known to suppress tumor progression and a cytokine IL-17A that may enhance tumor progression, considerable care must be observed in attempting to design strategies that target this cell type to modulate T cell function in the tumor microenvironment or elsewhere in normal tissues for therapeutic interventions.

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Disclosures

R.B.B. is president of Therapyx.

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