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*J Immunol* 2007; 178:5552-5562; doi: 10.4049/jimmunol.178.9.5552

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Characterization of Human Lung Tumor-Associated Fibroblasts and Their Ability to Modulate the Activation of Tumor-Associated T Cells¹

Michael R. Nazareth, Lori Broderick, Michelle R. Simpson-Abelson, Raymond J. Kelleher, Jr., Sandra J. Yokota, and Richard B. Bankert²

The tumor microenvironment of human non-small cell lung cancer (NSCLC) is composed largely of stromal cells, including fibroblasts, yet these cells have been the focus of few studies. In this study, we established stromal cell cultures from primary NSCLC through isolation of adherent cells. Characterization of these cells by flow cytometry demonstrated a population which expressed a human fibroblast-specific 112-kDa surface molecule, Thy1, α-smooth muscle actin, and fibroblast activation protein, but failed to express CD45 and CD11b, a phenotype consistent with that of an activated myofibroblast. A subset of the tumor-associated fibroblasts (TAF) was found to express B7H1 (PD-L1) and B7DC (PD-L2) constitutively, and this expression was up-regulated by IFN-γ. Production of cytokines and chemokines, including IFN-γ, monokine induced by IFN-γ, IFN-γ-inducible protein-10, RANTES, and TGF-β1 was also demonstrated in these cells. Together, these characteristics provide multiple opportunities for the TAF to influence cellular interactions within the tumor microenvironment. To evaluate the ability of TAF to modulate tumor-associated T cell (TAT) activation, we conducted coculture experiments between autologous TAF and TAT. In five of eight tumors, TAF elicited a contact-dependent enhancement of TAT activation, even in the presence of a TGF-β1-mediated suppressive effect. In the three other tumors, TAF had a net suppressive effect upon TAT activation, and, in one of these cases, blockade of B7H1 or B7DC was able to completely abrogate the TAF-mediated suppression. We conclude that TAF in human NSCLC are functionally and phenotypically heterogeneous and provide multiple complex regulatory signals that have the potential to enhance or suppress TAT function in the tumor microenvironment. *The Journal of Immunology, 2007, 178: 5552–5562.*

Lung tumors are the leading cause of cancer deaths in the United States (1). Human non-small cell lung cancers (NSCLC),³ including squamous cell carcinomas, large cell carcinomas, and adenocarcinomas, are the most prevalent type of lung tumor, and account for 80–90% of all lung cancer cases. Unfortunately, while lung cancer research progresses at a rapid pace, we are still short of understanding the causes and progression of this devastating disease. Typically, human tumors develop slowly over a prolonged period of time and they often progress and spread despite the presence, within the tumor microenvironment, of large numbers of CD4⁺ and CD8⁺ T cells with activated or memory cell phenotypes. The T cells in the microenvironment of human non-small cell lung tumors are hyporesponsive to stimulation via the TCR under conditions that fully activate T cells derived from the peripheral blood of normal donors or from cancer patients (2). A combination of factors both intrinsic and extrinsic have been identified that may contribute to the T cell hyporesponsiveness and to their inability to kill tumor cells (3). The hyporesponsiveness of memory T cells in the human NSCLC microenvironment was found to be partly due to TGF-β1 that is produced by and is present on cells within the tumor microenvironment (4). The arrest of T cell function was shown to be reversible both in vitro and in vivo by IL-12 (2).

Considerable effort has been given to identifying cells in the tumor microenvironment that are responsible for the nonresponsiveness of the tumor-associated T cells (TAT). This effort has been focused largely upon the tumor cells themselves, and has established that both soluble and membrane-associated factors have the capacity to suppress the antitumor response of T cells (5–7). Several different nonmalignant cell types within the tumor microenvironment have also been identified to have potent immunosuppressive effects upon TAT. These include subsets of both myeloid and plasmacytoid dendritic cells (8–10) and CD4⁺CD25⁺ regulatory T cells (11, 12). Another nonmalignant tumor-associated cell with potential immunomodulatory effects is the fibroblast. These cells are an extremely heterogeneous and multifunctional cell population that often constitute a majority of cells within the tumor microenvironment (13). Therefore, it is puzzling why this population of cells has received comparatively little attention as a potential modulator of TAT function (14).

Through their production of cytokines, chemokines, and other biologically active factors, it is known that fibroblasts play a significant role in attracting and retaining inflammatory leukocytes within sites of chronic inflammation (15). Fibroblasts in inflammatory
TAF and TAT isolation

A single-cell suspension was created from the tumor tissue by mechanical disruption of the tissue using a Teflon policeman to force cells through a size 50 mesh. Cell suspensions were layered over Ficoll-Paque Plus (Amersham Biosciences) and the resulting interface was removed and centrifuged at 1500 × g, and washed once in PBS. The interface, an enriched mononuclear cell population of CD45^+^ TAT, was resuspended in a freezing medium consisting of human serum albumin-PBS-DMSO (8:1:1) and was frozen at −80°C in a “Mister Frosty” slow freezing device before being transferred to liquid nitrogen storage tanks 1 day later. The pellet remaining after the Ficoll-Paque Plus separation, along with the tumor tissue, was put into culture in 6-well tissue culture treated plates to grow fibroblasts. Within 1–2 wk, confluent fibroblasts were removed by treatment with trypsin/EDTA for 5–10 min and seeded into tissue culture flasks. All fibroblast cultures were maintained in standard culture medium and were characterized and used in coculture experiments after four or five passages.
Table I. Summary of the histological diagnoses for each of the human non-small cell lung tumors from which TAF cultures were derived

<table>
<thead>
<tr>
<th>Histological Type of Tumor</th>
<th>Human Lung Tumor Patient Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>1, 2, 4, 6, 7, 12, 17, 18, 19, 26</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>3, 5, 8, 13, 16, 21, 22, 24, 25</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>15, 20</td>
</tr>
<tr>
<td>Bronchioloalveolar carcinoma</td>
<td>11</td>
</tr>
<tr>
<td>Unknown type of NSCLC</td>
<td>9, 14</td>
</tr>
<tr>
<td>Carcinoid tumor</td>
<td>10</td>
</tr>
<tr>
<td>Metastatic non-small cell</td>
<td>23</td>
</tr>
<tr>
<td>Lung carcinoma</td>
<td></td>
</tr>
<tr>
<td>Small cell lung carcinoma</td>
<td>27</td>
</tr>
</tbody>
</table>

Patient peripheral blood was also separated by centrifugation over a layer of Ficoll-Paque Plus. The interface was washed once in PBS and frozen in the same manner as the tumor-associated mononuclear cells.

**Phenotyping the primary fibroblast cultures**

Fibroblasts after four or five passages were isolated from culture flasks by treatment with trypsin/EDTA for 5–10 min. Fresh medium was then added to quench the trypsin and the cells were pelleted by centrifugation. Mouse IgG (for directly conjugated Abs) or goat IgG (for unconjugated Abs) was used to block nonspecific binding before the addition of the following primary mouse anti-human Abs: SM1214P (unconjugated) (Acris Antibodies), CD45 (FITC or allophycocyanin conjugated; BD Biosciences), HLA-A, -B, -C (class I; allophycocyanin conjugated; BD Biosciences), Thy1/CD90 (allophycocyanin conjugated; BD Biosciences), and fibroblast activation protein (FAP; unconjugated) (Calbiochem; EMD Biosciences). After incubation on ice, cells were washed in PBS and then pelleted by centrifugation. Goat anti-mouse Alexa 488 was then added to samples that contained unconjugated primary Abs, and the cells were incubated on ice, washed once in PBS, and then pelleted by centrifugation. A Fix and Perm Cell Permeabilization kit (Catag Laboratories; Invitrogen Life Technologies) was used to perform intracellular staining for α-smooth muscle actin (α-SMA; PE conjugated; R&D Systems), and mouse IgG was used as a block during the permeabilization steps. After completion of cell staining, all samples were fixed in 2% ultrapure formaldehyde. Flow cytometry was performed using a FACS Calibur flow cytometer (BD Biosciences; University at Buffalo, State University of New York) and analyzed in our laboratory by WinList software (Verity). Appropriate isotype control Abs were acquired from BD Biosciences and the results reflect the percentage of positive cells compared with the isotype controls.

**Assessment of TAF cytokine production**

Production of IFN-γ, MIG, IP-10, RANTES, IFN-α, TGF-β1, and IFN-β by TAF was assessed by intracellular flow cytometry and ELISAs as on the TAF culture supernatants. Intracellular flow cytometry was performed as described above and the following mouse anti-human Abs were used at a concentration of 1 μg/assay: IFN-γ, MIG, IP-10, RANTES (PE conjugated; BD Biosciences), and IFN-α (PE conjugated; ChromoProbe). Appropriate isotype controls for each of the Abs were also purchased from BD Biosciences. Results were analyzed in our laboratory by WinList software (Verity) and reflect the percentage of positive cells compared with the isotype controls. A human TGF-β1 Quantikine ELISA kit was acquired from R&D Systems (to assess TAF production of TGF-β1, and ELISAs for the presence of human IFN-γ, MIG, and IP-10 in the TAF culture supernatant fluids were also performed, using Abs from Endogen and 96-well plates from Nunc, on the TAF culture supernatant fluids.

**TAF expression of coregulatory molecules**

Fibroblasts were isolated from culture flasks as described above and stained with PE-conjugated primary Abs (eBioscience) that bind to the following B7 family coregulatory molecules: B7H1 (PD-L1), B7DC (PD-L2), B7H2 (B7h, B7RP1), B7H3, B7H4 (B7S1, B7x), PD-1, and inducible costimulator. All of the Abs were mouse anti-human, except for the B7H3 Ab, which was rat anti-mouse, though this particular Ab is also cross-reactive with human B7H3. Flow cytometry and analysis were performed as previously described.

**Immunohistochemistry for TGF-β1 in tumor tissue sections**

Immunohistochemistry was performed on fixed tissue sections of original lung tumors to assess the presence of TGF-β1. Fresh pieces of tumor were processed by the University at Buffalo Histology Service Laboratory as described above and stained with PE-conjugated primary Abs (eBioscience) that bind to the following B7 family coregulatory molecules: B7H1 (PD-L1), B7DC (PD-L2), B7H2 (B7h, B7RP1), B7H3, B7H4 (B7S1, B7x), PD-1, and inducible costimulator. All of the Abs were mouse anti-human, except for the B7H3 Ab, which was rat anti-mouse, though this particular Ab is also cross-reactive with human B7H3. Flow cytometry and analysis were performed as previously described.

**FIGURE 2.** Flow cytometry for fibroblast surface markers to define their phenotype and coregulatory molecule expression. A, Primary cultures of TAF were stained extracellularly with the human fibroblast-specific SM1214P Ab, HLA-A, -B, -C (class I), CD45, CD11b/Mac-1, or Thy1/CD90. B, They were also stained for FAP, α-SMA, B7H1 (PD-L1), or B7DC (PD-L2) both before and after 24 h treatment with IFN-γ. In the representative histograms, the unshaded region represents the isotype control or autofluorescence and the shaded region represents the human-specific Ab tested. The percentage of positive cells is shown for each Ab tested.
previously described and numerous serial sections of paraffin-embedded tissue blocks were generated on glass slides for use with immunohistochemistry protocols. A DakoCytomation HRP kit (DakoCytomation) and an unconjugated mouse anti-human TGF-β1 mAb (BioSource International; Invitrogen Life Technologies) were used to stain different tumors, and an appropriate isotype control was also run on a serial section. The slides were assessed by light microscopy and the expression of TGF-β1 was monitored in the stroma, tumor cells, fibroblasts, and lymphocytes in the section. Images were taken with a Sony color video camera CCD SSC-S20 mounted on an Olympus BX40 light microscope using Snappy Video Snapshot 4.0 software (Play).

**Coculture experiments of autologous TAF and TAT**

Coculture experiments were performed between eight autologous sets of TAF and TAT. Most autologous pairs were not available due to the inability to culture TAF from every tumor, the limited amount of TAT due to a lack of sufficient tumor tissue, and the use of TAT for other experiments. Five of the eight TAT used in these experiments were analyzed by FACS for their expression of CD3, CD4, CD8, and CD20. The percentage of CD3+ T cells in these populations ranged from 15 to 33%, and included both CD4+ (6–21%) and CD8+ (3–15%) T cells. In four of the cell preparations, the percentage of CD20+B cells was 1.5% or below. A more complete phenotypic analysis of two of the TAT populations revealed that the T cells had a phenotype of memory cells, i.e., CD3+, CD45RO+, CXCR3+, CD11a+, CD44+, IL-12Rβ7+, CD28+, and CD62L+. Previous studies from our laboratory have more extensively characterized the phenotype of T cells isolated from six different NSCLC (including three of the major histologic types) and showed them to express a similar phenotype that is associated with effector memory T cells, i.e., CD3+, CD45RO+, CXCR3+, CD11a+, CD44+, IL-12Rβ7+, CD28+, and CD62L+ with a subset expressing CD161 (23, 24). Less than 1% of the cells derived from the tumor tissues were CD56+ and monocytes ranged from 0.4 to 8.1% of the cells in the TAT population. TAF were split into 12-well tissue culture-treated plates and grown to confluence in DMEM/F12 plus 10% FCS medium. The population of enriched CD45+ mononuclear cells containing the TAT was thawed and added to the wells, along with 20 µl of DynaBeads coated with anti-CD3 and anti-CD28 (T Cell Expander kit; Dynal Biotech; Invitrogen Life Technologies). The total volume in each well was 2 ml and fresh medium was added back at each time point to maintain the same volume throughout the experiment. In some experiments, additional factors were added to the coculture conditions, including anti-TGF-β1 function blocking Ab (5 µg/ml; R&D Systems), recombinant human TGF-β1 (10 ng/ml; R&D Systems), anti-B7H1 (5 µg/ml; eBioscience), or anti-B7DC (5 µg/ml; eBioscience). Aliquots of each well were taken at days 3, 5, and 7, and the various factors that were being tested were also added, in addition to medium, on days 3 and 5.

Coculture experiments were also performed in the same manner with Transwell plates (Corning; Costar). To maintain cell separation, a polyester membrane with a 0.4-µm pore size was used. TAF were grown to confluence in the lower chamber and TAT and DynaBeads were added to either the upper or lower chamber. At the start of the coculture experiments, 1 ml of medium was added to the upper chamber and 1 ml to the lower chamber. Aliquots were taken from both the upper and lower chambers at the various time points. All other conditions, including other factors added to the conditions, were the same as for the regular 12-well plates, as described previously. Culture samples were assayed for IFN-γ production by ELISA as previously reported (25).

**Results**

**Establishment of primary TAF cultures**

The human non-small cell lung tumor microenvironment is a complex tissue involving dynamic interactions between a number of cellular and noncellular components (Fig. 1, A and B). Tumor cells are characterized by their large irregular shape with heterochromatic nuclei. Stromal cells including lymphocytes, plasma cells, blood vessels and fibroblasts are labeled in the sections (Fig. 1). The fibroblast is one of the major cellular constituents of the tumor microenvironment in tumors of the breast, colon, stomach, and pancreas (13). To demonstrate the predominance of fibroblasts in our lung tumor specimens, a trichrome stain, for collagen and fibroblasts, was applied to NSCLC tissue sections. The dominant presence of fibroblasts and ECM and their juxtaposition to the tumor cells in all of the NSCLC studied was clearly established in the trichrome images (Fig. 1, C and D).

To characterize and study the role of TAF in the lung tumor microenvironment, we established primary cell cultures directly from 27 different lung tumor biopsy tissues. The adherent cells, which possessed a fibroblast-like morphology (i.e., adherent, flat, elongated cells with cytoplasmic extensions and flat, oval-shaped nuclei) were successfully cultivated from NSCLC tissue including adenoc, squamous, and large cell carcinomas. Table 1 shows the histological diagnosis for each of the 27 tumors from

**FIGURE 3.** TAF constitutively produce IFN-β, IFN-γ, and IP-10. The culture supernatant fluids of a number of TAF cultures was collected and assessed by ELISA for the presence of IFN-β, IFN-γ, IP-10, and MIG. TAF constitutively produced and secreted IFN-β, IFN-γ, and IP-10, although MIG (*) was below the level of detection in the collected supernatant fluids.

**FIGURE 4.** TAF constitutively produce IFN-γ, IP-10, MIG, and RANTES intracellularly. Cells from several TAF cultures were assayed for the presence of intracellular cytokines by flow cytometry. TAF constitutively produced IFN-γ, IP-10, RANTES, and low, but detectable, levels of MIG. The results presented are representative of three different cultures, that yielded average values of 14.3 ± 2.84% for IFN-γ, 67.62 ± 11.62% for IP-10, 8.05 ± 7.63% for MIG, and 40.66 ± 1.46% for RANTES.
which we derived primary TAF cultures. There were 26 non-
small cell tumors, including squamous cell carcinomas, adeno-
carcinomas, and large cell carcinomas, in addition to the 1 small
cell carcinoma. To confirm that these cells were indeed fibro-
blasts, and not contaminated with tumor cells or tumor-associated
leukocytes, the adherent cells were assayed by flow cy-
tometry and immunofluorescence microscopy using the
SM1214P Ab, which is specific for a 112-kDa molecule found
on the surface of human fibroblasts (26–28). As shown in Fig.
A, the adherent cells stained positively with the SM1214P Ab
by flow cytometry in each of the 15 primary cultures tested.
Immunofluorescence microscopy with the SM1214P Ab dem-

Table II. Constitutive presence of TGF-β1 detected in TAF culture
supernatant fluids by ELISA

<table>
<thead>
<tr>
<th>Primary TAF Culture Supernatant</th>
<th>TGF-β1 (pg/ml/24 h) as Detected by ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluid, Patient No.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>292.3</td>
</tr>
<tr>
<td>6</td>
<td>473.9</td>
</tr>
<tr>
<td>7</td>
<td>182.4</td>
</tr>
<tr>
<td>8</td>
<td>197.3</td>
</tr>
<tr>
<td>9</td>
<td>168.7</td>
</tr>
<tr>
<td>10</td>
<td>101.1</td>
</tr>
<tr>
<td>27</td>
<td>144.9</td>
</tr>
</tbody>
</table>

Characterization of TAF based upon their expression of other
cell surface markers

Fibroblasts found in normal tissues are known to be heterogeneous
with respect to their function and phenotype. One of the major
distinguishing cell surface phenotypic markers of a subset of hu-
man and murine fibroblasts is Thy1 or CD90 (29, 30). Thy1+ and
Thy1− subpopulations can be isolated from fibroblasts derived
from the orbit, spleen, female reproductive tract, and the lung (30–
34). Thy1+ and Thy1− fibroblasts differ in their ability to produce
cytokines and express MHC class II peptides, though they do not
differ in terms of growth rate. All 15 of the primary TAF cultures
tested were shown to stain positively for Thy1 by flow cytometry
(92–100%) (Fig. 2A), suggesting they have the potential to play an
active role in the tumor microenvironment.

We also assessed the expression of FAP, a serine protease that
can potentiate tumor growth, and α-SMA, a characteristic marker
associated with differentiated myofibroblasts that have been re-
ported to be present in other tumor microenvironments (35). Twen-
ty-three to 39% of the cells in the TAF cultures were positive for
FAP, while the majority of the fibroblasts (65%) were positive for
α-SMA (Fig. 2B). These results suggest that the fibroblasts from
the human lung tumor microenvironment are less heterogeneous
than fibroblasts derived from normal tissues and express proteins
found in other tumor microenvironments. Having established and
characterized primary cultures of human TAF that were not con-
taminated with other cell types, we set out to determine whether
the lung TAF express B7 family coregulatory molecules and pro-
duce biologically active molecules which have the potential to al-
ter lymphocyte function.

Table III. TGF-β1 detected in situ by immunohistochemistry in original
NSCLC tissue sections

<table>
<thead>
<tr>
<th>Histological Diagnoses of Tumor</th>
<th>TGF-β1 Expression as Detected by IHC*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fibroblasts</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>+</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>+</td>
</tr>
<tr>
<td>Squamous</td>
<td>+</td>
</tr>
<tr>
<td>Unknown type</td>
<td>−</td>
</tr>
<tr>
<td>Unknown type</td>
<td>−</td>
</tr>
<tr>
<td>Squamous</td>
<td>−</td>
</tr>
<tr>
<td>Unknown type</td>
<td>+</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>+</td>
</tr>
</tbody>
</table>

* There was no positive staining with a mouse IgG1 isotype control Ab.

FIGURE 5. Immunohistochemistry demonstrates the in situ presence of
TGF-β1 in non-small cell lung tumor-derived fibroblasts and ECM, but not
tumor cells or lymphocytes. A. A ×100 image of one representative tumor,
showing fibroblasts/ECM, tumor cells, and lymphocytes. B. Dense stain-
ing, indicating the presence of TGF-β1, is seen in this ×400 image of
fibroblasts/ECM. C. In this ×400 image of the tumor cells, there is no
positive staining for TGF-β1. D. As is the case with tumor cells, no stain-
ing for TGF-β1 is visible in this ×400 image of the tumor-associated
lymphocytes. Although not shown, a mouse IgG1 isotype control was also
run. There was no nonspecific staining with the isotype control Ab. Tu,
tumor cells; ly, lymphocytes; fb, fibroblasts.
either constitutively or inducibly following treatment with IFN-γ (data not shown). The lung TAF also did not express B7 family receptors, such as PD-1 or inducible costimulator (data not shown). The expression of B7H1 and B7DC on the TAF represents one potential mechanism by which fibroblasts in the tumor microenvironment could be regulating lymphocyte function. This possibility is addressed later.

TAF production and secretion of cytokines and chemokines

The expression of Thy1, shown to be associated with cytokine expression and cell differentiation, and B7 regulatory molecules suggests that the TAF could play an active role in the tumor microenvironment. Both intracellular expression using FACS and ELISA analysis of culture supernatants were used to evaluate cytokine production by TAF. Fibroblasts are known to produce type I IFN. Consistent with this finding, the TAF produced low levels of IFN-β (Fig. 3). Surprisingly, the lung TAF produced IFN-γ (Fig. 3), a type II IFN previously believed to be produced primarily if not exclusively by lymphocytes. Secretion of IP-10 by TAF was also observed (Fig. 3). The lung TAF were further assayed for chemokines production by FACS analysis. A significant percentage of TAF stained positively for IFN-γ (12.5%), RANTES (40.2%) and IP-10 (60.2%), and fewer cells were positive for MIG (6.2%) (Fig. 4). The presence of these chemokines in the TAF, particularly IP-10 and MIG, are considered significant, as they are responsible for attracting and retaining activated T cells into the tumor microenvironment (45).

In addition to the production of the proinflammatory cytokines and chemokines, the TAF were evaluated for production or immunosuppressive cytokines, specifically TGF-β1. This cytokine, shown to have potent immunosuppressive effects upon T cell function in the tumor microenvironment (46, 47), was produced by TAF, as shown in Table II. Although TGF-β was produced by all of the 10 different TAF tested so far, this cytokine was produced by only two of four NSCLC tumor cell lines that have been assayed and the amount produced by the tumor cell line was 2- to 5-fold less than that produced by TAF (data not shown).

Because the in vitro cultivation of the TAF and tumor cell lines may have influenced the cells ability to produce TGF-β, the fibroblasts and tumor cells were monitored for the expression of this cytokine before being put into culture. The in situ production of TGF-β1 by fibroblasts, tumor cells, and lymphocytes was assayed by immunohistochemistry of fresh lung tumor biopsy tissues. TGF-β1 was present in the fibroblasts or the ECM in seven of eight tumors tested (Table III). Images for a representative tumor on which immunohistochemistry for TGF-β1 was performed are...
shown in Fig. 5. A ×100 image is shown in Fig. 5A, which illustrates the microenvironment of this tumor, and higher magnification images (×400) from this section containing fibroblasts/ECM, tumor cells, and lymphocytes are shown in Fig. 5, B–D, respectively. In this particular tumor, positive staining for TGF-β1 was present in the fibroblasts and the ECM (Fig. 5B), but not in the tumor cells (Fig. 5C) or lymphocytes (Fig. 5D). Tumor cells stained positive for TGF-β1 in only two of the eight tumors tested, and no TGF-β1 staining of lymphocytes was observed in any of the tumors tested (Table III). No staining was observed in any of the tumors using an isotype control Ab (data not shown). These results, that fibroblasts in the tumor microenvironment rather than the tumor cells, are the major cell type producing TGF-β in addition to their close association of fibroblasts with lymphocytes, strongly support the potential of fibroblasts to modulate lymphocyte function within the microenvironment of human non-small cell lung tumors.

Coculture of TAF with TAT has an enhancing effect upon the response of the T cells to activation via the TCR

To establish whether fibroblasts had the capacity to modulate lymphocyte function, coculture experiments were initiated. CD45+ cells were enriched from single-cell suspensions derived from fresh human non-small cell lung tumor biopsy tissues. These cell suspensions, which contained CD3+CD4+ and CD3+CD8+ T cells with an effector memory phenotype (24), were frozen and stored in liquid nitrogen until primary TAF cultures were established from the same tumor biopsy tissues. At this point, the enriched CD45+ population of cells was thawed, and the response of the TAT to activation via the TCR was assessed either with or without cocultivation of the TAT with autologous TAF. Activation of the TAT within the CD45+ cell suspension was initiated by the persistent cross-linking of CD3 and CD28 on the TAT by anti-CD3/anti-CD28 Abs immobilized on synthetic beads. The response to this prolonged activation signal was monitored by quantifying the level of IFN-γ in the culture supernatant fluids (picograms per milliliter) at days 3, 5, and 7 following activation. In five of the eight coculture pairs tested, TAF enhanced the response of TAT to activation via the TCR. Fig. 6A depicts all five of the patient tumors in which TAF enhanced autologous TAT activation. The increase in the level of IFN-γ was observed at all three time intervals (i.e., days 3, 5, and 7). Each panel in Fig. 6A represents a single experiment with a pair of TAT and TAF derived from a different tumor. Although the amount of IFN-γ produced in response to TCR activation varied in each coculture, the increase in IFN-γ when TAF were present was observed in all five pairs of cells and this pattern was repeated at least twice with each of the cell pairs. Differences in the amount of IFN-γ produced was likely due in part to both the percentage of T cells (which ranged from 15 to 20%) and the viability of the cells that varied from 26 to 33% at the end of each experiment. Less than 1% of the CD45+ enriched cells were NK cells and 0.4–8% were monocytes. All of the fibroblast lines expressed B7H1 and B7DC and produced TGF-β1.

Consistent with the assumption that the response being monitored is that of the T cells to TCR stimulation, no IFN-γ was detected when TAT, either alone or in coculture with TAF, were cultured without the anti-CD3/anti-CD28 stimulus. We conclude that TAF from the majority of lung tumors tested have the capacity to enhance the response of T cells derived from the same tumor. No correlation was observed with the ability of the TAF to augment the TAT response and the histological type of the NSCLC. To obtain further insight with respect to how the fibroblasts were able to modulate the lymphocyte response to activation, a protocol was designed to determine whether the enhancing effect was due to a diffusible factor or required a direct cell-to-cell interaction.

**TAF enhancement of TAT IFN-γ production in response to TCR stimulation requires direct cell contact**

To determine whether the enhancing response was cell contact dependent, the coculture experiments with TAF and TAT were repeated in Transwell plates with the two cell types separated by a cell-impermeable, 0.4-μm polyester membrane. It was determined that the enhancing effect of TAF on TAT response to TCR stimulation was only observed when the two cell types were in direct cell contact, as the enhancing effect was totally abrogated when they were separated by the Transwell membrane (Fig. 6B). In fact, suppression was sometimes noted when the two cell types were separated by the Transwell membrane. This observed suppression suggests that a soluble factor may mediate a down-regulation of the T cell activation potential that is not recognized when the TAF
and TAT are in direct contact due to the dominant-enhancing effect that is dependent on cell-to-cell contact. This possibility was addressed and our findings are presented in the last section of the results.

**Coculture of TAF with TAT can also have an inhibitory effect upon the response of TAT to activation via the TCR**

As indicated above, TAF derived from five of eight tumors tested had a net enhancing effect upon the response of autologous TAT to activation. However, TAF derived from three of the eight tumors had a suppressive effect upon T cell activation when cocultured with the TAT derived from the same tumor. The three panels in Fig. 7A represent data derived from a single experiment with a pair of TAT and TAF derived from three different tumors. Each of these experiments were repeated at least two times and the same pattern (i.e., inhibition of IFN-γ) was again observed in each of the three coculture experiments. We were unable to establish a correlation of the inhibitory activity of the fibroblasts and their expression of coregulatory molecules and the production of TGF-β1. The percentage of T cells in the CD45+−enriched population varied from 14 to 33% and the viability of the cells ranged from 14 to 46%. Again, no correlation was observed with the ability of the TAF to suppress the TAT response and the histological type of the NSCLC.

In one of the three tumors for which suppression of the TAT response by TAF was noted, we observed that this inhibition was reversed by anti-B7H1- and anti-B7DC-specific function-blocking Abs (Fig. 7B). This indicates that one of the mechanisms by which TAF can suppress TAT activation involves interactions among members of the B7 family of coregulatory ligands and their cognate receptor(s).

**Inhibitory effects of TGF-β on TAT response to TCR stimulation when cocultured with autologous TAF**

Because we had established that TAF constitutively produce significant quantities of the inhibitory cytokine TGF-β1, we set up experiments to assess whether this cytokine could be dampening the contact-dependent enhancing effect of TAF in coculture with the autologous TAT. Although it was observed that coculture of TAT with autologous TAF resulted in an increased IFN-γ response by TAT (in the presence of TCR stimulation) when the cells were in direct contact, the response could be partially suppressed by the addition of exogenous TGF-β1 (Fig. 8). Blocking endogenous TGF-β1 using a function blocking Ab was able to further enhance the response (Fig. 8). This suggests that TAF-produced TGF-β1 is capable of exerting an inhibitory effect on TAT activation, despite the fact that a net enhancement is observed in five autologous pairs of TAT and TAF. This scenario further illustrates the fact that the interaction of TAF with TAT is complex and that there are coexisting positive and negative factors that are present enabling fibroblast modulation of T cell activation and function.

**Discussion**

We establish here, for the first time, that fibroblasts derived from the microenvironment of human lung tumors have the capacity to modulate the function of T cells derived from the same tumor microenvironment. The effect of the TAF on the response of TAT to a strong and persistent stimulation via the TCR is complex and includes both enhancing and inhibiting activities. In coculture experiments of autologous TAF and TAT, a net enhancing effect on the activation of TAT was observed with five of the eight pairs of cells tested. The augmentation of the response was manifested by an increase in the amount of IFN-γ that was produced in response to the cross-linking of CD3 and CD28. This enhanced response persisted for at least 7 days and was dependent on the direct interaction of the TAF with the TAT. When the TAF and TAT cells were separated in coculture experiments in Transwell plates, augmentation of TAT activation was eliminated.

Superimposed on the contact-dependent net augmentation of the TAT response, we observed an inhibitory effect of the TAF that was mediated by TGF-β1. This was determined from the observation that IFN-γ levels were increased when the activated TAT were cocultured with TAF in the presence of function blocking anti-TGF-β1 Abs. This increase due to the blockade of endogenous TGF-β1 in the coculture system was observed whether the TAT and TAF were incubated together or separated by a Transwell cell impermeable membrane. Furthermore, the addition of exogenous TGF-β1 was also able to decrease the IFN-γ levels observed in the TAF/TAT cocultures. Previous studies established that T cells isolated from the microenvironment of human lung tumors are hyporesponsive to a brief stimulation of the TCR (2). This T cell hyporesponsiveness was determined to be due to endogenous TGF-β1 present within the tumor microenvironment (4). We conclude that TAF have the capacity to modulate the response of the TAT to activation and that this fibroblast effect includes both providing positive signals that require close association between TAF and TAT and negative signals mediated, at least in part, by soluble factors including TGF-β1, to the TAT.

A consistent net inhibitory effect was observed in coculture experiments with three of the autologous TAF and TAT pairs. Two coregulatory molecules, B7H1 (PD-L1) and B7DC (PD-L2), were observed on all of the TAF tested, and their expression was enhanced by treatment with IFN-γ. Because both of these members of the B7 family have been shown to suppress T cell activity (7), the possibility that they could be involved in the TAF-mediated suppression of TAT was addressed in two of the autologous TAF/TAT pairs for which the net inhibitory effect was observed. In one of these pairs, this inhibitory effect was completely reversed when the TAF and TAT were cocultured with either anti-B7H1 or anti-B7DC function blocking Abs. Although this reversal of the TAF inhibition was observed in only one of the two TAF/TAT pairs tested, it demonstrates yet another potential mechanism by which fibroblasts can alter the response of T cells within the tumor microenvironment and highlights the complexity of the influence that stromal fibroblasts have on T cells within the tumor microenvironment. Previous studies have demonstrated the abundant presence of B7H1 on human carcinomas of the lung, ovary, breast, head/neck, and colon, in addition to melanomas (39, 42, 48, 49). These studies have also established the ability of coregulatory molecules to promote the apoptotic cell death of activated tumor Ag-specific T cells in vitro (42, 48). We also observed the expression of...
both B7H1 and B7DC, in addition to other B7 family coregulatory molecules such as B7H4 and B7H2, on tumor cell lines derived from our primary human non-small cell lung tumors (data not shown). The finding of B7DC on both fibroblasts and tumor cell lines is significant, because this coregulatory molecule has been thought to be restricted to monocytes, macrophages, dendritic cells, and other cells of the myeloid lineage (50–53). The failure of anti-B7H1 and anti-B7DC function blocking Abs to reverse the inhibition in one of the TAF/TAT pairs tested may reflect differences in the expression of PD-1 or other putative receptors for these ligands on the surface of the TAT. The amount of tumor biopsy tissue obtained and the number of TAT derived differs for each of the lung tumors and these variables limit the number of conditions that can be tested in each experiment. We intend to explore the role of PD-1 in future experiments using ovarian tumors and tumor ascites where we are able to obtain greater numbers of TAT from most patients.

Our findings that fibroblasts have pleiotropic (i.e., both enhancing and inhibitory) effects on the T cells in the tumor microenvironment are not unexpected given the heterogeneity of the TAF with respect to membrane-associated and soluble factors produced by these cells. The phenotype of all of the fibroblasts that we derived from numerous human non-small cell lung tumors was very similar, as they expressed molecules previously associated with fibroblasts derived from chronic inflammatory tissues and other tumors. This phenotype includes a combination of FAP and α-SMA (54). Fibroblasts with this phenotype are termed myofibroblasts and these cells are also distinguished by their positivity for Thy1/CD90 (55). Myofibroblasts from sites of chronic, persistent inflammation have been shown to produce a wide variety of potentially immunomodulatory factors, including tenacin C, thrombospondin-1, many different peptide growth factors, PGs, and high levels of cytokines and chemokines, including IL-1, IL-6, IL-8, IL-10, TNF-α, and MCP-1 (16, 56). Relatively few studies so far have examined whether fibroblasts are a relevant source of cytokines or chemokines in tumors (14). We demonstrate here that the TAF are producing at least three significant chemokines, IP-10, MIG, and RANTES, in addition to cytokines including TGF-β, TNF-α, and IP-10.

Our finding that TAF produce IFN-γ raises the question of whether IFN-γ detected in the TAF and TAT coculture experiments is produced by the TAT or both the TAT and the TAF. Two things support the notion that the IFN-γ in the coculture experiments is produced by the TAT. First, control wells containing TAF alone run as part of the coculture experiments did not test positive for IFN-γ production, and, second, in coculture experiments with TAF and TAT, no IFN-γ was produced unless TCR stimulation (by anti-CD3/anti-CD28-coated DynaBeads) was provided. Although these findings suggest that the IFN-γ that we measure in the cocultures is derived from TCR-activated T cells it remains possible that other cell types in the CD45−/CD14+ population, such as phagocytes or TAF in response to soluble factors produced by the activated lymphocytes are contributing to the production of IFN-γ.

TAF production and secretion of the chemokines IP-10 and MIG is potentially highly significant for two reasons. Both of these glutamate-leucine-arginine-negative CXC chemokines have the capacity to attract and retain activated T cells (58, 59), and they both have been shown to have significant antngiotic activity that contributes to the inhibition of human NSCLC tumor angiogenesis and spontaneous metastases (60, 61). Collectively, these findings illustrate the importance and potential capacity of fibroblasts in the tumor microenvironment to attract, retain, and modulate the activation of tumor-associated lymphocytes and to directly and indirectly alter the local progression and metastasis of human tumors. In view of the multiple biologically active molecules produced by fibroblasts and their recognized heterogeneity, our observation of both enhancing and suppressing effects of TAF on autologous TAT is not surprising. Although we are not aware of any other reports on the cocultivation of human TAF and TAT, there are several reports on the cocultivation of TAF with tumor cells in vitro and in vivo. These studies have revealed that fibroblasts can both inhibit and enhance tumor growth. The cocultivation of fibroblasts with mammary carcinoma cells exhibits a more orderly histological differentiation (62, 63) and basal cell carcinoma cells grown in culture with stromal cells demonstrated a loss of their malignant properties (64). In contrast, other studies revealed that TAF have a growth-stimulating effect upon tumors in vitro (65–67) and in vivo (68–70). These studies support the concept that TAF contribute directly to the promotion of tumor growth but also have the capacity to inhibit tumor growth.

The accumulating evidence that fibroblasts are able to modulate the behavior of malignant cells has provoked interest in developing novel cancer therapeutic strategies that target TAF (71). Clinical trials that 1) target tumor signals responsible for the recruitment or development of TAF, 2) target TAF signals that initiate and promote tumor growth and facilitate invasion and metastasis, and 3) eliminate the TAF to abolish all of the fibroblast activities, have already been instituted or are at the preclinical development stage (54). In view of the results presented here that some TAF are able to enhance the function of TAT and produce and secrete chemokines that are known to inhibit the progression of human tumors, caution should be exercised in the development and testing of these new therapeutic approaches. This would be particularly true for those therapies that are designed to completely eliminate TAF.

Although much remains unknown about the actual mechanisms by which fibroblasts in the tumor microenvironment, and other sites of chronic inflammation as a whole, regulate lymphocytic responses, efforts will be made to further elucidate the complex possibilities. We show here a variety of ways in which TAF can affect the function of TAT and we expect that additional mechanisms that govern the TAF/TAT interactions will be discovered. Based upon differences in function and the varying expression levels of cytokines, chemokines, and coregulatory molecules in TAF, we expect that subsets of fibroblasts exist within the tumor microenvironment with different functional capacities enabling them to enhance or suppress T cell activation. Efforts are currently underway with the production of mAbs to fibroblasts that will help to define these subsets by surface markers that would be unique to each subset. We do not believe that fibroblast heterogeneity and their interaction with lymphocytes are properties unique to the tumor microenvironment, and, as such, we expect that fibroblasts from other tissues, particularly at sites of chronic inflammation, will have similar characteristics in terms of their phenotype and functional ability to regulate lymphocytes. This could partially be attributable to the conditioning of fibroblasts by inflammatory signals within a microenvironment or to the recruitment of specific subsets of fibroblasts to sites of inflammation, wound repair, or...
References


