T Cells and Stromal Fibroblasts in Human Tumor Microenvironments Represent Potential Therapeutic Targets

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Abstract The immune system of cancer patients recognizes tumor-associated antigens expressed on solid tumors and these antigens are able to induce tumor-specific humoral and cellular immune responses. Diverse immunotherapeutic strategies have been used in an attempt to enhance both antibody and T cell responses to tumors. While several tumor vaccination strategies significantly increase the number of tumor-specific lymphocytes in the blood of cancer patients, most vaccinated patients ultimately experience tumor progression. CD4⁺ and CD8⁺ T cells with an effector memory phenotype infiltrate human tumor microenvironments, but most are hyporesponsive to stimulation via the T cell receptor (TCR) and CD28 under conditions that activate memory T cells derived from the peripheral blood of the cancer patients or normal donors. Attempts to identify cells and molecules responsible for the TCR signaling arrest of tumor-infiltrating T cells have focused largely upon the immunosuppressive effects of tumor cells, tolerogenic dendritic cells and regulatory T cells. Here we review potential mechanisms by which human T cell function is arrested in the tumor microenvironment with a focus on the immunomodulatory effects of stromal fibroblasts. Determining *in vivo* which cells and molecules are responsible for the TCR arrest in human tumor-infiltrating T cells will be necessary to formulate and test strategies to prevent or reverse the signaling arrest of the human T cells *in situ* for a more effective design of tumor vaccines. These questions are now

addressable using novel human xenograft models of tumor microenvironments.

Keywords Cancer · Fibroblast · Immunotherapy · Stromal cell · T lymphocyte · TCR signal transduction · Tumor microenvironment · Xenograft model

Abbreviations

DC	dendritic cell
TAF	tumor-associated fibroblast
MDSC	myeloid-derived suppressor cell
MSC	mesenchymal stem cell
TIL	tumor-infiltrating T cells
TCR	T cell receptor
TGF- β 1	Transforming growth factor- β 1

Introduction

The presence of tumor-infiltrating lymphocytes (TIL) in the microenvironment of spontaneous human tumors remains a histopathological variable that is notable and potentially exploitable, but not yet fully understood [1–4]. The type, density and location of immune cells within human tumors have been shown to be a valuable prognostic indicator and suggested to be superior to and independent of factors currently used to stage tumors [5]. Moreover, there is compelling evidence demonstrating in both animals and humans that tumors are able to provoke adaptive anti-tumor immune responses mediated by B cells and CD4⁺ and CD8⁺ T cells and to initiate innate immune responses mediated by NK or NKT cells [6–12]. For example, the tumor-associated antigens NY-ESO-1 and LAGE-1 have been shown to be expressed on epithelial ovarian cancers and have been used in tumor vaccines to induce both anti-

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mental transplantable mouse tumors [18] and significant differences between human and mouse immune systems exist and have been well documented [21].

In this review we begin with a histological, immunohistochemical and flow cytometric characterization of the human tumor microenvironment. This is followed by a summary and discussion of the literature on the hyporesponsiveness of the TIL and how this relates to the TCR signaling cascade. We will also review our data derived largely from the *in vitro* study of lymphocytes present in the microenvironment of human non-small cell lung tumors demonstrating that a significant portion of the tumor-infiltrating memory T cells are hyporesponsive or nonresponsive to activation via the CD3/CD28 signaling pathway and that this arrest is reversible. Next we review a diverse array of studies showing that fibroblasts have the ability to significantly alter T lymphocyte function and survival and we discuss the possibility that tumor-associated fibroblasts contribute to the regulation of T cells in the tumor microenvironment. In the final segment of this review, we discuss the design and use of human xenograft models of tumor microenvironments that are

being used to study *in vivo* the dynamic interplay of human TIL with stromal cells and tumor cells *in situ* in human tumor microenvironments.

Inflammatory Leukocytes in the Tumor Microenvironment

Human tumors are infiltrated by a wide variety of different leukocytes including macrophages, granulocytes, natural killer cells, myeloid dendritic cells, plasmacytoid dendritic cells, and lymphocytes [22]. The vast majority of infiltrating leukocytes in advanced human solid tumors are lymphocytes including B lymphocytes, plasma cells and different subsets of T lymphocytes. Lymphocytes are found in juxtaposition to tumor cells and are often found to infiltrate directly into the tumor parenchyma (as indicated with the blue arrow in Fig. 2). Note in this figure the very dense accumulation of lymphocytes directly surrounding tumor nodules in both a human non-small cell squamous lung carcinoma and ovarian epithelial papillary serous adenocarcinoma (Fig. 2). This intimate association of the

Fig. 2 Human Tumor Microenvironment Histology. (a) and (b) Original tumor from a patient with non-small cell lung squamous cell carcinoma. (c) and (d) Original tumor from a patient with ovarian

papillary serous carcinoma. Red arrow indicates tumor, blue arrow indicates lymphocytes. Sections are stained with hematoxylin and eosin and magnification is 100X

lymphocytes with the human tumor shown here is in contrast to several reports by others with animal tumor models suggesting that lymphocytes are restricted to the periphery of the tumor and fail to infiltrate tumor tissue.

Phenotypic analysis by flow cytometry of the lymphocytes derived from the tumors indicates that the majority of the CD45⁺ cells present within human lung non-small cell tumors are T cells with a phenotype that is consistent with effector memory T cells (i.e. CD45RO⁺, CD4⁺ or CD8⁺, CD44⁺, CXCR3⁺, CD28⁺ and negative for CD27, CD45RA and CD62L) [23]. This same phenotype has been seen in CD45⁺ cells from ovarian tumors (Bankert et al., unpublished). The dominance of effector memory T cells in tumor microenvironments has been shown in a variety of different murine and human tumors [23–25], and these cells are the focus of this review. While these memory T cells can be further divided into phenotypically and functionally distinct subsets, including CD4⁺ cells that are distinguished by the cytokines they produce and CD8⁺ cytotoxic cells, they all appear to be sustained in the tumor microenvironment in a viable but hyporesponsive state.

The dominant presence of memory T cells within the microenvironment of human tumors raises at least three important questions. First, how can these cells persist in the face of continuous antigen stimulation? Next, why do these cells fail to attack and kill tumor cells? Finally, what is responsible for attracting and retaining these cells to and in the tumor microenvironment? Insights into the first two questions may be derived from murine studies where the ability of T cells to remain viable in a functionally inactive or hyporesponsive state in the face of persistent antigen stimulation has been shown [26]. This T cell quiescence, termed adaptive tolerance [26], avoids excessive tissue damage and prevents the loss of antigen-specific T cells that may otherwise occur in the presence of persistent antigen stimulation. Such an antigen-driven senescence could ultimately lead to an unacceptable partial depletion of the T cell repertoire. The evidence for the hyporesponsiveness of the tumor-associated memory T cells and the several possible mechanisms responsible for the functional arrest of T cells are discussed later. The possible role of tumor-associated fibroblasts in preventing memory T cell apoptosis, thereby sustaining their presence in the tumor microenvironment, and the production of chemokines and cytokine receptors that are responsible for the specific attraction of effector memory T cells (both CD4⁺ and CD8⁺) to the tumor are also addressed below.

CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells (Treg) represent another subset of T cells that are known to control all aspects of the immune response [27–31]. This subset of cells has been found in human solid tumors, tumor ascites fluids and hematological malignancies [32–34], and their presence correlated in human ovarian cancer with a poor

prognosis [32, 33]. Indirect evidence suggests that Tregs are able to suppress tumor-specific immunity [34–42], but definitive evidence of the role of this T cell subset in the immunopathogenesis of human cancer is still lacking.

Although we know much about what inflammatory cells can do when removed and studied independently from the tumor microenvironment *in vitro*, it remains unclear how the cells may contribute to supporting or curtailing the development and spreading of spontaneously arising human tumors. It has been impossible to distinguish, by morphology, the inflammatory cell networks that contribute to tumor eradication from those that either have no effect or contribute to the promotion of tumor growth. The inflammatory infiltrates in tumors therefore remain a histopathological variable that is notable and potentially exploitable therapeutically, but not yet sufficiently defined. However, in spite of our incomplete knowledge with respect to how inflammatory cells influence tumor progression, a careful *in situ* analysis of tumor-infiltrating immune cells has become a valuable prognostic tool. By characterizing tumor-infiltrating immune cells in a large cohort of human colorectal cancers by gene expression profiling, and *in situ* by immunohistochemical staining, the type, density and location of immune cells within tumors were found to be a better predictor of patient survival than methods currently used to stage this cancer [5].

These studies are consistent with the notion that immunocompetent cells within the tumor may contribute to control of the growth and spread of mature tumors. Most studies in both mouse and humans have focused upon CD8⁺ cytotoxic T cells that kill tumors directly and T_H1 CD4⁺ T cells (producing IL-2, and IFN- γ) that contribute directly or indirectly to tumor killing [43]. While T_H1 remains the dominant T_H subset in tumors within the memory T cell pool, another T cell subset has recently been found in both mouse and human tumors that may play a significant role in the pathogenesis of tumors [44–46]. This subset is a CD4⁺ T cell that secretes IL-17A and has been labeled as the T_H17 cell [47, 48]. These cells and the cytokines they produce (including IL-17A, IL-17F and IL-22) have been shown to play important roles in the pathogenesis of autoimmune diseases and inflammation [49, 50]. Their biological role in the tumor microenvironment is poorly understood, and at present suggested to be both beneficial and detrimental to the cancer patient [51]. The generation and regulation of T_H17 cells in ovarian cancer have been documented [45] and the possible function and clinical relevance of these cells in human tumor microenvironments reported [46]. It has been postulated that T_H17 cells contribute to protective human tumor immunity by inducing the production by tumor cells of two chemokines that attract and recruit effector T cells and effector memory T cells that express CXCR3, the

receptor for the T_H17 induced chemokines CXCL9 (MIG) and CXCL10 (IP10) [46]. This could help to explain the dominance and sustained presence of the effector memory T cells in the microenvironment of human lung and ovarian tumors. However, as will be apparent from the discussion that follows of the complex and dynamic interactions of the memory T cells with the other inflammatory and stromal cells in the tumor microenvironment, it is not sufficient to recruit and retain memory T cells to control tumor growth. A significant portion of the effector memory T cells appear to undergo a functional arrest upon entering the tumor microenvironment [20, 52]. With an understanding of how this functional arrest occurs it should be possible to design approaches to reverse it and thereby recover full function of these cells. The T cells would then be able to orchestrate the killing of tumor cells locally and systemically. These important issues are the focus of the remainder of this review.

Functional Arrest of Tumor-Infiltrating T Lymphocytes

Our studies and those of others suggest that the inability of T cells to respond to and eradicate tumors is due to a functional arrest in the T cells present in the tumor microenvironment. This hyporesponsiveness of the effector memory T cells is caused by a regulatory arrest in the activation of signaling molecules in the TCR/CD28 pathways (Fig. 3).

Role of Signaling Molecules in T Cell Hyporesponsiveness

Insufficient T cell function has been well documented among cancer patients and several studies have attempted to evaluate the molecular changes that occur with carcinogenesis. Using an *in vivo* murine colon carcinoma model,

Fig. 3 T Cell Receptor and CD28 Signaling Cascade in T Lymphocytes. Crosslinking of TCR/CD28 results in activation of the Src family kinases Lck and Fyn. Lck and Fyn phosphorylate the ITAMs and the ζ chain of the CD3 complex. The phosphorylated ζ chain binds Zap-70 which is then phosphorylated by Lck. Zap-70 phosphorylates LAT and SLP-76 and the LAT-SLP-76 complex acts as a platform for other signaling molecules including Grb2, Vav, and ITK. ITK phosphorylates PLC- γ which cleaves PIP2 to yield DAG and IP3. IP3 stimulates the release of calcium into the cytosol. Calcium binds to calmodulin, which binds to and activates calcineurin. Calcineurin dephosphorylates NFAT which results in the translocation of NFAT into the nucleus. DAG activates PKC θ and RasGRP. PKC θ activates IKK through the phosphorylation of the

CARMA1, Bcl10, and MAL1 complex. Once phosphorylated by IKK, I κ B dissociates from NF- κ B, thereby allowing NF- κ B to translocate into the nucleus. RasGRP, through its stimulation of Ras, activates ERK1/ERK2 which subsequently induces the transcription and synthesis of Fos. Vav activates JNK and JNK phosphorylates Jun which results in the translocation of Jun into the nucleus. The transcription factor AP-1 is composed of Fos and Jun. The CD28 signaling component is only briefly highlighted here demonstrating its role in NF- κ B translocation. Upon ligation of CD28, the p85 subunit of PI3K binds to the intracellular motif of CD28. The CD28-PI3K complex promotes the activation of AKT, followed by NF- κ B. Figure and text adapted from Simpson-Abelson, 2008, EOTT Review [85]

Mizoguchi and colleagues showed that the CD8⁺ T cells isolated from tumor bearing mice had impaired cytotoxicity, TNF- α production and granzyme expression [53]. Furthermore, the T cells from tumor-bearing mice upon activation had a reduction in the phosphorylation of Lck, and a reduction in the expression of CD3 γ and CD3 ζ , when compared to non-tumor bearing controls. Reduced expression or complete loss of the CD3 ζ chain of the TCR complex has been reported in both murine [53] and human carcinoma [54]. Rodriguez et al. further demonstrated that loss of CD3 ζ resulted in a reduction in T cell proliferation and cytokine production, and that the loss of CD3 ζ was due to a reduction in the levels of L-arginine, an essential amino acid that has been implicated in the regulation of T cell responses in cancer [55].

On the other hand, Agrawal et al. showed that the lack of activation of T cells in cancer could not be explained by defects in CD3 ζ [24]. By comparing the tumor-infiltrating T cells isolated from B cell non-Hodgkin's lymphomas (NHL) with T cells from non malignant lymphoid organs these investigators found that in contrast to the T cells from the non-malignant lymphoid organs, the TIL failed to proliferate when stimulated with anti-CD3. However, bypassing the upstream TCR signaling cascade with PMA and CD28 enhanced proliferation.

In a murine adenocarcinoma model, Frey and colleagues [56] identified two populations of tumor-derived CD8⁺ T cells that differed in their ability to lyse target cells. The non-lytic TIL had deficiencies in the ability to flux calcium and tyrosine phosphorylation of the proximal TCR signaling molecules Zap-70 and LAT was significantly reduced when compared to the lytic TIL. The investigators specifically localized this proximal signaling defect to a lack of Lck activation that was associated with a localization of SHP-1 to Lck and an inability of Lck to be phosphorylated by ERK, which in the non-lytic TIL was sequestered away from Lck.

SHP-1, a tyrosine phosphatase, has been shown to directly dephosphorylate the subunits of the TCR, as well as Zap-70, PI3-K, PLC- γ and LAT thereby leading to defects in activation and subsequent downstream signaling [57]. Its direct interaction with SLP-76 and the Vav-Grb2 complex suggests a role of SHP-1 in modulating T cell cytoskeletal structure and further downstream signaling. SHP-1 has also been implicated in the regulation of the Ras/MAPK pathway. Loss of SHP-1 enhances CTL function and increases the formation of CD8⁺ T cell-APC contact [58]. In addition to its direct effects on TCR signaling, SHP-1 can associate with the suppressive co-regulatory molecule programmed death 1 molecule (PD-1, CD279), which also inhibits T cell activation [59].

Several recent reports in both human and murine models have identified the upregulation of PD-1 on antigen specific

CD8⁺ T cells in chronic infection [60–62]. Blockade of PD-1 with an antibody to its ligand PD-L1 (B7-H1, CD274) resulted in an enhancement of the T cell response [61]. PD-L1 and another PD-1 ligand called PD-L2 (B7-DC, CD273) are both expressed on a wide variety of cells such as DCs and macrophages [62], all of which are present in the tumor microenvironment. PD-L1 is present on tumors and its elevated expression has been correlated with poor prognosis [62, 63]. Our laboratory has recently identified both PD-L1 and PD-L2 on stromal tumor-associated fibroblasts in non-small cell lung carcinoma [64]. One mechanism by which PD-1 and its ligands can inhibit T lymphocyte responses is by directly targeting the TCR pathway, although PD-1 ligation may also act indirectly by transducing a signal that leads to the recruitment of SHP-1 whose effects are described above.

Two independent investigators have observed that diacylglycerol kinase- α (DGK- α) is upregulated in anergic T cells [65, 66], indicating that there is a link between the TCR signaling molecule DAG and T cell responsiveness. As depicted in Fig. 3, upon TCR ligation PLC- γ generates diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3) from phosphatidyl-inositol-4,5-bisphosphate (PIP2). DAG is responsible for the partial activation of the MAPkinase pathway and the activation of PKC- θ leading to NF- κ B translocation. Defects in Ras have resulted in a reduction in the MAP kinases Erk, Jun and the subsequent trans-activation of AP-1. Therefore, DAG induced activation of PKC θ and Ras is absolutely essential for transcriptional activation of AP-1 and NF- κ B. Co-stimulation with CD28 also greatly amplifies this signal. Phosphorylation of the hydroxyl group on DAG, catalyzed by DGK- α , converts it to phosphatidic acid. Koretzky and colleagues demonstrated, using microarray analysis, that DGK- α was upregulated in anergic cells and downregulated in normally responsive cells [65].

Transcription Factor Translocation as an Indicator of T Cell Activation

Other investigators have evaluated activation by assessing the translocation of transcription factors, NFAT and NF- κ B. Uzzo et al. demonstrated that T cells isolated from human renal cell carcinomas (RCC) failed to translocate the transcription factor NF- κ B after exposure to PMA and ionomycin which bypass the proximal tyrosine kinase events in the TCR pathway that are required for NFAT and NF- κ B translocation [67]. Gangliosides, which are commonly secreted from RCC, were found to be responsible for the suppression of NF- κ B translocation.

We have also assessed the activation of memory T cells derived from the human lung tumor microenvironment by

evaluating the translocation of NF- κ B and NFAT. In most resting cells, NF- κ B is sequestered in the cytoplasm bound to its inhibitor I κ B. Upon CD3/CD28 stimulation there is a cascade of tyrosine phosphorylation of various signaling and adapter proteins [68, 69] that results in the ubiquitination and degradation of I κ B (Fig. 3). Upon its dissociation from I κ B, NF- κ B enters the nucleus to initiate the transcription of genes involved in proliferation, survival, adhesion, migration and overall cellular function.

NFAT, like NF- κ B, translocates from the cytoplasm into the nucleus with T cell receptor signaling (see Fig. 3). When calcium is released from the endoplasmic reticulum and/or from extracellular stores, it binds to calmodulin activating calcineurin [70], which dephosphorylates NFAT allowing its translocation from the cytosol into the nucleus.

We have demonstrated that unlike memory T cells derived from the peripheral blood of normal donors or from patient peripheral blood, T cells isolated from primary non-small cell lung tumors are unable to translocate both NF- κ B and NFAT into the nucleus following CD3 and CD28 cross-linking. This signaling defect appears to be specific to the CD3/CD28 pathway as these TIL are responsive to TNF- α , which induces the translocation of NF- κ B by an alternative signaling pathway [52].

Role of Transforming Growth Factor- β in TIL Hyporesponsiveness

In an effort to reverse the inability of the TIL to translocate these transcription factors we identified two molecules that play a role in regulating their activation. Transforming growth factor- β (TGF- β 1) was found to be at least partially responsible for inducing the hyporesponsiveness [20] and interleukin-12 (IL-12) could reverse this TCR/CD28 signaling arrest [52].

TGF- β 1 can regulate the development and function of all leukocytes, including T regulatory cells, dendritic cells (DC), NK cells and B cells [71]. Both the differentiation state of the cells and the presence of immunomodulatory molecules, including co-stimulatory molecules and cytokines, influence the effect of TGF- β 1 on T cell function.

The importance of TGF- β 1 in regulating T cell function was first demonstrated in mice that had a T cell specific blockade in TGF- β 1 signaling. These animals had widespread inflammatory leukocyte infiltration into multiple organs and died prematurely [72–74]. High levels of TGF- β 1 are produced by various tumor types including breast, colon, stomach, liver [75] and fibroblasts [64]. High levels of TGF- β 1 have also been associated with poor prognosis [76]. Since TGF- β 1 is highly expressed in the tumor microenvironment and has been shown to suppress both the

acquisition and expression of T cell effector function [77], it is likely that it may be at least partially responsible for the failure of TIL to control tumor progression [71].

Although the inhibitory effect of TGF- β 1 on naïve and effector T cells has been established, very little is known about its effect upon human memory T cells particularly in the tumor microenvironment. One study showed that TGF- β 1 suppressed the acquisition and expression of effector function of human memory T cells that were reactive against melanoma [77]. Byrne et al. evaluated the effect of TGF- β 1 on macrophages in the tumor microenvironment and found that TGF- β 1 was responsible for the recruitment and retention of macrophages at the tumor and that this enabled the tumor to escape from the immune system [78].

These studies on TGF- β 1 and T cell function suggest that TGF- β 1 mediates a suppressive regulatory mechanism on memory T cells in the tumor. They also demonstrate that TGF- β 1 has the potential to prevent the uncontrolled activation of T cells that may be present in an environment of chronic antigen stimulation.

Given the suppressive role of TGF- β 1 in the tumor microenvironment, a significant question was whether TGF- β 1 may be playing a role in the hyporesponsiveness of TIL to CD3/CD28 activation. Utilizing a TGF- β 1 specific function blocking antibody we demonstrated that the hyporesponsiveness of the TIL could be reversed following TGF- β 1 blockade [20]. Neither TGF- β 1 nor anti-TGF- β 1 had any noticeable effect upon the activation of T cells derived from the peripheral blood of normal donors or cancer patients. Several cells within the tumor microenvironment have been shown to secrete TGF- β 1. These include the immune and tumor cells, as well as the tumor-associated fibroblasts [64]. However, it is not clear how tumor-derived versus other cell-derived TGF- β modulates the activation of the TIL. We further evaluated the effects of TGF- β 1 by pulsing the TIL with a low pH buffer which should remove not only TGF- β 1, but all molecules bound to the surface of the cell. After an overnight recovery period, the TIL cells treated with the low pH buffer responded normally to activation by TCR/CD28 induced cross-linking [20]. We established that this was at least partially modulated by membrane-bound TGF- β 1 by demonstrating that the hyporesponsiveness was re-established by the addition of exogenous TGF- β 1 to the cell suspension, after low pH treatment.

In order to completely understand the direct role of TGF- β 1 on T cell activation, it is necessary to evaluate and elucidate its effects on the TCR signaling components. Chen et al. demonstrated that TGF- β 1 inhibited the phosphorylation and activation of Itk in activated CD4⁺ T cells [79]. In addition, intracellular Ca⁺⁺ was reduced and NFAT translocation was inhibited. TGF- β 1 did not affect

Lck and Zap70 phosphorylation, but did inhibit the phosphorylation of ERK [79]. TGF- β 1 has also been shown to induce I κ B α and inhibit NF- κ B translocation [71]. It will ultimately be necessary to determine how the TGF- β 1 signaling pathways intersect with and modulate the TCR signaling pathways.

Recent studies have evaluated anti-TGF- β 1 therapies targeted to both the tumor, tumor microenvironment and other systemic effects related to tumorigenesis [76]. Evidence from these studies strongly suggests that blocking TGF- β 1 and/or TGF- β 1 mediated signaling may be a therapeutic approach to treating cancer, although neither the cell(s) that are producing it nor the mechanism(s) by which TGF- β 1 is able to inhibit TCR activation and signaling are completely clear. However, because membrane-associated TGF- β 1 is suppressive to tumor development early on, complete loss of TGF- β 1 could be detrimental [80].

Role of Interleukin-12 in TIL Hyporesponsiveness

Over the last few years it has become increasingly evident that IL-12 has the potential to be a powerful therapeutic agent or adjuvant for cancer immunotherapy. IL-12 is a cytokine that plays a critical role in both the innate and adaptive immune systems [81]. IL-12 induces NK and T cells to proliferate and produce IFN- γ [81, 82]. Studies with IL-12 have validated its role in the reactivation and survival of memory CD4⁺ T cells [82, 83]. These studies have validated the multifunctional role of IL-12 and its potential utilization as an anti-cancer agent [82, 84, 85].

Until recently, the mechanism by which IL-12 provoked T cell mediated anti-tumor immunity was unknown. Studies in our laboratory have demonstrated that hyporesponsive human TIL can be reversed and the T cells reactivated by IL-12 *in vivo*. This IL-12 induced reactivation of the TIL resulted in their proliferation, production of IFN- γ and eradication of tumor cells [23, 86–88]. While it was evident that IL-12 could reverse the T cell hyporesponsiveness, the mechanism of this reactivation was unknown. We evaluated the effect of IL-12 on TCR responsiveness by incubating TIL *in vitro* with a brief pulse (3 h) of IL-12, followed by an overnight incubation without IL-12. Following overnight incubation, the T cells were stimulated with anti-CD3 and anti-CD28 cross-linking antibodies for 1 h, and the translocation of NFAT monitored by confocal immunofluorescence microscopy. The TIL pretreated with IL-12 responded normally to the TCR activation protocol whereas the control TIL (no IL-12 exposure) remained unresponsive to activation via CD3/CD28 [52]. Therefore, IL-12 is able to reverse the arrest in the TCR signaling cascade of the TIL. The mechanism by which IL-12 reverses the TIL

hyporesponsiveness has yet to be elucidated. Studies have demonstrated that one likely candidate that may be responsible for T cell hyporesponsiveness is the Tec family kinase Tec [89]. IL-12 treatment of CD4 + T cells in peripheral blood has been shown to increase the expression of the Tec kinases [90]. Studies *in vivo* have also demonstrated that IL-12 can enhance synapse formation and unresponsive T cells once treated with IL-12 were able to respond to weak peptides or self peptides [91]. The ability to reverse the TCR signaling arrest in TIL derived from malignant tissues indicates that these cells are not in a state of permanent immunosuppression, and that they can be reactivated to respond to stimulation through the CD3/CD28 pathway. It will be critical to determine how other cells in the tumor microenvironment, such as tumor cells, regulatory T cells, myeloid suppressor cells, monocytes, dendritic cells, granulocytes and fibroblasts, are able to modify T cell activity within the tumor microenvironment.

Fibroblasts as Contributors to TIL Hyporesponsiveness

Among the cells in the tumor stroma, T regulatory cells and myeloid-derived suppressor cells have received much attention in the literature for their role in downregulating immune responses to tumor cells, but many of the mechanisms by which these cells have been hypothesized to function within the tumor microenvironment have also been found in fibroblasts. The potentially immunosuppressive mechanisms which have been found on fibroblasts include the production of soluble products such as TGF- β 1 or hepatocyte growth factor (HGF or scatter factor), hormones such as prostaglandin E₂ (PGE₂), the co-regulatory surface molecules like PD-L1 (B7-H1), and the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO). Fibroblasts are also known to produce a host of soluble factors that influence angiogenesis, such as vascular endothelial growth factor (VEGF), and chemokines that orchestrate the influx of immune cells (Fig. 4). Therefore, fibroblasts have the potential to be much more than inert structural cells and do dynamically interact with the other cell types within the tumor microenvironment. Myofibroblasts frequently make up the bulk of the tumor microenvironment yet research is only recently beginning to dissect the role of this prominent cell type in the pathogenesis of cancer as well as in other pathologic settings. Fibroblasts have been implicated as tumor cell-promoting through their ability to produce factors that support angiogenesis as well as their ability to support tumor cell metastasis (reviewed in [92–94]). Here, we review ways in which fibroblasts have been reported to interact with and influence the activation of T lymphocytes.

Fig. 4 Fibroblast-T Lymphocyte Crosstalk. Through production of a diverse array of chemokines, cytokines, and extra-cellular matrix molecules, fibroblasts alter the trafficking and activation status of T lymphocytes directly and indirectly through the angiogenesis or activation of vascular endothelial cells. Conversely, T lymphocytes produce factors which alter the amount and types of molecules produced by the fibroblast

Fibroblast Heterogeneity

Fibroblasts are the heterogeneous mesenchymal cells of connective tissue and are identified morphologically by their cell processes giving them a spindle or stellate shape and by their adherence to cell culture plates *in vitro*. Molecular markers for fibroblasts include an 112 kD cell surface protein recognized by the D7-FIB monoclonal antibody (also called SM1214P) [64, 94], CD90 (Thy-1), fibroblast-specific protein 1 (FSP-1) [95], vimentin, and prolyl 4-hydroxylase (an enzyme for collagen synthesis). Myofibroblasts, which can be found in the tumor microenvironment, represent activated fibroblasts capable of increased levels of extracellular matrix (ECM) production and are characterized by their production of α -smooth muscle actin (reviewed in [96]).

However, no marker has thus far been identified that is found only on fibroblasts and expression of the current punitive markers differs among fibroblast subsets. Mesenchymal stem cells (MSC) are fibroblast-like progenitor cells usually derived from the bone marrow stroma with the capacity for self-renewal and for adipogenic, chondrogenic, and osteogenic differentiation. Like fibroblasts, MSC can be isolated from the bone marrow based on D7-FIB antibody recognition of surface protein and these isolated cells are negative for CD45 while positive for CD90. MSCs differ from dermal fibroblasts in that MSCs express the phenotypic markers LNGFR, STRO-1, and HLA-DR [97]. Based on their spindle-shaped morphology, adherence, and expression of D7-FIB antigen and CD90, MSCs represent a

subset of fibroblasts with the capability of differentiation into other cell types. Like fibroblasts, MSC suffer from a lack of a specific marker, since even markers that distinguish MSC from differentiated fibroblasts are also found on other cell types. Thus, there is still considerable debate within the field as to what constitutes MSC and whether they represent true self-renewing, pluripotent stem cells.

Fibroblasts & Extracellular Matrix

Fibroblasts are responsible for the production of ECM components such as collagen, fibronectin, and laminin. Cell adhesion to the ECM is necessary for the organization of tissues and cell trafficking and is mediated primarily through integrins. Signaling via α 2 β 1 integrin increases T lymphocyte IFN- γ production [98] and culturing activated T cells on ECM protein-coated plates has been shown to increase their proliferation [99, 100]. Thus, the fibroblast-produced ECM itself can serve as a means by which T lymphocytes receive co-stimulation and drive the inflammatory response.

The Fibroblast and MSC as Immunomodulatory Cells

Despite the known capability of fibroblasts to produce collagen and fibronectin, which have been shown to act as co-stimulatory molecules for T lymphocyte activation via

the T cell receptor [100, 101], a number of studies have reported that MSC and mature fibroblasts derived from a variety of sources demonstrate immunosuppressive effects on T lymphocytes. Human MSC, rheumatoid arthritis synovial membrane fibroblasts, normal dermal fibroblasts, and articular chondrocytes have all been shown to inhibit proliferation of allogeneic peripheral blood T cells and reduce IFN- γ production [102–104]. MSC constitutively produce COX-2, PGE₂, TGF- β 1 and HGF and were able to suppress both MLR and concanavalin A driven T lymphocyte proliferation. PD-L1 and IDO expression could be induced and PGE₂ and HGF expression increased by IFN- γ addition to MSC [105]. Uccelli's group found that this MSC-induced T cell unresponsiveness was a state of anergy that was reversible with administration of IL-2 [106]. Similarly, human colonic myofibroblasts were shown to suppress the proliferation of anti-CD3/CD28 activated peripheral blood lymphocytes and IL-2 production via a contact-mediated mechanism and exogenous IL-2 could reverse this fibroblast-mediated inhibition [107].

The PD-1 ligands, PD-L1 and PD-L2, have been shown to be expressed constitutively on human fibroblasts and to a greater extent on myofibroblasts from normal colon biopsies [107]. As mentioned previously, both are considered to be negative co-regulatory molecules of T lymphocyte function. IFN- γ has been shown to increase PD-L1 expression levels in both malignant [64] and normal fibroblasts [108]. Since fibroblasts express PD-L1, they have the potential to influence PD-1⁺ T lymphocyte activation.

Fibroblast Products as Promoters of Inflammation

A number of laboratories have cocultured fibroblasts and lymphocytes as a means of investigating the interactions between these cell types. Most human coculture studies to date have utilized fibroblasts and peripheral blood lymphocytes derived from different patients. Since T lymphocytes recognize and respond to alloantigens, conclusions drawn from allogeneic coculture systems are open to question. The fact that in most studies T lymphocytes fail to respond to the alloantigens of MSC has added to the notion that MSC are immunologically inert or even immunosuppressive and some have extended this idea to include all adult human fibroblasts [104]. However, it has been reported that fibroblasts activate neither autologous nor allogeneic T lymphocytes when cultured together [109], but either autologous or allogeneic resting T cells are able to activate transcription of several relevant genes in fibroblasts. Thus, results derived from experiments using allogeneic coculture systems may

be as physiologically relevant as those from autologous systems.

Our experiments culturing anti-CD3/CD28 activated lung tumor-infiltrating T lymphocytes or patient peripheral blood T lymphocytes with tumor-derived fibroblasts indicate that fibroblasts have the ability to modulate T cell activation as monitored by IFN- γ production (Fig. 5). In most cases, IFN- γ levels are significantly increased in T cells activated with these tumor-derived fibroblasts over those T cells activated alone. In a few instances, suppressive fibroblasts were derived from non-small cell lung tumors and this suppression could be reversed by addition of PD-L1 blocking antibody [64]. Fibroblasts were also shown to produce TGF- β . While these candidate molecules may explain the immunosuppression observed by three fibroblast lines, they cannot account for the much more frequently observed effect of an increase in IFN- γ levels found in coculture supernatants.

Fibroblasts produce many molecules with the potential to alter lymphocyte function and vice versa. For example, skin, bone marrow, and RA synovial fluid fibroblasts all spontaneously make IL-6 [110–112]. Increased levels of TNF- α , IL-6, IFN- γ [113] and IL-17A [114] were found when synovial fibroblasts were cultivated with T cells. Dermal fibroblasts also increase their level of IL-6 production when cultivated with T cells and this was even more pronounced with allogeneic cultures than when autologous T cells were used [115]. TNF- α , lymphotoxin (TNF- β) [110, 116], IFN- γ [117], TGF- β 1 [117] and IL-17A [117] represent T lymphocyte-produced cytokines that have been shown to

Fig. 5 Fibroblast Modulation of T Lymphocyte IFN- γ Production. Primary fibroblasts established from the lung tumor of a human cancer patient enhanced the production of IFN- γ by CD3/CD28 antibody-activated autologous tumor-infiltrating lymphocytes over the level of IFN- γ produced by these T cells when they were activated without fibroblasts present

upregulate fibroblast production of IL-6. IL-6 is important in the differentiation of naïve T lymphocytes into T_H17 cells that produce inflammatory IL-17A.

RA synovial fibroblasts have been shown to express surface IL-15 that could induce TNF- α , IFN- γ , and IL-17A in T lymphocytes [118]. PGE₂, which can be produced by fibroblasts, was shown to have anti-proliferative effects on T lymphocytes and to decrease IFN- γ levels, but more recent evidence has shown that PGE₂ augments T_H17 cell differentiation [119] and this may serve as another pathway by which fibroblasts could contribute to inflammatory disorders. Conversely, IL-17A has been shown to cause an increase in fibroblast PGE₂ production [120].

Because fibroblasts represent a heterogeneous and mostly undefined cell type, different populations of fibroblasts derived from the same patient may be responsible for the suppression or enhancement of the T cell function. Alternatively, individual cells may have the capacity to produce both lymphocyte activation-enhancing molecules (such as collagen) and inhibiting molecules (TGF- β). The altered levels of IFN- γ or proliferation observed in T lymphocyte-fibroblast cocultures represent the sum of these conflicting actions.

Fibroblasts & T Lymphocyte Apoptosis

While fibroblast-produced factors may be categorized as suppressive or enhancing to T cells, other fibroblast effects are less easily classified, but nevertheless modulate the immune response. For instance, human synovial fibroblasts and mouse lung fibroblasts prevent T lymphocyte apoptosis [102, 121].

Fibroblast produced type I interferons, particularly IFN- β , have been shown to rescue activated peripheral blood T lymphocytes from apoptosis when they are deprived of IL-2 common γ chain receptor cytokines (IL-2, IL-4, IL-7, IL-9, IL-15) [110, 122]. In our hands, we have been unable to detect IFN- β production by ELISA in the media supernatants of cocultured lung tumor fibroblasts and T lymphocytes.

Activation-induced cell death, a form of apoptosis occurring via the expression of Fas Ligand when T cells are restimulated through the TCR, can be decreased by fibroblast-produced PGE₂, which inhibits the activation of caspase-8 [121]. IL-6 can also prevent T cell activation-induced cell death through anti-CD3 stimulation by decreasing the expression of FasL on T lymphocytes [123]. Components of the extracellular matrix, such as fibronectin, are also anti-apoptotic, particularly for memory T cells [99, 104]. By influencing apoptosis, fibroblasts have the ability to both mold the T cell repertoire and allow for inflammation to persist chronically.

Fibroblasts & Trafficking: Chemokines & Angiogenesis

Fibroblasts have been shown to increase the trafficking of T lymphocytes directly through their production of chemokines such as the CXCR3 ligands, CXCL9 (MIG) and CXCL10 (IP10), and the CXCR4 ligand, CXCL12 (SDF-1 α), as well as indirectly through angiogenesis promoting factors or through increasing the adhesiveness of nearby endothelial cells to bind circulating lymphocytes. In addition to their role as chemoattractants for leukocytes, chemokines (including macrophage inflammatory protein-1 α and β , RANTES, and MCP-1) can serve as costimulators of T cell activation to induce further proliferation [124].

Fibroblasts and endothelial cells are frequently in close proximity to one another in a number of different microenvironments. Thus, fibroblasts are in a prime position for altering the nearby vasculature. The manner in which fibroblasts alter the adhesiveness of endothelial cells has been shown to be in part dependent upon the milieu from which the fibroblast was derived. For instance, rheumatoid arthritis-derived synovial fibroblasts increased adherence of lymphocytes to endothelial cells while those taken from uninflamed microenvironments (dermal fibroblasts) actually decreased adherence of lymphocytes [125]. Fibroblasts themselves express adhesion molecules with most expressing ICAM-1 particularly in the presence of T cells and with even greater levels of expression in the presence of activated T cells [113].

While fibroblasts are capable of producing chemokines, they are also regulated by them. For instance, CCL2 (monocyte chemoattractant protein-1 or MCP-1), CCL5 (RANTES), and CXCL12 (SDF-1) have all been shown to enhance IL-6 and IL-8 production by RA synovial fibroblasts [111]. IL-8 is a chemoattractant for neutrophils and thus a regulator of acute, innate inflammation, but also a promoter of angiogenesis. Cytokines differentially regulate synovial fibroblast production of chemokines. TGF- β 1 induces CXCL12 production while TNF- α and IL-1 β give rise to CCL5 production. All three of these cytokines lead to enhanced CCL2 expression [111]. The cytokine milieu thus alters which cell types will be summoned to an inflammatory site, including the tumor microenvironment, based on which chemokines are released.

Through chemokines, cytokines, and modification of the vasculature, fibroblasts coordinate the movement of immune cells throughout the tissues. Fibroblasts can directly modify the activation and differentiation states of T lymphocytes through co-regulatory receptors, cytokine, and extracellular matrix production. Once T cell activation has occurred, fibroblasts influence which T lymphocytes remain and which go through apoptotic and survival pathways. All of these networks have been shown to exist within the tumor microenvironment and thus fibroblasts

themselves may have much more impact on what constitutes the inflammatory infiltrate within tumors than has previously been recognized and thus warrant closer scrutiny.

Mouse/Human Xenograft Models to Study in vivo Dynamic Interactions of Lymphocytes with Stromal Cells and Tumor Cells in situ in the Tumor Microenvironment

Most of what has been learned and is discussed above with respect to the functional arrest of T cells in human tumors has come from in vitro studies of single cell suspensions of lymphocytes, stromal cells and tumor cells. While this knowledge could potentially help to design more effective immunotherapeutic strategies for cancer, it will first be essential to establish in vivo which of the immunosuppressive mechanisms identified in vitro are involved in the T cell arrest, and which of the cells and cell products contribute most significantly to the induced hyporesponsiveness of the TIL in situ. This must be achieved by monitoring events within structurally intact human tumor microenvironments. By implanting non-disrupted pieces of human tumor tissue into severe combined immunodeficient (SCID) mice, our laboratory has been able to establish xenografts in which the microenvironment of the human tumor tissue remained physically and functionally intact with a sustained presence of the inflammatory leukocytes, stromal fibroblasts and tumor cells [87, 126]. Initial studies with this first SCID-human tumor xenograft model confirmed that the TIL were hyporesponsive and that the functional arrest of the T cells could be reversed by the local and sustained release of IL-12 or IL-12 and GM-CSF delivered directly into the tumor by microspheres [23, 87] or liposomes [127]. A major limitation of the early xenograft model was the presence of the host innate immune response that ultimately resulted in the invasion of host innate immune cells and the eradication of the xenograft [128]. To overcome this pitfall, a number of different strategies have been used to selectively eliminate or block the innate immune cell-mediated disruption of the xenograft. One approach was to deplete the SCID mouse of natural killer (NK) cells with a polyclonal antibody to asialo-GM1 or a monoclonal antibody directed against the β chain of the IL-2 receptor [128]. However, the effects of this treatment were transient and NK cells eventually were replenished and xenografts rejected. An alternative method for the elimination of NK cells was a further genetic alteration of the SCID mouse that was achieved by crossing the SCID mouse with the non-obese diabetic (NOD) mouse which has decreased NK cell function, lack of hemolytic complement and has functionally immature macrophages

[129]. While there was an initial improved engraftment of human tissues in the NOD-SCID mice, xenografts were eventually invaded and rejected by the host cells due to residual NK cell activity.

The initial limitations of the SCID and NOD-SCID mice for the implantation and long term survival of human normal and neoplastic tissues have now been overcome by the generation of a new generation of NOD-SCID mouse with a null mutation of the IL-2 common γ chain receptor [130–132]. These NOD-SCID IL2R γ^{null} mice are called NOG mice, and the IL-2R mutation results in a defect of the high affinity receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 that blocks the development of NK cells and further impairs innate immunity [130, 133]. The implantation of non-disrupted pieces of human tumor tissues into NOG mice results in the successful engraftment and long term survival of human tumor microenvironments with little or no invasion of the xenograft for up to 9 weeks post engraftment [134]. The presence of viable cytokeratin positive tumor cells that are actively dividing (Ki67+) are observed in xenografts of human non-small cell lung tumor tissues for up to 12 weeks post-engraftment [134]. The cytokeratin and Ki67 staining of a human squamous cell carcinoma xenograft established in a NOG mouse is shown in Fig. 6. The tumor is surrounded by stromal fibroblasts with a diffuse presence of inflammatory CD45+ leukocytes including CD3+ T cells and CD138+ plasma cells (Fig. 7). The majority of the T cells in the tumor xenografts are CD45RO+ and fewer CD20+ B cells and CD68+ monocytes are present in the xenografts. The entire xenograft tissue stains positively for HLA, indicating the tissue is of human origin without any noticeable infiltration of host murine cells [134]. The T cells remain viable and responsive to IL-12 as shown by their proliferation and production of IFN- γ . The presence of human immunoglobulin in the sera of the xenograft bearing NOG mice suggests that B cells and plasma cells also remain viable and functional for prolonged periods following tumor tissue implantation.

The NOG mice have also been shown to be a reliable model to demonstrate and evaluate human NK cell mediated antibody-dependent cellular cytotoxicity (ADCC) directed in vivo to Hodgkin lymphoma [135]. Another finding of considerable interest with this same lymphoma tumor model in the NOG mice was the ability of a monoclonal antibody directed against CCR4 (a chemokine receptor on Treg cells) to significantly reduce the number of Tregs that infiltrate the tumor xenograft [135].

The ability of tumor specific T cells to overcome the immunosuppressive effects of the tumor microenvironment was demonstrated in another NOG mouse tumor xenograft model by the direct injection of T cells (engineered to express a chimeric receptor with high affinity for a tumor antigen) into pre-established large tumor xenografts [136].

Fig. 6 NOG Lung Tumor Xenograft Immunohistochemistry. Intact tumor tissue pieces from a human non-small cell lung adenosquamous carcinoma were engrafted subcutaneously into SCID/NOD/IL-2R $\gamma^{-/-}$ (NOG) mice. Mice were treated with IL-12 microspheres on day 7

post-engraftment, sacrificed on day 53 post-engraftment. Sections are shown stained with hematoxylin and eosin or antibodies to cytokeratin (to mark the tumor cells), Ki67 (to show proliferation of the tumor), or HLA (to mark human cells)

The retargeted tumor-specific T cells survived and significantly reduced tumor burden, and, in some cases, resulted in the complete eradication of large pre-established mesothelioma xenografts [136]. It was further shown in this tumor xenograft model that the incorporation of the CD137 signaling domain into the chimeric receptor, significantly enhanced the persistence of the engineered T cells in the tumor-bearing mice following intratumoral or intravenous administration.

The ability of NOG mice to support the long term engraftment of human tumor microenvironments and the demonstrated utility of this model to study the interaction of

lymphocytes with stromal cells and tumor cells in situ provides an opportunity for the first time to study and determine which of the cells and factors shown to contribute to the arrest of T cell function in vitro are also found to be operating in vivo in the tumor microenvironment.

Conclusions and Future Directions

Immunotherapy either by itself or in combination with other treatments for cancer has great potential. However, to achieve full therapeutic benefits of immunotherapy, many

Fig. 7 NOG Lung Tumor Xenograft Immunohistochemistry. Intact tumor tissue pieces from a human non-small cell lung adenosquamous carcinoma were engrafted subcutaneously into SCID/NOD/IL-2R $\gamma^{-/-}$ (NOG) mice. Mice were treated with IL-12 microspheres on day 7

post-engraftment, sacrificed on day 53 post-engraftment. Sections are shown stained with hematoxylin and eosin or antibodies to CD45 (to identify hematopoietic immune cells), CD3 (to identify T lymphocytes) or CD138 (to identify plasma cells)

important questions about how the cells and molecules within the tumor microenvironment interact with each other need to be addressed and answered. In particular, it will be essential to gain insights into how tumor stromal cells and TIL modulate each other's function, and to identify potential molecular targets that can be exploited therapeutically to reverse the arrest of T cell signaling that occurs upon the entry of lymphocytes into the tumor microenvironment. While some of these issues have already been addressed by taking a reductionist approach in which different cell types from the tumor microenvironment are cocultured together *in vitro*, eventually it will be necessary to study the T cell and stromal cell interactions within the intact tumor microenvironment. Because of the differences between experimental mouse tumors and spontaneous human malignancies, and the increasing awareness of the differences between mouse and human immune systems, an increased emphasis needs to be placed upon the study of human tumor microenvironments *in situ* and *in vivo*. There are many obstacles both intrinsic and extrinsic to such human studies. Difficulties in obtaining human tissues, the genetic diversity of the study population, variability within and between tumor types and stage of tumor development, and the necessary limitations to the design and conduction of clinical trials to protect patients are just a few examples that have contributed to the impediments to conducting human studies. Nevertheless, these challenges must be addressed if we are to gain the insights into the complex cellular and molecular events occurring in human tumor tissues that are required to design ways to improve upon current immunotherapeutic approaches to cancer.

The use of new and improved immunodeficient mice for the establishment of intact human tumor microenvironment xenografts as discussed above has provided an opportunity to study human tumors *in vivo* for prolonged periods, and to test paradigms that develop from *in vitro* studies. It is expected that additional improvements in these xenograft models must and will be made to more closely approximate the native tumor microenvironment and the dynamic interactions between the tumor and tumor-associated cells that occur over time in patients' tumors. An example of such improvements is the design of orthotopic tumor implantations that progress and undergo spontaneous metastases to other organ sites that is now in development (Bankert et al. unpublished). The generation of Class I and Class II HLA transgenic NOD-scid IL-2 γ^{null} mice represents an exciting and potentially very important advance in humanized mice for studying T cell responses to tumor antigens in tumor microenvironments. By adoptively transferring human hematopoietic stem cells from an HLA-A2 donor into an HLA-A2 transgenic mouse it was possible to generate human MHC restricted antigen specific CD8⁺ T

cells 7 days after infection with a Dengue virus [137]. The use of these new humanized mice that are engineered to express both Class I and II HLA should ultimately provide an opportunity to pre-clinically evaluate tumor vaccination strategies in which both the generation of MHC restricted tumor-specific T cells and their effect upon the arrest of tumor growth can be monitored and quantified. Using these mice it will be possible to test the paradigm that upon entry into the tumor microenvironment from the periphery T cells become anergic, and subsequently to preclinically test strategies that are designed to reverse the signaling arrest of TIL and determine whether by reactivating these T cells the tumors are eradicated. The application of newer assay technologies such as two-photon laser-scanning microscopy will make it possible to visualize in tumor xenografts the dynamic changes in the movement, expansion, and contraction of lymphocytes, fibroblasts and tumor cells *in vivo* at the single cell level following perturbations to the tumor microenvironment that are designed to reactivate quiescent T cells [138, 139]. A combination of fine needle biopsies and real time PCR will make it possible to monitor changes in gene expression patterns and alteration in tumor progression without sacrificing the xenograft bearing mice.

The insights that will be gained from using human tumor xenografts to study the cellular and molecular dynamics in the tumor microenvironment are expected to lead to the design of novel approaches to enhance the therapeutic efficacy of cancer immunotherapies. These new approaches will ultimately need to be tested in clinical trials with cancer patients.

The discovery that IL-12 is able to reverse the hyporesponsiveness of T cells in the human tumor microenvironment provides a rationale for designing future clinical trials. While previous clinical trials with subcutaneous or intravenous IL-12 at doses of 500–1,000 ng/kg resulted in unacceptable toxicities [140], we predict (based upon the studies with human tumor xenografts) that ultra low and sustained levels of IL-12 delivered directly into the tumors will be effective at re-activating tumor-infiltrating T cells without the toxic effects that have been observed following large doses of this cytokine systemically. It is anticipated that by re-activating the T lymphocytes, these cells will orchestrate a cascade of events that result in the killing of tumor locally and that with the release of tumor antigens from the dead and dying tumor cells there will be an induction of a systemic anti-tumor immunity. This expectation has been confirmed in two animal tumor models [141, 142] and several clinical trials have shown that low doses of IL-12 injected locally into tumors show little or no toxicity (reviewed in [143]). An Investigational New Drug (IND) application for a local sustained delivery device for the introduction of IL-12 into tumor nodules has recently been approved by the FDA and a Phase I

clinical trial that will address the safety and later the efficacy of this in situ tumor vaccination strategy is to be initiated shortly.

Future efforts must be focused upon determining how IL-12 is able to reverse the hyporesponsiveness of tumor-infiltrating memory T cells. Armed with the knowledge of the mechanisms by which this TCR signal transduction is arrested, it should be possible to design and exploit additional methods to reverse the T cell hyporesponsiveness and by understanding how stromal fibroblasts modulate T cell function within the tumor microenvironment we predict that novel therapeutic approaches will be developed that target the fibroblasts and the biologically active factors produced by these cells.

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References

- Berd D, Mastrangelo MJ, Lattime E, Sato T, Maguire HC Jr (1996) Melanoma and vitiligo: immunology's Grecian urn. *Cancer Immunol Immunother* 42:263–267
- Barnhill RL, Mihm MC (1992) Histopathology of malignant melanoma and its precursor lesions. In: Balch CM, Houghton AN, Milton GW, Sober AJ, Soong S (eds) *Cutaneous melanoma*, 2nd edn. JB Lippincott, Oxford
- Balch CM, Soong S, Shaw HM, Urist MM, McCarthy WH (1992) An analysis of prognostic factors in 8500 patients with cutaneous melanoma. In: Balch CM, Houghton AN, Milton GW, Sober AJ, Soong S (eds) *cutaneous melanoma*, 2nd edn. JB Lippincott, Oxford
- Cook MG (1992) The significance of inflammation and regression in melanoma. *Virchows Arch A Pathol Anat Histopathol* 420:113–115
- Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313:1960–1964
- Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 3:991–998
- Smyth MJ, Godfrey DI, Trapani JA (2001) A fresh look at tumor immunosurveillance and immunotherapy. *Nat Immunol* 2:293–299
- Melief CJ, Toes RE, Medema JP, van der Burg SH, Ossendorf F, Offringa R (2000) Strategies for immunotherapy of cancer. *Adv Immunol* 75:235–282
- Pardoll D (2003) Does the immune system see tumors as foreign or self? *Annu Rev Immunol* 21:807–839
- Finn OJ (2003) Cancer vaccines: between the idea and the reality. *Nat Rev Immunol* 3:630–641
- Khong HT, Restifo NP (2002) Natural selection of tumor variants in the generation of “tumor escape” phenotypes. *Nat Immunol* 3:999–1005
- Gilboa E (2004) The promise of cancer vaccines. *Nat Rev Cancer* 4:401–411
- Odunsi K, Jungbluth AA, Stockert E, Qian F, Gnjjatic S, Tammela J, Intengan M, Beck A, Keitz B, Santiago D, Williamson B, Scanlan MJ, Ritter G, Chen YT, Driscoll D, Sood A, Lele S, Old LJ (2003) NY-ESO-1 and LAGE-1 cancer-testis antigens are potential targets for immunotherapy in epithelial ovarian cancer. *Cancer Res* 63:6076–6083
- Jager E, Karbach J, Gnjjatic S, Neumann A, Bender A, Valmori D, Ayyoub M, Ritter E, Ritter G, Jager D, Panicali D, Hoffman E, Pan L, Oettgen H, Old LJ, Knuth A (2006) Recombinant vaccinia/fowlpox NY-ESO-1 vaccines induce both humoral and cellular NY-ESO-1-specific immune responses in cancer patients. *Proc Natl Acad Sci USA* 103:14453–14458
- Valmori D, Souleimanian NE, Tosello V, Bhardwaj N, Adams S, O'Neill D, Pavlick A, Escalon JB, Cruz CM, Angiulli A, Angiulli F, Mears G, Vogel SM, Pan L, Jungbluth AA, Hoffmann EW, Venhaus R, Ritter G, Old LJ, Ayyoub M (2007) Vaccination with NY-ESO-1 protein and CpG in Montanide induces integrated antibody/Th1 responses and CD8 T cells through cross-priming. *Proc Natl Acad Sci USA* 104:8947–8952
- Zou W (2005) Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* 5:263–274
- Chiou SH, Sheu BC, Chang WC, Huang SC, Hong-Nerng H (2005) Current concepts of tumor-infiltrating lymphocytes in human malignancies. *J Reprod Immunol* 67:35–50
- Rangarajan A, Weinberg RA (2003) Opinion: comparative biology of mouse versus human cells: modelling human cancer in mice. *Nat Rev Cancer* 3:952–959
- Steinman RM, Mellman I (2004) Immunotherapy: bewitched, bothered, and bewildered no more. *Science* 305:197–200
- Broderick L, Bankert RB (2006) Membrane-associated TGF-beta1 inhibits human memory T cell signaling in malignant and nonmalignant inflammatory microenvironments. *J Immunol* 177:3082–3088
- Mestas J, Hughes CC (2004) Of mice and not men: differences between mouse and human immunology. *J Immunol* 172:2731–2738
- Schreiber H, Rowley DA (1999) Inflammation and cancer. In: Gallin JI, Smyderman R (eds) *Inflammation: basic principles and clinical correlates*, 3rd edn. Lippincott Williams & Wilkins, Philadelphia
- Broderick L, Yokota SJ, Reineke J, Mathiowitz E, Stewart CC, Barcos M, Kelleher RJ Jr, Bankert RB (2005) Human CD4+ effector memory T cells persisting in the microenvironment of lung cancer xenografts are activated by local delivery of IL-12 to proliferate, produce IFN-gamma, and eradicate tumor cells. *J Immunol* 174:898–906
- Agrawal S, Marquet J, Delfau-Larue MH, Copie-Bergman C, Jouault H, Reyes F, Bensussan A, Farcet JP (1998) CD3 hyporesponsiveness and in vitro apoptosis are features of T cells from both malignant and nonmalignant secondary lymphoid organs. *J Clin Invest* 102:1715–1723
- Radoja S, Saio M, Schaer D, Koneru M, Vukmanovic S, Frey AB (2001) CD8(+) tumor-infiltrating T cells are deficient in perforin-mediated cytolytic activity due to defective microtubule-organizing center mobilization and lytic granule exocytosis. *J Immunol* 167:5042–5051
- Schwartz RH (2003) T cell anergy. *Annu Rev Immunol* 21:305–334
- Beyer M, Schultze JL (2006) Regulatory T cells in cancer. *Blood* 108:804–811
- Shevach EM (2009) Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30:636–645
- Waldmann H, Cobbold S (2009) Regulatory T cells: context matters. *Immunity* 30:613–615
- Battaglia M, Roncarolo MG (2009) The fate of human Treg cells. *Immunity* 30:763–765

31. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D, Mathian A, Nakahata T, Yamaguchi T, Nomura T, Ono M, Amoura Z, Gorochoy G, Sakaguchi S (2009) Functional delineation and differentiation dynamics of human CD4⁺ T cells expressing the FoxP3 transcription factor. *Immunity* 30:899–911
32. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnjatic S, Ambrosone C, Kepner J, Odunsi T, Ritter G, Lele S, Chen YT, Ohtani H, Old LJ, Odunsi K (2005) Intraepithelial CD8⁺ tumor-infiltrating lymphocytes and a high CD8⁺/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci* 102:18538–18543
33. Curiel T, Zou W, Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10:942–949
34. Hilchey SP, De A, Rimsza LM, Bankert RB, Bernstein SH (2007) Follicular lymphoma intratumoral CD4 + CD25 + GITR + regulatory T cells potently suppress CD3/CD28-costimulated autologous and allogeneic CD8 + CD25- and CD4 + CD25- T cells. *J Immunol* 178:4051–4061
35. Shevach EM (2002) CD4⁺ CD25⁺ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2:389–400
36. Wood KJ, Sakaguchi S (2003) Regulatory T cells in transplantation tolerance. *Nat Rev Immunol* 3:199–210
37. von Herrath MG, Harrison LC (2003) Antigen-induced regulatory T cells in autoimmunity. *Nat Rev Immunol* 3:223–232
38. Bach JF (2003) Regulatory T cells under scrutiny. *Nat Rev Immunol* 3:189–198
39. Read S, Powrie F (2001) CD4(+) regulatory T cells. *Curr Opin Immunol* 13:644–649
40. Dieckmann D, Plottnner H, Berchtold S, Berger T, Schuler G (2001) Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med* 193:1303–1310
41. Ng WF, Duggan PJ, Ponchel F, Matarese G, Lombardi G, Edwards AD, Isaacs JD, Lechler RI (2001) Human CD4(+) CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood* 98:2736–2744
42. Levings MK, Sangregorio R, Roncarolo MG (2001) Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 193:1295–1302
43. Boon T, Coulie PG, Van den Eynde BJ, van der Bruggen P (2006) Human T cell responses against melanoma. *Annu Rev Immunol* 24:175–208
44. Kryczek I, Wei S, Zou L, Altuwaijri S, Szeliga W, Kolls J, Chang A, Zou W (2007) Cutting edge: Th17 and regulatory T cell dynamics and the regulation by IL-2 in the tumor microenvironment. *J Immunol* 178:6730–6733
45. Miyahara Y, Odunsi K, Chen W, Peng G, Matsuzaki J, Wang RF (2008) Generation and regulation of human CD4⁺ IL-17-producing T cells in ovarian cancer. *Proc Natl Acad Sci USA* 105:15505–15510
46. Kryczek I, Banerjee M, Cheng P, Vatan L, Szeliga W, Wei S, Huang E, Finlayson E, Simeone D, Welling TH, Chang A, Coukos G, Liu R, Zou W (2009) Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood* 114:1141–1149
47. Weaver CT, Hatton RD, Mangan PR, Harrington LE (2007) IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 25:821–852
48. Chen Z, O'Shea JJ (2008) Regulation of IL-17 production in human lymphocytes. *Cytokine* 41:71–78
49. Miossec P, Korn T, Kuchroo VK (2009) Interleukin-17 and type 17 helper T cells. *N Engl J Med* 361:888–898
50. Gaffen SL (2009) Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol* 9:556–567
51. Murugaiyan G, Saha B (2009) Protumor vs antitumor functions of IL-17. *J Immunol* 183:4169–4175
52. Broderick L, Brooks SP, Takita H, Baer AN, Bernstein JM, Bankert RB (2006) IL-12 reverses anergy to T cell receptor triggering in human lung tumor-associated memory T cells. *Clin Immunol* 118:159–169
53. Mizoguchi H, O'Shea JJ, Longo DL, Loeffler CM, McVicar DW, Ochoa AC (1992) Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science* 258:1795–1798
54. Whiteside T (2004) Down-Regulation of z-chain expression in T cells: a biomarker of prognosis of cancer? *Cancer Immunol Immunother* 53:865–878
55. Rodriguez PC, Ochoa AC (2006) T cell dysfunction in cancer: role of myeloid cells and tumor cells regulating amino acid availability and oxidative stress. *Semin Cancer Biol* 16:66–72
56. Koneru M, Schaer D, Monu N, Ayala A, Frey AB (2005) Defective proximal TCR signaling inhibits CD8⁺ tumor-infiltrating lymphocyte lytic function. *J Immunol* 174:1830–1840
57. Kosugi A, Sakakura J, Yasuda K, Ogata M, Hamaoka T (2001) Involvement of SHP-1 tyrosine phosphatase in TCR-mediated signaling pathways in lipid rafts. *Immunity* 14:669–680
58. Sathish JG, Dolton G, Leroy FG, Matthews RJ (2007) Loss of Src homology region 2 domain-containing protein tyrosine phosphatase-1 increases CD8⁺ T cell-APC conjugate formation and is associated with enhanced in vivo CTL function. *J Immunol* 178:330–337
59. Chemnitz JM, Parry RV, Nichols KE, June CH, Riley JL (2004) SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol* 173:945–954
60. Trautmann L, Janbazian L, Chomont N, Said EA, Gimmig S, Bessette B, Boulassel MR, Delwart E, Sepulveda H, Balderas RS, Routy JP, Haddad EK, Sekaly RP (2006) Upregulation of PD-1 expression on HIV-specific CD8⁺ T cells leads to reversible immune dysfunction. *Nat Med* 12:1198–1202
61. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R (2006) Restoring function in exhausted CD8 T cell during chronic viral infections. *Nature* 439:682–687
62. Freeman GJ, Wherry EJ, Ahmed R, Sharpe AH (2006) Reinvigorating exhausted HIV-specific T cells via PD-1-PD-1 ligand blockade. *J Exp Med* 203:2223–2227
63. Thompson RH, Dong H, Lohse CM, Leibovich BC, Blute ML, Cheville JC, Kwon ED (2007) PD-1 is expressed by tumor-infiltrating immune cells and is associated with poor outcome for patients with renal cell carcinoma. *Clin Cancer Res* 13:1757–1761
64. Nazareth MR, Broderick L, Simpson-Abelson MR, Kelleher RJ Jr, Yokota SJ, Bankert RB (2007) Characterization of human lung tumor-associated fibroblasts and their ability to modulate the activation of tumor-associated T cells. *J Immunol* 178:5552–5562
65. Olenchok BA, Guo R, Carpenter JH, Jordan M, Topham MK, Koretzky GA, Zhong XP (2006) Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat Immunol* 7:1174–1181

66. Zha Y, Marks R, Ho AW, Peterson AC, Janardhan S, Brown I, Praveen K, Stang S, Stone JC, Gajewski TF (2006) T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase- α . *Nat Immunol* 7:1166–1173
67. Uzzo RG, Rayman P, Kolenko V, Clark PE, Cathcart MK, Bloom T, Novick AC, Bukowski RM, Hamilton T, Finke JH (1999) Renal cell carcinoma-derived gangliosides suppress nuclear factor- κ B activation in T cells. *J Clin Invest* 104:769–776
68. Weil R, Israel A (2006) Deciphering the pathway from the TCR to NF- κ B. *Cell Death Differ* 13:826–833
69. Nel AE, Slaughter N (2002) T-cell activation through the antigen receptor. Part 2: role of signaling cascades in T-cell differentiation, anergy, immune senescence, and development of immunotherapy. *J Allergy Clin Immunol* 109:901–915
70. Nel AE (2002) T-cell activation through the antigen receptor. Part 1: signaling components, signaling pathways, and signal integration at the T-cell antigen receptor synapse. *J Allergy Clin Immunol* 109:758–770
71. Li MO, Sanjabi S, Wan YY, Robertson AL, Flavell RA (2006) Transforming growth factor- β of immune responses. *Annu Rev Immunol* 24:99–146
72. Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S (1993) Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci USA* 90:770–774
73. Letterio JJ, Roberts AB (1998) Regulation of immune responses by TGF- β . *Annu Rev Immunol* 16:137–161
74. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D et al (1992) Targeted disruption of the mouse transforming growth factor- β 1 gene results in multifocal inflammatory disease. *Nature* 359:693–699
75. Wrzesinski SH, Wan YY, Flavell RA (2007) Transforming growth factor- β and the immune response: implications for anticancer therapy. *Clin Cancer Res* 13:5262–5270
76. Saunier EF, Akhurst RJ (2006) TGF β inhibition for cancer therapy. *Curr Cancer Drug Targets* 6:565–578
77. Ahmadzadeh M, Rosenberg SA (2005) TGF- β 1 attenuates the acquisition and expression of effector function by tumor antigen-specific human memory CD8 T cells. *J Immunol* 174:5215–5223
78. Byrne SN, Knox MC, Halliday GM (2008) TGF β is responsible for skin tumour infiltration by macrophages enabling the tumours to escape immune destruction. *Immunol Cell Biol* 86:92–7
79. Chen CH, Seguin-Devaux C, Burke NA, Oriss TB, Watkins SC, Clipstone N, Ray A (2003) Transforming growth factor beta blocks Tec kinase phosphorylation, Ca²⁺ influx, and NFATc translocation causing inhibition of T cell differentiation. *J Exp Med* 197:1689–1699
80. Leivonen SK, Kahari VM (2007) Transforming growth factor-beta signaling in cancer invasion and metastasis. *Int J Cancer* 121:2119–2124
81. Colombo MP, Trinchieri G (2002) Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev* 13:155–168
82. Del Vecchio M, Bajetta E, Canova S, Lotze MT, Wesa A, Parmiani G, Anichini A (2007) Interleukin-12: biological properties and clinical application. *Clin Cancer Res* 13:4677–4685
83. Yoo JK, Cho JH, Lee SW, Sung YC (2002) IL-12 provides proliferation and survival signals to murine CD4⁺ T cells through phosphatidylinositol 3-kinase/Akt signaling pathway. *J Immunol* 169:3637–3643
84. Tatsumi T, Takehara T, Yamaguchi S, Sasakawa A, Miyagi T, Jinushi M, Sakamori R, Kohga K, Uemura A, Ohkawa K, Storkus WJ, Hayashi N (2007) Injection of IL-12 gene-transduced dendritic cells into mouse liver tumor lesions activates both innate and acquired immunity. *Gene Ther* 14:863–871
85. Simpson-Abelson M, Bankert RB (2008) Targeting the TCR signaling checkpoint: a therapeutic strategy to reactivate memory T cells in the tumor microenvironment. *Expert Opin Ther Targets* 12:477–490
86. Egilmez NK, Hess SD, Chen FA, Takita H, Conway TF, Bankert RB (2002) Human CD4⁺ effector T cells mediate indirect interleukin-12- and interferon- γ -dependent suppression of autologous HLA-negative lung tumor xenografts in severe combined immunodeficient mice. *Cancer Res* 62:2611–2617
87. Hess SD, Egilmez NK, Bailey N, Anderson TM, Mathiowitz E, Bernstein SH, Bankert RB (2003) Human CD4⁺ T cells present within the microenvironment of human lung tumors are mobilized by the local and sustained release of IL-12 to kill tumors in situ by indirect effects of IFN- γ . *J Immunol* 170:400–412
88. Kilinc MO, Aulakh KS, Nair RE, Jones SA, Alard P, Kosiewicz MM, Egilmez NK (2006) Reversing tumor immune suppression with intratumoral IL-12: activation of tumor-associated T effector/memory cells, induction of T suppressor apoptosis, and infiltration of CD8⁺ T effectors. *J Immunol* 177:6962–6973
89. Tomlinson MG, Heath VL, Turck CW, Watson SP, Weiss A (2004) SHIP family inositol phosphatases interact with and negatively regulate the Tec tyrosine kinase. *J Biol Chem* 279:55089–55096
90. Berg LJ, Finkelstein LD, Lucas JA, Schwartzberg PL (2005) Tec family kinases in T lymphocyte development and function. *Annu Rev Immunol* 23:549–600
91. Markiewicz MA, Wise EL, Buchwald ZS, Cheney EE, Hansen TH, Suri A, Cemerski S, Allen PM, Shaw AS (2009) IL-12 enhances CTL synapse formation and induces self-reactivity. *J Immunol* 182:1351–1361
92. Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. *Nat Rev Cancer* 6:582–601
93. Silzle T, Randolph GJ, Kreutz M, Kunz-Schughart LA (2004) The fibroblast: sentinel cell and local immune modulator in tumor tissue. *Int J Cancer* 108:173–180
94. Bagloli CJ, Ray DM, Bernstein SH, Feldon SE, Smith TJ, Sime PJ, Phipps RP (2006) More than structural cells, fibroblasts create and orchestrate the tumor microenvironment. *Immunol Invest* 35:297–325
95. Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, Neilson EG (1995) Identification and characterization of a fibroblast marker: FSP1. *J Cell Biol* 130:393–405
96. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G (2007) The myofibroblast: one function, multiple origins. *Am J Pathol* 170:1807–1816
97. Jones EA, Kinsey SE, English A, Jones RA, Straszynski L, Meredith DM, Markham AF, Jack A, Emery P, McGonagle D (2002) Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheum* 46:3349–3360
98. Boisvert M, Gendron S, Chetoui N, Aoudjit F (2007) Alpha2 beta1 integrin signaling augments T cell receptor-dependent production of interferon- γ in human T cells. *Mol Immunol* 44:3732–3740
99. Sturm A, Krivacic KA, Fiocchi C, Levine AD (2004) Dual function of the extracellular matrix: stimulatory for cell cycle progression of naive T cells and antiapoptotic for tissue-derived memory T cells. *J Immunol* 173:3889–3900
100. Davis LS, Oppenheimer-Marks N, Bednarczyk JL, McIntyre BW, Lipsky PE (1990) Fibronectin promotes proliferation of

- naive and memory T cells by signaling through both the VLA-4 and VLA-5 integrin molecules. *J Immunol* 145:785–793
101. Rao WH, Hales JM, Camp RD (2000) Potent costimulation of effector T lymphocytes by human collagen type I. *J Immunol* 165:4935–4940
 102. Jones S, Horwood N, Cope A, Dazzi F (2007) The antiproliferative effect of mesenchymal stem cells is a fundamental property shared by all stromal cells. *J Immunol* 179:2824–2831
 103. Bocelli-Tyndall C, Barbero A, Candrian C, Ceredig R, Tyndall A, Martin I (2006) Human articular chondrocytes suppress in vitro proliferation of anti-CD3 activated peripheral blood mononuclear cells. *J Cell Physiol* 209:732–734
 104. Haniffa MA, Wang XN, Holtick U, Rae M, Isaacs JD, Dickinson AM, Hilkens CM, Collin MP (2007) Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. *J Immunol* 179:1595–1604
 105. English K, Barry FP, Field-Corbett CP, Mahon BP (2007) IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett* 110:91–100
 106. Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, Giunti D, Ceravolo A, Cazzanti F, Frassoni F, Mancardi G, Uccelli A (2005) Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* 106:1755–1761
 107. Pinchuk IV, Saada JI, Beswick EJ, Boya G, Qiu SM, Mifflin RC, Raju GS, Reyes VE, Powell DW (2008) PD-1 ligand expression by human colonic myofibroblasts/fibroblasts regulates CD4+ T-cell activity. *Gastroenterology* 135:1228–1237, 1237.e1–2
 108. Lee SK, Seo SH, Kim BS, Kim CD, Lee JH, Kang JS, Maeng PJ, Lim JS (2005) IFN-gamma regulates the expression of B7-H1 in dermal fibroblast cells. *J Dermatol Sci* 40:95–103
 109. Young E, Stark WJ (1988) In vitro immunological function of human corneal fibroblasts. *Invest Ophthalmol Vis Sci* 29:1402–1406
 110. Filer A, Parsonage G, Smith E, Osborne C, Thomas AM, Curnow SJ, Rainger GE, Raza K, Nash GB, Lord J, Salmon M, Buckley CD (2006) Differential survival of leukocyte subsets mediated by synovial, bone marrow, and skin fibroblasts: site-specific versus activation-dependent survival of T cells and neutrophils. *Arthritis Rheum* 54:2096–2108
 111. Nanki T, Nagasaka K, Hayashida K, Saita Y, Miyasaka N (2001) Chemokines regulate IL-6 and IL-8 production by fibroblast-like synoviocytes from patients with rheumatoid arthritis. *J Immunol* 167:5381–5385
 112. Georganas C, Liu H, Perlman H, Hoffmann A, Thimmapaya B, Pope RM (2000) Regulation of IL-6 and IL-8 expression in rheumatoid arthritis synovial fibroblasts: the dominant role for NF-kappa B but not C/EBP beta or c-Jun. *J Immunol* 165:7199–7206
 113. Bombara MP, Webb DL, Conrad P, Marlors CW, Sarr T, Ranges GE, Aune TM, Greve JM, Blue ML (1993) Cell contact between T cells and synovial fibroblasts causes induction of adhesion molecules and cytokines. *J Leukoc Biol* 54:399–406
 114. Thiele K, Riemann D, Navarrete Santos A, Langner J, Kehlen A (2000) Cell-cell contact of human T cells with fibroblasts changes lymphocytic mRNA expression: increased mRNA expression of interleukin-17 and interleukin-17 receptor. *Eur Cytokine Netw* 11:53–58
 115. Sporri B, Bickel M, Limat A, Waelter ER, Hunziker T, Wiesmann UN (1997) Autologous versus allogeneic T cell-stimulated IL-6 production by dermal fibroblasts. *Inflammation* 21:371–378
 116. Akashi M, Loussarian AH, Adelman DC, Saito M, Koeffler HP (1990) Role of lymphotoxin in expression of interleukin 6 in human fibroblasts. Stimulation and regulation. *J Clin Invest* 85:121–129
 117. Hwang SY, Kim JY, Kim KW, Park MK, Moon Y, Kim WU, Kim HY (2004) IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-kappaB- and PI3-kinase/Akt-dependent pathways. *Arthritis Res Ther* 6:R120–R128
 118. Miranda-Carus ME, Balsa A, Benito-Miguel M, Perez de Ayala C, Martin-Mola E (2004) IL-15 and the initiation of cell contact-dependent synovial fibroblast-T lymphocyte cross-talk in rheumatoid arthritis: effect of methotrexate. *J Immunol* 173:1463–1476
 119. Boniface K, Bak-Jensen KS, Li Y, Blumenschein WM, McGeachy MJ, McClanahan TK, McKenzie BS, Kastelein RA, Cua DJ, de Waal MR (2009) Prostaglandin E2 regulates Th17 cell differentiation and function through cyclic AMP and EP2/EP4 receptor signaling. *J Exp Med* 206:535–548
 120. Yamamura Y, Gupta R, Morita Y, He X, Pai R, Endres J, Freiberg A, Chung K, Fox DA (2001) Effector function of resting T cells: activation of synovial fibroblasts. *J Immunol* 166:2270–2275
 121. Yarovsky TO, Hunninghake GW (2001) Lung fibroblasts inhibit activation-induced death of T cells through PGE(2)-dependent mechanisms. *Am J Physiol Lung Cell Mol Physiol* 281:L1248–L1256
 122. Pilling D, Akbar AN, Girdlestone J, Orteu CH, Borthwick NJ, Amft N, Scheel-Toellner D, Buckley CD, Salmon M (1999) Interferon-beta mediates stromal cell rescue of T cells from apoptosis. *Eur J Immunol* 29:1041–1050
 123. Ayroldi E, Zollo O, Cannarile L, DA F, Grohmann U, Delfino DV, Riccardi C (1998) Interleukin-6 (IL-6) prevents activation-induced cell death: IL-2-independent inhibition of Fas/fasL expression and cell death. *Blood* 92:4212–4219
 124. Taub DD, Turcowski-Corralles SM, Key ML, Longo DL, Murphy WJ (1996) Chemokines and T lymphocyte activation: I. Beta chemokines costimulate human T lymphocyte activation in vitro. *J Immunol* 156:2095–2103
 125. McGettrick HM, Smith E, Filer A, Kissane S, Salmon M, Buckley CD, Rainger GE, Nash GB (2009) Fibroblasts from different sites may promote or inhibit recruitment of flowing lymphocytes by endothelial cells. *Eur J Immunol* 39:113–125
 126. Williams SS, Chen FA, Kida H, Yokata S, Miya K, Kato M, Barcos MP, Wang HQ, Alosco T, Umemoto T, Croy BA, Repasky EA, Bankert RB (1996) Engraftment of human tumor-infiltrating lymphocytes and the production of anti-tumor antibodies in SCID mice. *J Immunol* 156:1908–1915
 127. Simpson-Abelson MR, Purohit VS, Pang WM, Iyer V, Odunsi K, Demmy TL, Yokota SJ, Loyall JL, Kelleher RJ Jr, Balu-Iyer S, Bankert RB (2009) IL-12 delivered intratumorally by multilamellar liposomes reactivates memory T cells in human tumor microenvironments. *Clin Immunol* 132:71–82
 128. Bankert RB, Egilmez NK, Hess SD (2001) Human-SCID mouse chimeric models for the evaluation of anti-cancer therapies. *Trends Immunol* 22:386–393
 129. Shultz LD, Ishikawa F, Greiner DL (2007) Humanized mice in translational biomedical research. *Nat Rev Immunol* 7:118–130
 130. Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, Koth M, Gillies SD, King M, Mangada J, Greiner DL, Handgretinger R (2005) Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* 174:6477–6489
 131. Sugamura K, Asao H, Kondo M, Tanaka N, Ishii N, Ohbo K, Nakamura M, Takeshita T (1996) The interleukin-2 receptor gamma chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID. *Annu Rev Immunol* 14:179–205

132. Asao H, Okuyama C, Kumaki S, Ishii N, Tsuchiya S, Foster D, Sugamura K (2001) Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. *J Immunol* 167:1–5
133. Shultz LD, Pearson T, King M, Giassi L, Carney L, Gott B, Lyons B, Rossini AA, Greiner DL (2007) Humanized NOD/LtSz-scid IL2 receptor common gamma chain knockout mice in diabetes research. *Ann N Y Acad Sci* 1103:77–89
134. Simpson-Abelson MR, Sonnenberg GF, Takita H, Yokota SJ, Conway TF Jr, Kelleher RJ Jr, Shultz LD, Barcos M, Bankert RB (2008) Long-term engraftment and expansion of tumor-derived memory T cells following the implantation of non-disrupted pieces of human lung tumor into NOD-scid IL2R-gamma(null) mice. *J Immunol* 180:7009–7018
135. Ito A, Ishida T, Yano H, Inagaki A, Suzuki S, Sato F, Takino H, Mori F, Ri M, Kusumoto S, Komatsu H, Iida S, Inagaki H, Ueda R (2009) Defucosylated anti-CCR4 monoclonal antibody exercises potent ADCC-mediated antitumor effect in the novel tumor-bearing humanized NOD/Shi-scid, IL-2Rgamma(null) mouse model. *Cancer Immunol Immunother* 58:1195–1206
136. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhai M, Suhoski MM, Varela-Rohena A, Haines KM, Heitjan DF, Albelda SM, Carroll RG, Riley JL, Pastan I, June CH (2009) Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc Natl Acad Sci USA* 106:3360–3365
137. Jaiswal S, Pearson T, Friberg H, Shultz LD, Greiner DL, Rothman AL, Mathew A (2009) Dengue virus infection and virus-specific HLA-A2 restricted immune responses in humanized NOD-scid IL2rgammanull mice. *PLoS ONE* 4:e7251
138. Bouso P, Robey EA (2004) Dynamic behavior of T cells and thymocytes in lymphoid organs as revealed by two-photon microscopy. *Immunity* 21:349–355
139. Hugues S, Fétler L, Bonifaz L, Helft J, Amblard F, Amigorena S (2004) Distinct T cell dynamics in lymph nodes during the induction of tolerance and immunity. *Nat Immunol* 5:1235–1242
140. Leonard JP, Sherman ML, Fisher GL, Buchanan LJ, Larsen G, Atkins MB, Sosman JA, Dutcher JP, Vogelzang NJ, Ryan JL (1997) Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood* 90:2541–2548
141. Hill HC, Conway TF Jr, Sabel MS, Jong YS, Mathiowitz E, Bankert RB, Egilmez NK (2002) Cancer immunotherapy with interleukin 12 and granulocyte-macrophage colony-stimulating factor-encapsulated microspheres: coinduction of innate and adaptive antitumor immunity and cure of disseminated disease. *Cancer Res* 62:7254–7263
142. Nair RE, Jong YS, Jones SA, Sharma A, Mathiowitz E, Egilmez NK (2006) IL-12 + GM-CSF microsphere therapy induces eradication of advanced spontaneous tumors in her-2/neu transgenic mice but fails to achieve long-term cure due to the inability to maintain effector T-cell activity. *J Immunother* 29:10–20
143. Broderick L, Bankert RB (2006) Memory T cells in human tumor and chronic inflammatory microenvironments: sleeping beauties re-awakened by a cytokine kiss. *Immunol Invest* 35:419–436