Human ovarian tumor ascites fluids rapidly and reversibly inhibit T cell receptor-induced NF-κB and NFAT signaling in tumor-associated T cells

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Human memory T cells present in ovarian tumor ascites fluids fail to respond normally to stimulation via the T cell receptor (TCR). This immunosuppression is manifested by decreases in NF-κB and NFAT activation, IFN-γ production, and cell proliferation in response to TCR stimulation with immobilized antibodies to CD3 and CD28. The anergy of the tumor-associated T cells (TATs) is mediated by soluble factors present in ovarian tumor ascites fluids. The nonresponsiveness of the T cells is quickly reversed when the cells are assayed in the absence of the ascites fluid, and is rapidly re-established when a cell-free ascites fluid is added back to the T cells. Based upon the observed normal phosphorylation patterns of the TCR proximal signaling molecules, the inhibition of NF-κB, and NFAT activation in response to TCR stimulation, as well as the ability of the diacylglycerol analog PMA and the ionophore ionomycin to bypass the ascites fluid-induced TCR signaling arrest, the site of the arrest in the activation cascade appears to be at or just upstream of PLC-γ. An identical TCR signaling arrest pattern was observed when T cells derived from normal donor peripheral blood were incubated with either malignant or nonmalignant (cirrhotic) ascites fluids. The immunosuppressive activity of ascites fluids reported here suggests that soluble factors acting directly or indirectly upon T cells present within tumors contribute to the anergy that has previously been observed in T cells derived from malignant and nonmalignant inflammatory microenvironments. The soluble immunosuppressive factors represent potential therapeutic targets for ovarian cancer.

Keywords: ovarian cancer, ovarian ascites, T cell anergy, T cell activation, TCR signaling

Introduction
A major conundrum with cancer is that human tumors progress in spite of the presence of T cells and other inflammatory leukocytes in the tumor microenvironment. Tumor-specific T cells have been identified both within the tumor itself and peripheral blood, proving that the immune system is capable of eliciting a response and that tumor escape is not due to immunological ignorance (1). Multiple and diverse explanations have been offered to explain the failure of immunocompetent cells to control tumor progression (2-7). For example, both cellular and soluble biological factors extrinsic to tumor-associated T cells (TATs) have been associated with their dysfunction. This includes two major immunosuppressive cells that accumulate in tumors: regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (8). The cells secrete the immunosuppressive cytokines IL-10, TGF-β, as well as reactive oxygen and nitrogen species, etc. Additionally, inhibitory B7 co-regulatory molecules expressed on tumor antigen-presenting cells (APCs), stromal cells, and tumor cells are implicated in the downregulation of T cell responsiveness (7-9).

There are also factors intrinsic to TATs responsible for their dysfunction. These cells directly express inhibitory receptors, which include programmed receptor 1 (PD-1/CD279) and lymphocyte activating gene 3 (LAG-3). The cells secrete the immunosuppressive cytokines IL-10, TGF-β, as well as reactive oxygen and nitrogen species, etc. Additionally, inhibitory B7 co-regulatory molecules expressed on tumor antigen-presenting cells (APCs), stromal cells, and tumor cells are implicated in the downregulation of T cell responsiveness (7-9).

Mitzoguchi and colleagues (10) determined the overall basal level of protein tyrosine phosphorylation in tumor-specific CD8+ T cells and reported that Lck was reduced in tumor-bearing mice. In addition, there was very little CD3γ expression and a complete absence of CD3ζ, which was replaced by the Fceγ chain. T cells isolated from patients with renal cell carcinoma (RCC) and in murine mouse models were unable to translocate the proinflammatory transcription factor nuclear factor-κB (NF-κB) in response to stimulation with PMA/ionomycin (11). Gangiosides, which are commonly secreted from RCC, were found to be responsible for the suppression of the NF-κB translocation.

The activation of memory T cells derived from human non-small cell lung carcinoma was assessed by monitoring the translocation of NF-κB, following activation via CD3/CD28. Fewer T cells derived from non-small cell lung tumors translocated NF-κB, as compared to T cells derived from patient or normal donor peripheral blood (12). An assessment
Figure 1

Activation of ovarian ascites T cells in ovarian ascites fluid. Leukocytes derived from tumor ascites fluid were thawed and allowed to recover overnight. The following day, the tumor ascites cells were left unstimulated or stimulated with immobilized antibodies to CD3 and CD28 for one hour in the presence of complete medium or 50% ascites fluid from ovarian cancer patients. The translocation of NF-κB was assessed by confocal microscopy and presented as the percentage of CD3+ T cells that translocate NF-κB. The figure shows the average and standard error of five independent experiments (*p < 0.05 compared with anti-CD3 and CD28 stimulated cells in the absence of ascites fluid.)

We establish here that memory T cells present in the ascites fluid of ovarian cancer patients fail to activate NF-κB and NFAT in response to activation via the T cell receptor (TCR). The anergy of these TATs is shown to be reversible and due to soluble factors present within the tumor ascites fluid that also rapidly and reversibly induce a similar TCR signaling arrest in T cells derived from normal donor peripheral blood. The ability of PMA and ionomycin to activate NF-κB and NFAT and the normal phosphorylation patterns of TCR proximal signaling molecules observed with cells in the ascites fluid, places the site of the ascites fluid-induced TCR signaling arrest at or just proximal to PLC-γ in both TATs and the T cells derived from normal donor peripheral blood.

Figure 2

(A) Freshly isolated CD45+ enriched cells from ovarian ascites fluid of four patients were allowed to recover overnight. The following day, cells were stimulated (circles) with antibodies to CD3 and CD28 or left unstimulated (triangles) for one hour in either serially diluted 100% ascites supernatant or complete medium and assessed for NF-κB translocation by multispectral imaging flow cytometry. At least 15,000 cells were collected and assessed for NF-κB translocation using the Similarity score and Rd value. The figure shows individual data points, average, and standard error of four independent experiments.

(B) CD45+ enriched populations containing T cells derived from ovarian ascites fluids were suspended in various concentrations of an ovarian ascites supernatant fluid diluted in complete medium. The cells were stimulated with antibodies to CD3 and CD28 for one hour and evaluated for NF-κB translocation, as above. The data are expressed as an average of at least three independent experiments with error bars representing the standard error. (*p < 0.05 compared with anti-CD3/anti-CD28 stimulated TATs suspended in 100% ascites fluid.)

Ovarian ascites fluid decreases the translocation of NF-κB in a dose-dependent manner. (A) Freshly isolated CD45+ enriched cells from ovarian ascites fluid of four patients were allowed to recover overnight. The following day, cells were stimulated (circles) with antibodies to CD3 and CD28 or left unstimulated (triangles) for one hour in either serially diluted 100% ascites supernatant or complete medium and assessed for NF-κB translocation by multispectral imaging flow cytometry. At least 15,000 cells were collected and assessed for NF-κB translocation using the Similarity score and Rd value. The figure shows individual data points, average, and standard error of four independent experiments. (*p < 0.05 compared with anti-CD3 and anti-CD28 stimulated TATs suspended in complete medium.) (B) CD45+ enriched populations containing T cells derived from ovarian ascites fluids were suspended in various concentrations of an ovarian ascites supernatant fluid diluted in complete medium. The cells were stimulated with antibodies to CD3 and CD28 for one hour and evaluated for NF-κB translocation, as above. The data are expressed as an average of at least three independent experiments with error bars representing the standard error. (*p < 0.05 compared with anti-CD3/anti-CD28 stimulated TATs suspended in 100% ascites fluid.)
Results

Receptor-induced activation of NF-κB in tumor-associated T cells is rapidly and reversibly inhibited by ovarian tumor ascites fluid

Inflammatory leukocytes coexist with tumor cells in the ascites fluid (supernatant) of ovarian cancer patients. The majority of the T cells present within the tumor ascites fluid have a phenotype that is consistent with effector memory T cells (CD45RO+, CD11a+, CD44+, CD 62L-) (Supplementary Figure 1). Because these T cells are in direct contact with the tumor cells, they have been termed tumor-associated T cells (TATs).

The activation potential of TATs was first assessed by incubating the ascites cells with immobilized anti-CD3 and CD28 antibodies, followed by the immunofluorescent staining of the cells with indirectly fluorochrome-labeled anti-CD3 and anti-NF-κB antibodies, and the cells then monitored for the presence of NF-κB in the nucleus by confocal microscopy. The percentage of CD3+ T cells with nuclear staining (activated cells) was recorded. The percentage of TATs with nuclear staining prior to the activation ranged from 0-3% and, in one hour after the activation, in the TATs present within the tumor ascites fluid, 11-35% of the cells were activated (Figure 1). This level of activation is significantly below the level of activation seen with T cells derived from multiple different normal donor peripheral blood leukocytes (PBLs) which typically ranges from 60-70% (data not shown). Of particular interest to us was the finding that when the cells were removed from the ascites fluid and activated in culture media, the percentage of TATs that were activated, rapidly and significantly increased to 44-62% (Figure 1). This finding has been repeated with 5 different sets of tumor ascites fluids and cells and suggests that a significant proportion of the TATs is hyporesponsive to activation via the TCR and that the decrease in the activation is reversible. Because the observed increase in the activation of the TATs in the absence of the ascites fluid was again reversed when cell-free ascites fluid was added back to the TATs derived from the ascites, we conclude that a soluble factor or multiple factors present in the ascites fluids contribute to the TCR signaling arrest.

To confirm and extend our observations made initially with immunofluorescent confocal microscopy, another technique was used in which the activation potential of larger numbers of TATs could be assessed objectively and, like the confocal analysis, analyzed without separating the T cells from the other inflammatory leukocytes and tumor cells present within the tumor ascites. Multispectral imaging flow cytometry (MIFC) has been validated previously for its ability to quantify the translocation of both NF-κB and NFAT in primary T cells as a measure of activation following a TCR stimulus. MIFC is a high-speed quantitative microscopy technique that enables statistically objective image analysis of thousands of cells per sample (19, 20). Representative MIFC fluorescent images of unstimulated control and TCR stimulated T cells derived from normal donor PBLs are shown in Supplementary Figure 2A and 2B. Nuclear translocation for each cell is measured using the Similarity score, and treatment-induced changes in nuclear translocation are measured using Fisher’s discriminant ratio (Rd), a statistic that quantifies the shift in Similarity score for a population of cells (described in Materials and methods). Increases in Similarity score and positive Similarity Rd values indicate that treatment has induced activation of nuclear translocation.

MIFC analysis of TATs reveals that NF-κB translocation is poorly activated when stimulus is applied in the presence of 100% ascites compared to stimulation performed in media (Figure 2A). We next titrated the tumor ascites fluid for its ability to inhibit the TAT activation. The data presented in Figure 2B establishes an ascites dose-dependent inhibition of TCR signaling and reveals a significant inhibition with as little as 1% tumor ascites fluid.

Ovarian ascites fluid decreases NF-κB translocation in normal donor (ND) peripheral blood T cells. (A) Cell populations containing CD45+ enriched cells derived from normal donor peripheral blood and ovarian ascites fluids were thawed and allowed to recover overnight. The following day, cells were stimulated with antibodies to CD3 and CD28 or left unstimulated for one hour in either AIM V medium or 50% ascites fluid. Cells were centrifuged and the pellet suspended in 50% ovarian ascites or complete medium and assessed for NF-κB translocation by multispectral imaging flow cytometry. Data show individual data points, average, and standard error of four independent experiments (* p < 0.05 compared with unstimulated TATs or ND-PBL-derived T cells.) (B) CD45+ enriched cells from normal donor peripheral blood were thawed and allowed to recover overnight. The following day, cells were stimulated with immobilized antibodies to CD3 and CD28 for one hour in the presence of 50% ascites fluid from 13 different ovarian cancer patients. The translocation of NF-κB was assessed by confocal microscopy and presented as the percentage of CD3+ T cells that translocate NF-κB. (* p < 0.05 compared with anti-CD3 and CD28 stimulated cells in the absence of ascites fluid.)
Ascites fluid from nonmalignant cirrhosis patients suppresses CD3+ T cells from ovarian ascites and normal donor peripheral blood. Cell populations containing CD45+ enriched cells from ovarian ascites fluids (A) and from normal donor peripheral blood (B) were thawed and recovered overnight. The following day, cells were stimulated with antibodies to CD3 and CD28 or left unstimulated for one hour in either AIM V medium or 50% cirrhotic ascites fluid (n = 3) and assessed for NF-κB translocation by multispectral imaging flow cytometry. Data are expressed as an average of at least three independent experiments with error bars representing the standard error. (*p < 0.05 compared with anti-CD3) and anti-CD28 stimulated TATs or ND-PBL-derived T cells in complete medium.)

MIFC and confocal microscopy. The T cells in normal donor PBLs and the TATs from the ascites fluid both had significantly decreased Rd values when activated in the presence of tumor ascites (Figure 3A). The tumor ascites fluids from 13 different ovarian cancer patients inhibited the TCR activation of T cells from the normal donor as determined by confocal microscopy (Figure 3B). These results establish that the inhibitory activity of tumor ascites fluids is not restricted to the effector memory tumor-associated T cells. We have also established that both CD4+ and CD8+ T cells are similarly inhibited in cells derived from the ascites fluid and normal donor PBLs (data not shown).

Since tumor cells are present in the tumor ascites fluid and are known to secrete many different immunosuppressive factors, as well as tumor-associated antigens, it was possible that one or more of the tumor factors was contributing to the TCR signaling arrest. Therefore, it was of interest to determine what effect ascites fluid derived from a nonmalignant source would have upon the activation potential of T cells. Three ascites fluids derived from patients with liver cirrhosis were obtained and tested for their effect upon the activation of T cells. All three of the ascites fluids from the nonmalignant source were found to significantly inhibit the activation of TATs and T cells from normal donor PBLs (Figure 4, A and B).

Ovarian tumor ascites fluids do not inhibit TNF-α or PMA/ionomycin-induced activation of NF-κB

One possible explanation for the inhibitory activity of both the tumor and cirrhotic ascites fluids would be the presence of cytotoxic factors. While there was no observable change in the viability of cells incubated in the ascites fluid for up to 48 hours, it was still considered possible for toxic factors to arrest the translocation of transcription factors without showing evidence of cell death. However, tumor ascites fluid was found to have no effect upon the ability of TNF-α or PMA/ionomycin to activate NF-κB despite its significant suppression of the response to anti-CD3 and anti-CD28 TCR stimulation (Figure 5). These results suggest that the inhibitory effects of the ascites are not due to non-specific cytotoxic effects, and that they are acting selectively to initiate an arrest within the TCR signaling pathway. Since PMA and ionomycin bypass the upstream TCR-tyrosine phosphorylation events, the data further suggest a block at or possibly just proximal to PLC-γ.

Ovarian tumor ascites fluids block translocation of NFAT and inhibit the phosphorylation of ERK, but have no effect upon the phosphorylation of more proximal TCR signaling molecules

If the tumor ascites were acting to block the TCR signaling pathway at or just upstream of PLC-γ, one would expect to see an inhibition of NFAT translocation in addition to the observed block in NF-κB translocation. Consistent with this expectation, TCR stimulation-induced translocation of NFAT and NF-κB were both inhibited by tumor ascites fluid in TATs and in T cells derived from normal donor PBLs (Table 1). In contrast, tumor ascites fluid had no impact on PMA/ionomycin-induced activation of NFAT and NF-κB in TATs and normal T cells.

Diacylglycerol (DAG), which is dependent upon PLC-γ and necessary for the activation of PKCδ and the translocation of NF-κB, is also required for the MAPK pathway. This pathway leads to the activation (phosphorylation) of ERK and subsequent transcription of Fos, which is a component of the AP-1 transcription factor in the T cell activation cascade. We determined that tumor ascites fluid inhibits the phosphorylation of ERK, consistent with an ascites-induced arrest in the MAPK pathway (Figure 6).

The phosphorylation of several TCR proximal signaling molecules following TCR activation of TATs was monitored in the presence and absence of tumor ascites fluid. No differences in the phosphorylation of CD3ζ, Lck, Zap70, or SLP76 were observed in the TATs incubated in tumor ascites or control media as determined by Phosflow analysis (Figure 7).

Table 1

<table>
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<th>Cells</th>
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<th>Condition</th>
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<td>+</td>
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<tr>
<td>+</td>
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<td>FRA + ionomycin</td>
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Figure 4

A. Culture Medium Control
   - Cirrhotic Ascites #1
   - Cirrhotic Ascites #2
   - Cirrhotic Ascites #3

B. Culture Medium Control
   - Cirrhotic Ascites #1
   - Cirrhotic Ascites #2
   - Cirrhotic Ascites #3
Normal donor peripheral blood T cells in ovarian ascites fluid translocate NF-κB with TNF-α or PMA/ionomycin stimulation. Cell populations containing CD45+ enriched cells from normal donor peripheral blood were stimulated with or without TNF-α (n = 3) or PMA/ionomycin (n = 5), in either AIM V medium or 50% ovarian ascites fluid, and evaluated for NF-κB translocation by multispectral imaging flow cytometry. Data show individual data points, average, and standard error of five independent experiments (n = 3 for TNF-α, *p < 0.05 compared with anti-CD3 and anti-CD28 stimulated peripheral blood lymphocytes stimulated in complete medium.)

Discussion

It has been established here that multiple different cell free ascites fluids derived from ovarian tumors or nonmalignant ascites fluids derived from cirrhotic patients reversibly suppress the TCR activation of human TATs and T cells derived from the peripheral blood of normal human donors. Our discovery that a soluble factor(s) present in both malignant and nonmalignant microenvironments is intriguing and has led us to consider the possibility that this represents an important conserved and physiologically relevant mechanism by which the activation of T cells is attenuated in environments where multiple proinflammatory cytokines and large numbers of dead and dying cells releasing self-proteins, lipids, and glycophospholipids. Such environments in which the T cells are persistently activated by tumor or microbial antigens without attenuation would result in their terminal differentiation and elimination. Persistent stimulation has been shown to result in T cell hyporesponsiveness to activation via the TCR in several different chronic viral infections and in local chronic inflammatory conditions such as nasal polyposis (13).

The ability of memory T cells to become unresponsive to persistent stimulation may well be important for at least two reasons. Firstly, this would avoid significant tissue damage resulting from an uncontrolled T cell proliferation and an overproduction of inflammatory cytokines and other biologically active factors. Secondly, the escape of memory T cells from persistent activation would prevent the loss of potentially important cells from the T cell repertoire.

A previous study found that ovarian tumor ascites fluids had highly variable effects upon the function of T cells derived from bulk ascites (23). Some ascites fluids enhanced and others suppressed T cell proliferation, while some other T cell functions were largely suppressed, including CD107a and CCL4 overproduction. It was determined with the TATs incubated in the ascites fluid, and the proliferation index (PI) was reduced production of IFN-γ by the TATs incubated in the tumor ascites fluid (Figure 8C).

We conclude that soluble factors present in both malignant and nonmalignant ascites fluids significantly inhibit the short- and long-term response of TATs and T cells derived from normal donor PBLs to a strong TCR stimulus and that this arrest in the TCR signaling cascade is rapidly induced and reversible.
Peripheral blood, in addition to their inhibition of TAT function, was unexpected and provided us with a sufficiently large number of easily obtained and isolated T cells to investigate the impact of the ascites fluids on the TCR signaling cascade in greater detail. The ascites fluids were shown to induce changes in the phosphorylation patterns of the distal, but not proximal, TCR signaling molecules, and to inhibit NF-κB and NFAT activation. Consistent with an anergic phenotype in the TATs, is the previous report that the signaling arrest of anergic T cells is reversed by PMA and ionomycyn (24). The collective findings that ascites fluids (a) inhibit TCR-induced, but not PMA/ionomycyn-induced, activation of NF-xB and NFAT and, (b) suppress the phosphorylation of ERK but not of the PLC-γ proximal signaling molecules, suggest that the site of the TCR signaling arrest is at or just proximal to PLC-γ. However, the precise mechanism and site of arrest within the TCR cascade remains to be determined.

The hyporesponsiveness of T cells is not restricted to tumor microenvironments. It has been observed in chronic viral infections where memory T cells are maintained in a functionally quiescent state (25). T cells derived from other nonmalignant chronic inflammatory tissues have also been found to be hyporesponsive to TCR signaling and exhibit a TCR arrest signature similar to what is induced by the ascites fluid reported here (12, 13). The ability to suppress T cell activation in both malignant and nonmalignant chronic inflammatory microenvironments appears to be a highly conserved mechanism. We report here that soluble inhibitory factors present in both malignant and nonmalignant environments contribute to the suppression of T cells. We have determined that the tumor ascites and cirrhotic ascites fluids contain several different cytokines and other biologically active proteins including IL-2, IL-4, IL-6, IL-8, IL-17, IFN-γ, IL-23, TNF-xα, MIP-1α, TGF-β1, IP-10, MIG, VEGF, angiogenin, and RANTES. Initial attempts with function blocking antibodies specific for IL-6 and TGF-β to identify protein factors that contribute to the TCR signaling arrest were unsuccessful. It was subsequently determined that 30 minutes in a boiling water bath had no effect upon the inhibitory activity of tumor ascites. Thus, we have concluded that the inhibitory factors are heat stable and are unlikely to include any of the known cytokines, chemokines, or other biologically active proteins present in either the tumor or cirrhotic ascites fluids. Our focus in the future will be upon small molecules and lipids that may be responsible for modulating the T cell function. We should emphasize that the inhibitory ascites fluids have been centrifuged and filtered to remove all cells and cell debris, and the pH of the ascites fluids was adjusted when necessary to a pH range of 7.2 – 7.4.

While our data have established that soluble factors in ascites fluids inhibit the activation of TATs, we have not yet determined whether the factors are acting directly or indirectly to block the TCR-induced signaling cascade. Because we have monitored the ascites fluid inhibitory activity on T cells that are in the presence of other inflammatory cells, it is possible that the effect upon T cells may be mediated indirectly by other immunoregulatory cells that are activated by the ascites fluids. One possibility that is currently being explored is that a subset of natural killer T (NKT) cells are responsible for the functional arrest of the TATs. Type I NKT cells upon activation by lipids presented in the context of CD1d have been shown to mediate the suppression of type I NKT cells as well as CD4+ and CD8+ T cells in a variety of different inflammatory conditions including cancer (26). We have established that the tumor ascites fluids inhibit both CD4+ and CD8+ T cells (data not shown). Polar lipids present in ovarian cancer-associated ascites have recently been shown to bind to and suppress mouse and human NKT cells (27). It remains to be determined whether lipids or possibly other molecules within the tumor ascites fluids acting directly or indirectly are responsible for the arrest of the tumor-associated CD4+ and CD8+ effector memory T cells.

Figure 7

Phosphorylation of upstream CD3 and CD28 signaling molecules is normal in the presence of ascites fluid. Cell populations containing CD45+ enriched cells from ovarian ascites fluids were allowed to recover overnight. The following day, cells were stimulated for one or three minutes, in either AIM V medium (A, C, E, and G) or ascites fluid (B, D, F, and H), with soluble antibodies to CD3/CD28, fixed, permeabilized, and stained for the phosphorylated form of the following signaling molecules and surface molecules: (A, B) CD3ζ (n = 2); (C, D) Lck (n = 2); (E, F) ZAP70 (n = 4); (G, H) SLP76 (n = 5), CD45RO, and CD45RA. Fold changes (Δ) in MFI were calculated between the unstimulated (dotted black line) and anti-CD3/CD28 stimulated (solid gray line) samples and a representative experiment is shown for each of the phosphorylated proteins.
The identification of the inhibitory factors present within human tumor ascites fluids and a determination of how they are able to arrest the function of the tumor-associated T cells, could lead to viable therapeutic targets designed to reverse the T cell anergy and, thereby, enhance the T cell eradication of tumors.

**Acknowledgements**

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**References**


Materials and methods
All experiments reported in this manuscript were performed in compliance with institutional guidelines and with IRB- and IACUC-approved protocols.

**Figure 8**

Ovarian ascites fluid decreases proliferation and IFN-γ production by T cells derived from ovarian ascites. The CD4+ enriched population from ascites was labeled with CFSE and stimulated with antibodies to CD3 and CD28 for four days. Cells were stained with LIVE/DEAD Violet, anti-CD4, and anti-CD8 and gated on live cells. Ovarian ascites fluid resulted in reduced proliferation index (PI) in both CD4+ T cell (A) and CD8+ T cell (B) populations derived from TATs. Data are representative of four independent experiments in which at least 50,000 events were collected. (C) Production of IFN-γ was assayed by ELISA after stimulation of CD45+ enriched cells from ovarian ascites by anti-CD3 and CD28 in the absence or presence of 25% or 50% ovarian ascites fluid. The figure shows the average and standard error of four independent experiments. (*p < 0.05 compared with cells stimulated with anti-CD3 and anti-CD28 in complete medium.)
Ascites fluid samples and cells

Ovarian ascites fluids and TATs were received from the Roswell Park Cancer Institute Tissue Procurement Facility. Ascites fluids from patients with hepatic cirrhosis were obtained from Dr. Thomas Russo at SUNY Buffalo. All fluids were depleted of cells by centrifugation and filtration. Normal donor PBLs were provided by the Flow and Image Cytometry Facility at Roswell Park Cancer Institute. All specimens were obtained under sterile conditions and using IRB-approved protocols.

Ovarian ascites fluid cell isolation

Ovarian ascites fluid was centrifuged and the cell pellet and ascites fluid were isolated separately. The cell-free ascites fluid (also referred to as supernatant) was filtered using a 0.22 um millipore filter and placed at either 4°C or frozen at -80°C until further use. The cell pellet was washed once in RPMI 1640 and centrifuged on a Ficoll-Paque Plus gradient (GE Healthcare) to enrich for viable CD45+ cells. The enriched CD45+ cell population, which contains many other inflammatory cells, including tumor cells and fibroblasts, was either used directly in experiments or frozen and stored in liquid nitrogen using Freezing Media (Invitrogen). Thawed or freshly isolated cells from the tumor ascites fluid or from normal donor PBLs, were allowed to recover overnight in either complete medium (RPMI containing 10% fetal bovine serum, or AIM V) or ascites fluid.

Anti-CD3/anti-CD28 stimulation of isolated cells

Antibodies were immobilized on either 24-well tissue culture plates or maxisorb 12 x 75 mm tubes (Nunc) by incubating 0.1 μg of purified anti-CD3 (Bio X Cell) and 5 μg of purified anti-CD28 (Invitrogen) in 500 μl of PBS, at 4°C overnight. PBLs from normal donors or leukocyte-enriched cells from the tumor ascites fluid from ovarian cancer patients were thawed, resuspended in RPMI 1640 + 1% human serum albumin, and approximately 5x10^5 total cells were incubated in anti-CD3/anti-CD28 in coated plates or plates at 37°C/5% CO2 for the duration of activation (1 or 2 hours for NF-kB or NFAT activation, the cells were removed from plates or tubes and stained with 3 μg/ml of normal mouse Ig for 10 minutes and stained with anti-human CD3-PE (BD Biosciences) in 0.1% Triton/3% FBS for 20 minutes. Cells were washed and resuspended in 2% UPF. The nuclear stain DRAQ5 (Invitrogen) was added before acquisition on the ImageStream (Amnis Corporation).

ImageStream acquisition and analysis of nuclear translocation

High speed collection of spectrally isolated, spatially correlated images of CD3, DRAQ5, NF-kB or NFAT, and brightfield was performed using the ImageStream multispectral imaging flow cytometer. The images were compensated on a pixel-by-pixel basis to compensate for spectral overlap and analyzed for nuclear translocation using Image Data Exploration and Analysis Software (IDEAS, Amnis Corporation). Analysis was performed on single, in focus, DRAQ5+ (nuclear stain), live, CD3+ cells as follows. Single cells were discriminated from debris and multicellular aggregates by gating events with intermediate area and high-aspect ratio. In-focus cells have high nuclear contrast and were gated as those events with high DRAQ5+ Gradient Max values, which measures the magnitude of the largest pixel intensity slope over the whole image (20). Live cells were gated as those events with low LIVE/DEAD Violet staining intensity (Invitrogen). Finally, CD3+ events were gated. Nuclear translocation was quantified as previously described (19, 20).

Supplementary Figure 2A (unstimulated) and 2B (anti-CD3/anti-CD28 stimulated) show individual CD3 (red), nuclear (blue), and NF-kB (green) fluorescent images and the composite of these images for selected cells. Note that unstimulated cells show an extranuclear (cytoplasmic) distribution of NF-kB, while NF-kB localizes to the nucleus in stimulated cells. For each cell, translocation of NF-kB was assessed using the Similarity score. The Similarity score is a log transform of the Pearson’s correlation coefficient (p) of individual pixel intensities from the nuclear and corresponding NF-kB images (20). With increasing nuclear translocation, the nuclear and NF-kB images appear more similar to one another, resulting in higher pixel-by-pixel correlation and, therefore, higher Similarity values.

After the Similarity scores were computed [Similarity = ln [(1+p) / (1-p)], Fisher’s discriminant ratio (Rd) was used to quantify the degree of NF-kB translocation between experimental samples. The Rd value quantifies the statistical separation between the similarity scores of two sample distributions. For NF-kB translocation, the Rd value was calculated according to the following equation: Rd = (MeanS - MeanU) / (Standard deviationS + Standard deviationU), where S = stimulated sample and U = unstimulated control. Therefore, the separation increases as the difference in the means increases, and the sum of the standard deviations decreases. In the example shown in Supplementary Figure 2C, stimulation did not result in a significant shift in the NF-kB Similarity score, resulting in an Rd value close to zero, indicating that treatment did not induce nuclear translocation. In contrast, stimulation conditions in Supplementary Figure 2D caused a right shift in the Similarity score distribution, resulting in a positive Rd value, indicating that treatment induced nuclear translocation of NF-kB.

Statistical significance for all multispectral imaging was determined by applying the Wilcoxon matched-pairs signed rank and Mann-Whitney tests and p values < 0.05 were
considered significant.

**CFSE for proliferation**

Leukocyte-enriched cells were thawed and recovered in complete medium overnight. The following day, cells were suspended at a concentration of $10^7$ cells/mL in PBS. A 5 mM stock solution of CellTrace™ CFSE (Invitrogen) was prepared by dissolving CFSE in ultrapure DMSO. Cells were stained at a concentration of 5 μM and incubated at 37°C/5% CO₂ for 15 minutes. Staining was quenched by addition of 1 volume of cold heat-inactivated FCS and centrifuged at 1000 rpm for 10 minutes at 4°C. Cells were resuspended in fresh medium and incubated at 37°C for 30 minutes. Cells were washed twice in 1X PBS, added to the 24-well plate, and either stimulated for 4 days or left unstimulated. After the 4 days of stimulation, cells were harvested, stained phenotypically for LIVE/DEAD Violet, CD45, CD4, and CD8. At least 100,000 events were collected on the LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star) and ModFit software (Verity).

**Phosflow analysis of the CD3/CD28 signaling molecules**

Cells were suspended in ice cold AIM V medium or ascites fluid and placed into polypropylene tubes. Samples were incubated for 1 hour at 37°C, centrifuged at 4°C, and resuspended in residual buffer. Purified anti-CD3 (Bio X Cell) and anti-CD28 (Invitrogen) were added (1 μg of each) per 1.0x10⁶ cells, and cross-linked with 2 μg goat anti-mouse Ig on ice. Samples were washed, resuspended in warm medium or ascites fluid, and activated at 37°C for 1, 3, 5, or 10 minutes. Samples were incubated with warmed Cytofix buffer (BD Biosciences) after stimulation. Samples were washed and permeabilized on ice with Permeabilization Buffer III (BD Biosciences) for 30 minutes. Samples were washed with Stain Buffer (BD Biosciences), transferred to polystyrene tubes, blocked with normal mouse Ig, and stained for 30 minutes at RT with antibodies to signaling molecules and cell surface markers. Signaling molecules were assessed with antibodies against phosphotyrosine-SLP76, phosphotyrosine-ZAP70, phosphotyrosine-Lck, phosphotyrosine-CD3ζ, and phosphotyrosine-ERK (BD Biosciences), and surface markers CD45RO and CD45RA (BD Biosciences). Data were collected on a FACSCalibur flow cytometer (BD Biosciences) and the data analyzed using FlowJo (Tree Star) software. Three hundred thousand events or more were collected for each sample.

**Immunofluorescence staining for confocal microscopy**

After activation, the cells were attached to alcian blue coverslips (21) for 10 minutes in a humid chamber. The cells were then fixed with 2% ultrapure formaldehyde in PBS for 40 minutes. After fixation, the cells were washed twice with PBS and once with block/perm buffer (5% normal goat serum in PBS + 0.4% Triton X-100). The cells were incubated with NGlG to block and then stained for intracellular CD3 (anti-huCD3, BD Biosciences). After washing once with blocking buffer (PBS + 5% normal goat serum), the cells were incubated with the secondary antibody, goat anti-mouse Alexa Fluor 568 (Invitrogen, Molecular Probes). After another wash with blocking buffer, the cells were incubated with 2 μg/ml purified anti-NF-xB p65 (Santa Cruz Biotechnology) or a 1:25 dilution of purified anti-NFAT 1 polyclonal antibody (Cell Signaling) in block/perm buffer for 1 hour. After washing twice with blocking buffer, the cells were incubated with goat anti-rabbit Alexa Fluor 488 (Invitrogen, Molecular Probes) for 30 minutes. The cells were washed twice with blocking buffer and twice with PBS before mounting the coverslips on glass slides with VECTASHIELD Mounting Medium (Vector Laboratories). Cells were then observed on a Zeiss LSM 510 Confocal Microscope with at least 100 CD3+ cells counted per condition.

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**Supplemental data**

Supplementary Figure 1. The majority of CD3+ T cells derived from TATs possess an effector memory T cell phenotype.

Download from [http://www.cancerimmunity.org/130314-suppl-fig-1.pdf](http://www.cancerimmunity.org/130314-suppl-fig-1.pdf) (0.18 MB PDF file).

Supplementary Figure 2. Multispectral imaging flow cytometry for assessing NF-xB and NFAT translocation.