Tumor-associated neutrophils induce apoptosis of non-activated CD8 T-cells in a TNFα and NO-dependent mechanism, promoting a tumor-supportive environment

Janna Michaelia,†, Merav E. Shaula,†, Inbal Mishaliana, Avi-Hai Hovavb, Liran Levya, Lidia Zolotriova, Zvi Granotc, and Zvi G. Fridlendera

‡Institute Of Pulmonary Medicine, Hebrew University Hadassah Medical Center, Jerusalem, Israel; bFaculty of Dental Medicine, Institute of Dental Sciences, Hebrew University Hadassah Medical Center, Jerusalem, Israel; cDepartment of Developmental Biology and Cancer Research, The Institute for Medical Research Israel-Canada, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

ABSTRACT

The role of neutrophils in tumor progression has become in recent years a subject of growing interest. Tumor-associated neutrophils (TANs), which constitute an important portion of the tumor microenvironment, promote immunosuppression in advanced tumors by modulating the proliferation, activation and recruitment of a variety of immune cell types. Studies which investigated the consequences of manipulating TAN polarization suggest that the impact of these neutrophils on tumor progression is considerably mediated by and dependent on the presence of CD8 T-cells. It has been previously shown that granulocytic myeloid regulatory cells, i.e. TANs and granulocytic myeloid-derived suppressor cells (G-MDSCs) are capable of suppressing CD8 T-cell proliferation and affect their activation. In the current study, we find that in addition, TANs isolated from different models of murine cancer promote immunosuppression by strongly inducing CD8 T-cell apoptosis. We demonstrate that the TNFα pathway in TANs is critical for the induction of apoptosis, and that the mechanism through which apoptosis is induced involves the production of NO, but not ROS. In the absence of pre-activation, TANs are capable of activating CD8 T-cells, but specifically induce the apoptosis of non-activated CD8⁺CD69⁻ cells. Despite this contradictive effect on T-cell function, we show in vivo that TANs suppress the anti-tumor effect of CD8 T-cells and abolish their ability to delay tumor growth. Our results add another important layer on the understanding of the possible mechanisms by which TANs regulate the anti-tumor immune response mediated by CD8 T-cells, therefore promoting a tumor-supportive environment.

Introduction

It is now well accepted that non-malignant immune cells present in the tumor modulate the tumor microenvironment to promote “immunotolerance” and support tumor growth.1,2 Myeloid cells such as neutrophils, macrophages, dendritic cells (DCs) and myeloid derived suppressor cells (MDSCs), represent a significant portion of the tumor microenvironment and impact tumor development at multiple levels, from the remodeling of the extracellular matrix to malignant transformation, angiogenesis and modulation of other tumor-infiltrating cells.3

Among the variety of leukocyte subsets reported to infiltrate the tumor microenvironment,3 CD8 cytotoxic T-cells (CTLs) have been attributed an anti-tumor function since their presence in the tumor environment provided a positive prognosis for patients.4-6 Nevertheless, multiple studies have shown that various mechanisms are activated in the tumor microenvironment leading to CTL exclusion,7 therefore impairing their potential anti-tumor effect. Along with the expression of FasL by tumor cells8 which was first associated with high levels of apoptosis in intra-tumoral T-cells, it is now clear that a significant part of the inhibitory mechanisms directed toward CD8 T-cells results from their interactions with other non-cancerous stromal cells, including tumor-infiltrating immune cells, and among them myeloid regulatory cells (MRCs).9-11

Cancer-related neutrophils were demonstrated by multiple studies to correlate poorly with patients outcomes12 and affect various aspects of cancer biology.13,14 There has been in recent years a growing interest in characterizing tumor-associated neutrophils’ (TANs) interactions with other tumor-infiltrating immune cell types and understanding how these might impact tumor progression.14,15 The interaction of T-cells with TANs has only recently been investigated.16-18 We and others previously reported that depending on their polarization, pro-tumor (N2) or anti-tumor (N1) TANs can differently impact T-cell subsets.17-20 TANs’ polarization is modulated by cytokines such as TGFβ or IFNγ,20,21 is modified upon entering the tumor microenvironment and changes with tumor progression.22 Treatment of tumor-bearing mice with TGFβ inhibitors was shown to promote the recruitment and activation of TANs with anti-tumor (N1) properties21 which correlated with a marked increase in CD8 T-cells anti-tumor activity.23 In contrast, deple-
driven increase in intra-tumoral neutrophil infiltration.\textsuperscript{21} In accordance with these findings, TGFβ inhibition shows a markedly decreased efficiency in immunodeficient SCID mice.\textsuperscript{23} Recently Eruslanov et al.\textsuperscript{16} demonstrated that TANs isolated from early stage human lung tumors are able to stimulate T-cell proliferation and activation. Altogether, these data support the notion that TANs’ impact on tumor progression is at least in part mediated through their interaction with CD8 T-cells.\textsuperscript{21}

The vast majority of the studies which have examined the modulation of T-cell function by cancer-related MRCs, such as MDSCs, tumor associated macrophages (TAMs) or TANs, have emphasized the suppressive effects of MRCs on T-cell proliferation, polarization or recruitment.\textsuperscript{9,24,25} Although a clear discrimination between G-MDSCs and TANs remains a subject of debate,\textsuperscript{26,27} the mechanisms by which TAN modulate CD8 T-cells anti-tumor function are still vague. In the present study, we sought to investigate the impact of TANs on CD8 T-cell survival, as a mechanism by which TANs can impair CD8 T-cells anti-tumor effect, therefore promoting a permissive environment.

Using an in vitro platform, we find that TANs isolated from established tumors markedly induce apoptosis in CD8 T-cells in a contact-dependent manner. This induction in apoptosis level was found to be TNFα-dependent and mediated via the release of NO. Surprisingly, TANs specifically induce apoptosis of non-activated CD8 T-cells, although we show that TANs are capable of activating them. Finally, we use in vivo models to show that TANs abolish the anti-tumor effect of CD8 T-cells and their ability to limit tumor growth. Our results add another important layer to the understanding of the different mechanisms by which TANs regulate the anti-tumor immune response mediated by CD8 T-cells. This dual and potentially conflicting regulation further supports the understanding that MRCs have many different regulatory effects on the immune system, controlling the anti-tumor activity of the host’s immune system. TANs can therefore exert a dual effect on CD8 T-cells, by simultaneously inducing apoptosis but at the same time promoting their activation.

Results

TANs induce CD8 T-cell apoptosis in 3 different models of thoracic malignancies

We first found that TANs significantly induce CD8 T-cell apoptosis. TANs isolated from primary AB12, LKRK or LLC thoracic tumor models, were co-cultured with CD8 T-cells isolated from the spleen of the same mice, and the rate of CD8 T-cell apoptosis was measured by AnnexinV-positive PI-negative staining. A representative gating of the flow cytometry results is showed in Fig. 1A. The presence of TANs dramatically increased the rate of CD8 T-cell apoptosis, in all 3 models (Fig. 1B), from an average of ~5–10% basal spontaneous apoptosis (CD8 cells alone) to 25–40% apoptosis in the presence of TANs. In contrast, naïve bone marrow neutrophils (BMN) had no effect on the apoptosis rate (Fig. 1B). This effect was also noted when TANs were incubated with T-cells isolated from naïve mice, although the amplitude of induction was smaller (Fig. 1C).

The collagenase treatment and isolation of the neutrophils did not appear to contribute for the differential effects of TAN and BMN on CD8 T-cells, as collagenase treatment had no impact on BMNs morphology and did not alter BMN incapacity to induce CD8 T-cell apoptosis (Supplementary Fig. 1).

TANs’ ability to mediate CD8 T-cell death did not appear to be influenced by the source or type of tumor model from which the cells were isolated. We found that TANs derived from AB12 (mesothelioma) or 4T1 (breast Ca.) tumors promoted apoptosis independently of the tumor type of origin (Supplementary Fig. 2A). Nevertheless, we found a clear difference in the amplitude of apoptosis driven by TANs originating from different tumors, as TANs isolated from LLC and AB12 tumors induced a significantly higher apoptosis percentage compared with TANs originating from 4T1 tumors, independent of the source of the T-cells (Fig. 1B and Supplementary Fig. 2).

Interestingly, we found a striking correlation between tumor progression and the level of CD8 T-cell apoptosis induced by TANs (Fig. 1D). TANs isolated from larger AB12 tumors showed higher induction of apoptosis, possibly reflecting a stronger tumor-permissive phenotype acquired by TANs as the tumor progresses, as we have previously reported.\textsuperscript{22} We then further evaluated TAN morphology, to better characterize the nature of the intra-tumoral Ly6G-positive cells. The H&E staining of TANs showed that these cells present fragmented nuclei (Fig. 1E), supporting the notion that most of TANs have a mature, neutrophil-like morphology and are not G-MDSCs.\textsuperscript{21}

NO production by TANs is required for them to induce T-cell apoptosis

Major pathways involved in the killing of target cells by neutrophils include the release of reactive oxygen species (ROS) such as hydrogen peroxide (H2O2) and O2\textsuperscript{−} (via the activation of the NADPH oxidase complex) and production of NO (following induction of iNOS). To assess whether ROS secretion by TANs is involved in the killing of CD8 T-cells, TANs and CD8\textsuperscript{+} cells were incubated in the presence or absence of the enzyme catalase (for the breakdown of H2O2) or apocynin (NADPH Oxidase complex blocker which blocks the production of O2\textsuperscript{−}, H2O2 and HOCl). Surprisingly, these exogenous inhibitors did not prevent CD8 T-cell apoptosis, and the percentage of apoptosis did not change significantly between the different experiments (Fig. 2A-B). These results indicate that the induction of T-cell apoptosis does not occur via oxidative burst and the activation of the NADPH oxidase complex.

We next tested whether NO production is involved in this induced apoptosis. Quantification of nitrite (NO\textsubscript{2−}) levels revealed that whereas TANs and CD8 T-cells cultured separately produced similar background NO levels, TAN co-cultured with CD8 T-cells produced significantly higher levels of NO. The addition of an iNOS inhibitor to the co-culture significantly inhibited the percentage of T-cell apoptosis by 7-fold compared with cellular death without inhibitor (Fig. 2C).

TANs induce CD8 T-cell apoptosis in a contact-dependent manner

Multiple studies have demonstrated granulocytes can impact target cells either through direct contact between the cells or via secretion of soluble factors (cytokines).\textsuperscript{28} To investigate
Figure 1. TANs Induce CD8 T-cell apoptosis in 3 different models of lung malignancies. TANs and bone marrow neutrophils (BMN) isolated from 3 different models of thoracic tumors (AB12, LKRM and LLC) were co-cultured at a ratio of 1:1 with CD8 T-cells. Apoptosis levels in the CD8\(^+\) population were then evaluated by flow cytometry using an AnnexinV-PI staining. (A) Representative gating of CD8 T-cell apoptosis at basal levels (left panel), following co-culture with TAN (middle panel) and following co-culture with BMN (right panel). (B and C) TANs isolated from AB12, LKRM and LLC tumors significantly induced apoptosis in CD8 T-cells isolated either from the spleen of the same tumor-bearing mice (CD8\(_T\)) (B) or from naïve animals (CD8\(_N\)) (C). (D) TANs isolated from advanced AB12 tumors of increasing sizes were co-culture at a ratio of 1:1 with CD8 T-cell and the level of apoptosis in CD8\(^+\) cells was assessed by flow cytometry using AnnexinV-PI staining. TANs’ ability to induce CD8 T-cell apoptosis positively correlates with tumor size. (E) Representative photographs of isolated TANs. The cells were centrifuged in cytopsin, fixed and stained using Hematoxylin&Eosin staining. Data represent the mean ± SEM from at least 3 independent experiments (n = 5–9), \({ }^{* *}<0.01,{ }^{* * *}<0.0001.\)
whether TANs-driven T-cell apoptosis requires direct cellular contact, we co-cultured TANs with CD8 T-cells using a trans-well assay system which would block any contact between the 2 cell types, yet allowing soluble factors to diffuse between the 2 chambers. We found apoptosis to be significantly inhibited when the contact between the cells was blocked, with T-cell apoptosis decreasing to levels comparable to the spontaneous rates seen in T-cells alone or in co-cultured with BMN (Fig. 2D). Similar results were obtained whether the cells were isolated from an AB12 (Fig. 2D) or an LKRM tumor (data not shown). These results show that direct contact between the cells is required to drive TAN’s induced CD8 T-cell apoptosis.

Surprisingly, blocking contact between the cells did not affect the levels of NO produced even tough apoptosis was significantly inhibited in this system (Fig. 2E). Together, these results point toward a paracrine, cytokine-dependent mechanism, in which cytokine(s) secreted by the T-cells induce NO production in TANs, whereas the induction of apoptosis itself requires close contact to take place.

**TNFα, but not IFNγ, mediates the production of NO and induction of CD8 T-cell apoptosis by TAN**

The involvement of IFNγ, known to induce NO production, in mediating CD8 T-cell apoptosis was next tested. To our surprise, the addition of an anti-IFNγ antibody had no effect on apoptosis (Fig. 3A), and only a minor effect on NO levels (Fig. 3B), suggesting that although IFNγ induces or amplifies to some extent NO production in TANs, T-cell apoptosis is mediated in this context via another mechanism.

TNFα is an additional major mechanism inducing iNOS and NO secretion. We therefore tested whether TNFα production by TANs is implicated in the induction of CD8 T-cell apoptosis. To do this, we used a TNFα-KO mouse model (TNFKO) and compared the induction of T-cell apoptosis in co-culture with TANKO vs. TANs isolated from wild type mice (TANWT). TNFα knockout TANs (TANKO) lost most of their ability to induce CD8 T-cell apoptosis (Fig. 3C). The significant decrease in NO secretion noted following co-culture with TANKO (Fig. 3D) implies TNFα as a major stimulus inducing NO production in TAN and T-cells’ death. As previously reported, we find that TANs from different models of thoracic malignancies express TNFα at various levels (Fig. 3E). To further characterize the TNFα-expressing TAN population, we assessed the expression of ICAM-1 and CD62L, markers of adhesion and activation, respectively. We found that only TNFα-positive TANs express CD62L whereas ICAM-1 is expressed in both TNFα-negative and TNFα-positive populations (Fig. 3E).
Figure 3. TNFα is crucial for the induction of CD8 T-cell apoptosis. (A and B) CD8 T-cells were cultured with or without TANs, in the absence or presence of a neutralizing antibody against IFNγ (αIFNγ). Apoptosis levels in CD8 cells were then measured by flow cytometry using AnnexinV-PI staining (A) and NO secretion was measured using Griess assay (B). (C and D) CD8 T-cells were co-cultured with TANs isolated from wild type tumor-bearing mice (TANWT) or with TANs isolated from TNF-KO mice (TANKO). CD8 cells apoptosis (C) and NO secretion (D) were then measured. (E) TANs' expression of TNFα in different models of thoracic tumors and the expression levels of ICAM1 and CD62 L in the TNF-negative (light gray) and TNF-positive (dark gray) populations were assessed. Bar plots represent the mean ± SEM from at least 3 independent experiments (n = 5–10), * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. n.s.- not significant.
Altogether, these results demonstrate that TNFα, but not IFNγ, is the main pathway mediating the induction of NO production and CD8 T-cell apoptosis by TAN.

**TANs specifically induce the apoptosis of non-activated (CD8+ CD69-) T-cells**

CD8 T-cells co-cultured with TANs show a dramatic increase in CD69 expression, suggesting an activation of these cells by TANs (Fig. 4A and supplementary Fig. 3) as previously reported. This activation is mediated by a contact dependent mechanism as confirmed with a transwell assay, as the physical separation between the 2 cell types impaired the increase in CD8+ CD69+ fraction compared with co-cultured with TANs. Similar results were obtained in all 3 tumor models tested (data not shown).

To determine whether the cells undergoing activation are the same cells undergoing apoptosis, we performed additional staining with CD69 and AnnexinV/PI. Surprisingly, we found a clear separation in the induction of apoptosis between CD69- and CD69+ cells. Compared to CD8 T-cells alone, the presence of TANs did not change the percentage of CD69+ AnnexinV+ PI−, i.e., active apoptotic cells (Fig. 4B). In contrast, the fraction of CD8+ CD69- AnnexinV+ PI−, i.e., non-activated apoptotic cells, increased significantly (Fig. 4C). In other words, although TANs promote their activation, they preferably induce apoptosis of the non-activated fraction of CD8 T-cell cells.

**TANs inhibit in vivo CD8 T-cell anti-tumor activity**

Finally, we aimed at validating the implications of TANs’ impact on CD8 T-cells in vivo, using a modified Winn Assay. LKRM tumor cells were injected to the flank of NOD/SCID mice in combination with either TANs, CD8 T-cells, or together with both cell types (Fig. 5A). Compared to tumor cells injected alone (control), the injection of TANs with the tumor cells had no impact on tumor growth. In contrast, the injection of CD8 T-cells together with the tumor cells strongly delayed tumor growth, resulting in significantly smaller tumors 7 d after injection. However, this T-cell inhibitory effect was abolished when CD8 T-cells were injected together with TANs (Fig. 5B). In comparison, BMN injected together with CD8 T-cells and LKRM cells showed no impact on CD8 T-cells’ ability to delay tumor growth (Fig 5B and supplementary Fig 4). These results show that TANs, but not BMN, inhibit *in vivo* the anti-tumor effect of CD8 T-cells, therefore promoting a permissive environment for tumor growth.

**Discussion**

Over the past decade, it has become evident that a variety of immune cell types present in the tumor microenvironment greatly contribute to the development of a permissive environment supporting tumor growth. There has been a growing interest in characterizing and understanding the mutual impacts between the various immune cell types infiltrating the tumor, emphasizing the role of MRCs. In the present study, we aimed to uncover the direct impact of TAN on CD8 T-cell survival as a mechanism by which TANs impair these cells’ anti-tumor effect, therefore promoting a permissive environment.

In our study, TANs were shown to induce CD8 T-cell apoptosis in several thoracic tumor models (Fig. 1). Interestingly, there was a striking correlation between the magnitude of this phenomenon and the size of the tumor studied, suggesting that this effect is more prominent in advanced large tumors (Fig. 1D). We found that NO secretion, but not ROS production, is the dominant mechanism through which TANs induce CD8 T-cell apoptosis (Fig. 2). Two of the major pathways known to promote NO secretion in neutrophils are mediated through IFNγ, by activation of Stat transcription factors, and TNFα in an NFκB dependent manner. Although depletion of IFNγ in a co-culture model decreased NO secretion to a certain extent (Fig. 2), it was not sufficient to prevent apoptosis.

![Figure 4](image-url)

**Figure 4.** TANs specifically induce the apoptosis of non-activated (CD69- CD8+) T-cells. (A) Expression of the CD69 activation marker in CD8 T-cells following co-culture with TANs (CD8+ TAN) and when contact between the cells was prevented (CD8//TAN) using a transwell assay. (B and C) Following co-culture of CD8+ cells with TANs, apoptosis levels in activated (CD8+ CD69+) (B) and non-activated (CD8+ CD69−) (C) T-cells was assessed by flow cytometry using the combined staining for CD8, CD69 and AnnexinV-PI. Data represent the mean ± SEM from at least 3 independent experiments (n = 5–10), **** < 0.0001.
Activation of TNFα pathway in neutrophils plays a major role in the priming and increased activity of these cells. Using a TNF-KO mouse model, we found a significant reduction in the ability of TANs lacking expression of TNFα to induce apoptosis in CD8 T-cells, which correlated with a strong downregulation of NO production (Fig. 3). Interestingly, TANs isolated from the 4T1 breast tumor model, which showed a relatively low level of apoptosis induction (Supplementary Fig. 2B) also showed very low levels of TNFα (data not shown). Altogether, our data identifies the TNFα pathway as a key mechanism in the induction of the NO-dependent CD8 T-cell apoptosis by TAN (Fig. 6).

It is possible that different sub-populations among TANs can differentially affect CD8 T-cells. It has long been suggested by us and others that tumor-associated neutrophils may be a heterogeneous population composed of phenotypically different neutrophils, such as N1 (anti-tumor) and N2 (pro-tumor) TANs. Although a great effort is currently made in the field toward finding specific markers which will allow the identification of the different neutrophils sub-populations in the tumor itself (as well as in the circulation), we do not currently have such markers to fully segregate between possible sub-populations of TANs. In this study, we find that TNFα+ TANs (but not TNFα− TANs) express CD62L together with high levels of ICAM-1, suggesting an advanced activation of the TNFα-expressing neutrophils (Fig. 3E). More studies are still needed to shed light on the functional differences between possible neutrophil sub-populations in the tumor.

In the context of a regulatory effect of tumor neutrophils, it is important to address the relationship between the myeloid-derived suppressor cells (MDSCs) and TANs. MDSCs, defined as immature myeloid cells of both monocytic and granulocytic lineages, accumulate in the circulation of advanced cancer patients and in the spleen, bone and circulation of tumor-bearing animals. These cells were named based on their ability to suppress the proliferation of the innate and adaptive immune system. The immaturity of the Granulocytic MDSCs (G-MDSCs) is most commonly used as a major feature differentiating them from fully mature neutrophils. Nevertheless,
since both cell types are recognized by Ly6G antibodies, an exact discrimination and classification of TANs vs. G-MDSCs is still a matter of debate,\textsuperscript{26,34} and many studies have refer to these 2 populations in an interchangeable way. Although we cannot exclude the presence of some G-MDSCs in the neutrophil fraction isolated from the tumor, the differentiated and fragmented morphology observed in TANs (Fig. 1E)\textsuperscript{31} indicates that the majority of the Ly6G\textsuperscript{+} cells present in the tumor are mature neutrophils and probably not G-MDSCs.

The ability of MDSCs to suppress T-cell function in the context of cancer has been addressed in multiple studies.\textsuperscript{35} However, most of these studies have focused on particular functional aspects such as T-cell proliferation, polarization or recruitment.\textsuperscript{36} MDSCs, either as a whole, or specifically G-MDSCs, were demonstrated in the context of cancer\textsuperscript{37,38} (as well as models of chronic inflammation\textsuperscript{39,40}) to inhibit T-cell proliferation by producing high amount of reactive oxygen species (ROS) and nitric oxide (NO),\textsuperscript{39} depleting key nutrient factors such as arginine,\textsuperscript{41,42} and tryptophan\textsuperscript{3,42} needed for T-cell proliferation. MDSCs were also shown to block T-cell activation,\textsuperscript{45,46} promote a pro-tumor phenotype through the upregulation of TGF\beta and IL-10,\textsuperscript{47,48} and impair T-cell recruitment via nitration of chemokines.\textsuperscript{49}

Our current findings also reinforce some of the functional differences between the mechanisms used by TANs compared with MDSCs and TAMs to affect T-cell function. In contrast to M-MDSCs and G-MDSCs, which were shown to exert their suppressive effect by producing massive amounts of ROS and NO, here we find NO production (but not ROS) to be the main pathway by which TANs induce CD8 T-cell apoptosis. Although the induction of apoptosis required close contact between the cells, NO production by TAN was found to occur independently of contact (Fig. 2E). This is in contrast to reports showing that direct contact between the T-cells and other myeloid cells (MDSCs or TAMs) is necessary for NO production.\textsuperscript{9,39} The TNF\alpha pathway, but not IFN\gamma, was found in this study to play a crucial role in NO production in TANs, in contrast to studies linking IFN\gamma as a major stimulus for NO secretion in MDSCs and TAMs.\textsuperscript{50} Finally, similarly to what has been recently shown in humans,\textsuperscript{16} we find that TANs induce the activation of CD8 T-cells and promote their secretion of IFN\gamma (data not shown), in contrast to MDSCs which have been shown to suppress T lymphocytes activation.\textsuperscript{35,51}

TANs’ ability to activate CD8 cells was demonstrated by an increase in CD69 expression in these cells (Fig. 4 and Supplementary Fig. 3) and induction of IFN\gamma expression following co-culture (data not shown). This activation was specific to tumor-infiltrated neutrophils, since naïve bone marrow neutrophils has no impact on CD8\textsuperscript{+} cells activation levels. Eruslanov et al. recently reported that TAN did not affect the CD69 expression of pre-activated T-cells.\textsuperscript{16} In the current study, CD8 T-cells were not pre-activated with anti-CD3 before co-culturing with TANs and 95% of total the T-cells were found to be CD69\textsuperscript{+} before co-culturing. The activation showed after co-culturing TANs with T-cells suggests that TANs are capable of activating these cells in the absence of pre-activation. It is possible that TANs activate CD8 T-cells in a weaker way than CD3 activation and therefore no further activation can be seen if combined with anti-CD3 pre-activation.

Because preventing direct contact inhibited T-cell death but not NO production (Fig. 2), we conclude that TANs activation must first occur in a paracrine way, leading to the activation of the TNF\alpha pathway and NO production in TANs. Following TAN activation, TANs then bind specifically to non-activated T-cells and promote their apoptosis via NO secretion (Fig. 6). Similarly to the paracrine effect leading to TAN activation and NO production described here, the suppression of T-cell function by mesenchymal stromal cells was also found to be dependent on the co-localization of the 2 cell types, whereas the secretion of cytokines leading to NO production was independent of close cellular contact.\textsuperscript{52} Our results point in a similar way toward a paracrine mechanism mediating TANs activation and subsequent NO secretion, while close contact between TANs and CD8 T-cells is required for mediating T-cell death. We examined the possible involvement of multiple key contact antigens and cytokines in the process. The \textit{in vitro} inhibition of Gr-1, Ly-6G, CD11b, CD86 and CD54 antigens had no effect on the ability of neutrophils to induce T-cell death (Supplementary Fig. 5); We found IFN\gamma, IL1\beta and IL12 to be upregulated following co-culture of CD8 T-cells and TANs but \textit{in vitro} inhibition of these cytokines did not impact apoptosis levels (Supplementary Fig. 5 and 6).

It has been well documented that following activation, T-cells can undergo apoptosis through a program termed activation-induced cell death (AICD): exposure of activated T-cells to a second activation stimulus (e.g. CD3/TCR stimulation in the absence of CD28 co-stimulation) promotes de novo production of FasL, leading to both autocrine and paracrine Fas/Fasl-mediated T-cell apoptosis.\textsuperscript{53,54} Bronte et al have previously suggested that MDSCs production of peroxynitrite causes activated T lymphocytes to undergo apoptosis and Yu et al.\textsuperscript{55} demonstrated that CD33\textsuperscript{+} MDSCs isolated from primary breast cancer tissues are able to induce apoptosis in both CD3/CD28 and IL2-activated T-cells. Similarly, TAMs were also demonstrated to induce activated T-cells apoptosis in an NO-dependent pathway.\textsuperscript{9} In contrast to all the studies mentioned above, CD8 T-cells in this study were not pre-activated before co-culturing with TANs. In the absence of pre-activation, we find that TAN preferentially induce apoptosis of non-activated (CD8\textsuperscript{+}CD69\textsuperscript{−}) T-cells (Fig. 4). Further studies will be needed to identify the signals involved in the cross-talk between these cells promoting CD8 T-cell death.

In this study, we therefore show 2 seemingly opposite effects of neutrophils on CD8 T-cells, i.e., activation of T-cells aside induction of apoptosis. As demonstrated by previous studies,\textsuperscript{37,58} we also find that TANs have the capacity to inhibit T-cell proliferation (data not shown). It is therefore possible that although TAN can activate T cells, the added effect of inhibiting proliferation and inducing apoptosis leads \textit{in vivo} to TANs’ inhibition of CD8 T-cells cytotoxic effect and consequent increased tumor growth (Fig. 5B). We believe that neutrophils, as important regulators of the immune system, can have actions that seem contradicted to each other in the context of cancer (i.e., with pro- and anti-tumor effects).

In conclusion, in the current study we demonstrate for the first time that in established tumors, tumor-associated neutrophils induce CD8 T-cell apoptosis in a TNF\alpha- and NO-dependent mechanism. Although TANs can induce CD8 T-cell
activation, they preferably promote apoptosis in non-activated T-cells. Although NO secretion was found contact-independent, apoptosis itself requires close contact between TANs and CD8 T-cells (Fig. 6). We therefore show a different regulatory effect of neutrophils on the adaptive immune system – induction of CD8 T-cell apoptosis, and elucidate the mechanism of this effect. These findings support recent notions that the regulatory effects of TANs and other myeloid cells on the adaptive immune system and specifically on CD8 T-cells comprises a more complex combination of different and sometimes contradictory effects, e.g., in simultaneously promoting T-cell activation and inducing apoptosis in non-activated cells. Our results therefore identify a new mechanism by which TANs are capable of inhibiting the anti-tumor immune response mediated by CD8 T-cells.

Since neutrophils have been previously and in this study demonstrated to acquire tumor- permissive characteristics as the tumor progresses, it may be possible to modulate cytotoxic T-cell inhibition by either altering TANs pro- vs. anti-tumor phenotype or blocking NO secretion at the tumor site. Impairing TANs induced-CD8 T-cell death would be expected to have beneficial effect on the cytotoxic immune response against tumor cells.

Materials and methods

Animals

C57 BL/6 and BALB/c mice, 6–8 week of age, 20–25 g weight, were purchased from Harlan Laboratories (Jerusalem, Israel). For the LKRM model, 129/SVJ mice purchased from Jackson Laboratories were bred with C57 BL/6 mice and the first generation only was used in experiments. Mice were housed under specific pathogen-free conditions at the Hebrew University School of Medicine Animal Resource Center. All experiments were executed in compliance with institutional guidelines and regulations and the protocols were approved by the Animal Research Committee of the Hebrew University School of Medicine. In all experiments, animals were killed before surgery.

Cell lines and flank tumor injection

AB12 (a murine malignant mesothelioma cell line), LLC (Lewis Lung Carcinoma) cell, LKR-M (Lung K-Ras Metastatic tumor cell line) and 4T1 (breast tumor cells) were cultured and maintained in DMEM (41965, Gibco) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (04–127–1 A, Biological Industries), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 12.5 units/ml nystatin (03–032–1B, Biological Industries). The cultures were maintained at 37°C and 5% CO2. All cell lines were regularly tested and maintained negative for mycoplasma contamination (EZ-PCR Mycoplasma test kit, KI-50341, Biological Industries). Mice were injected on the flank with 1–2 × 10^6 tumor cells in the appropriate syngeneic host. Tumor growth was measured every 2–3 days, and tumor volume was calculated using the formula volume = length × width^2 × 3.14/6. After reaching a size of 200–800 mm^3 in the different experiments, flank tumors were harvested, minced and digested at 37°C for 1 h in L15 medium (L1518) containing 0.2 mg/ml collagenase type I (C0130), 0.05 mg/ml collagenase II (C6885), 0.2 mg/ml collagenase type IV (C5138) (all from Sigma-Aldrich), 0.025 mg/ml DNase I (10104159, Roche Applied Science) and 0.025 mg/ml Elastase (LS002292, Worthington Biochemicals).

Neutrophil and CD8 T-cell isolation

Tumors were harvested and digested as mentioned above. Ly6G+ cells were isolated using the EasySep PE positive Selection Kit (18554, STEMCELL Technologies) according to the manufacturer’s protocol. For isolation of bone marrow (BM) neutrophils, BM was harvested from femurs and tibias of BALB/c mice under sterile conditions and suspended in PBS. Red blood cells were lysed using RBC lysis buffer (01–888–1B, Biological Industries), and Ly6G+ cells were purified using the EasySep PE positive selection kit. Purification of neutrophils was confirmed with FACs analysis with PE-conjugated anti-Ly6G antibody (127608, BioLegend), showing a purity of above 90% for TAN and above 98% for BMN. Neutrophil survival measured by Annexin/PI staining showed survival of above 80% for TAN and 90% for BMN.

CD8 T-cells were isolated from spleens of tumor-bearing mice. Spleens were harvested into HBSS buffer (02–018–1 A, Biological Industries) with 2% PBS, crushed, filtered through a 40 µm filter and centrifuged at 300 g for 10 min at RT. RBCs were lysed using ACK buffer and following centrifugation, the pellet was resuspended in EasySep buffer and CD8 T-cells were isolated using the EasySep CD8 negative selection kit (19853, STEMCELL Technologies).

Morphology of neutrophils

Immediately following isolation, TANs were prepared at a concentration of 50,000 cells/ml, and 100 µl were centrifuged at 1,000 rpm, 5 min, RT in a Shandon cytocentrifuge (Thermo Fisher Scientific). The neutrophils on the slides were then immediately fixed and stained with hematoxylin and eosin using Hemacolor Rapid Staining kit (111957, Merck). Samples were then mounted and the photographs were taken using ImageJ software under light microscopy (Nikon).

Apoptosis and activation assays

Isolated CD8 T-cells were seeded in a 96-well plate (5 × 10^5 cells per well) in the presence of absence of TANs or BMNs, in a ratio of 1:1. Some experiments were conducted in the presence or absence of 1000 U/ml Catalase (C1345, Sigma-Aldrich), 1 mM Apocynin (4663, Tocris Bioscience) or 100 µM NOS inhibitor N^G,N^G-Dimethyl-L-arginine di(p-hydroxyazobenzene-p'-sulfonate (SC-224167, Santa-Cruz Biotechnology). After 18 h incubation, cells were harvested, stained and analyzed by LSRII flow cytometer, as described in “Staining and Flow Cytometry” section. To test the contribution of contact-dependent mechanisms, apoptosis and activation assays were repeated using a transwell assay with 0.4 µm (CA3413, Costar) semi-permeable membrane. 1 × 10^6 CD8 T-cells were seeded at the bottom of each well in a 24-wells plate, whereas TANs were seeded on top of the membrane. Following overnight
incubation, T-cells only were harvested and stained as described in the "Staining and Flow Cytometry" section.

**Staining and flow cytometry**

Following treatment and incubation, cells were resuspended in FACS buffer (PBS supplemented with 2% FBS, 1 mM EDTA and 0.01% sodium azide) and blocked with “FcBlock” (anti mouse CD16/CD32, 101302, BioLegend). Cells were then stained for 30 min on ice for various markers. Antibodies and matched isotype controls were purchased from BioLegend: PE or APC anti-mouse Ly6G (127608 and 127613, respectively), APC anti-mouse TNFα (506308), APC or VioBlue450 anti-mouse CD8 (100712 and 100725, respectively), APC or PE anti-mouse CD69 (104513 and 104504, respectively), FITC anti-mouse CD54 (116105). Anti-mouse CD62 L eFluor450 (48–0621–80) was purchased from Biotest Ltd.

For intracelular staining, the cells were first blocked with FcBlock and stained for membranal antigens. The cells were then washed with FACS buffer, and further fixed and permeabilized using the Intracellular Fixation and Permeabilization buffer kit (88–8824–00, eBioscience). Following additional wash, the cells were then stained for intracellular markers.

For apoptosis level assessment, CD8 T-cells were first stained with APC anti-mouse CD8 antibody, washed and then stained with the AnnexinV FITC-PI staining kit (4700, MBL) according to manufacturer’s instruction. Activation levels were measured by co-staining with APC anti-mouse CD8 and PE anti-mouse CD69 antibodies. Immunostained cells were analyzed with LSRII flow cytometry (BD Biosciences) using Flowjo software (Ashland, OR).

**Nitric Oxide production by TAN**

Isolated TANs were placed in a 96-well plate (5 × 10⁵ cells per well) in the presence of absence of CD8 T-cells, in a ratio of 1:1. After 18 h, the media were collected, and NO levels in the supernatants were evaluated using the Griess Reagent System (Promega) according to manufacturer’s instruction. Supernatants were collected, and NO levels in the well were measured by co-staining with APC anti-mouse CD8 antibody, washed and then stained with APC anti-mouse CD8 and PE anti-mouse CD69 antibodies.

**Modified WINN assay**

TANs and CD8 T-cells isolated from tumors and spleens of LKRM-tumor bearing mice were further injected in various combinations with LKRM tumor cells into NOD/SCID mice. The mice were divided into 4 groups (n = 6–7) and injected subcutaneously on the right flank. The first group was injected 1 × 10⁶ LKRM cells; The second group a mixture of 1 × 10⁶ LKRM cells and 3 × 10⁶ CD8 T-cells (ratio of 1:3); The third group a mixture containing 1 × 10⁶ LKRM cells and 3 × 10⁶ TANs (ratio of 1:3); The fourth group a mixture containing 1 × 10⁶ LKRM cells, 3 × 10⁶ CD8 T-cells and 3 × 10⁶ TANs (ratio of 1:3:3). Tumor size was measured every 2 d for 12 d using a caliper and calculated according to the formula [(length x width² x 3.1416) / 6 mm³]. We next assessed the effect of BMN on CD8 anti-tumor effect in vivo. Four groups of mice were again evaluated for tumor growth following subcutaneous flank injection simultaneously of LKRM cells, LKRM cells with CD8 T-cells, LKRM cells and BMNs and a mixture of LKRM cells, CD8 T-cells and BMNs at the same ratios.

**Disclosure of potential conflicts of interest**

The authors declare no potential conflict of interest.

**Acknowledgments**

This work was supported by a grant from the Chief Scientist of Israel Ministry of Health (Grant 3–1103), and by a grant from the Israel Cancer Association (Grant 20150132).

This work was kindly backed by the COST Action BM1404 Mye-EUNITER (http://www.mye-euniter.eu). COST is supported by the EU Framework Program Horizon 2020.

**References**

1. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol. 2012;12:253-68. doi:10.1038/nri3175. PMID:22437938

2. Kumar V, Patel S, Tcyganov E, Gabrilovich DI. The nature of myeloid-derived suppressor cells in the tumor Microenvironment. Trends Immunol. 2016;37:208-20. doi:10.1016/j.it.2016.01.004. PMID:26858199

3. Grivennikov SI, Greten FR, Karin M. Inflammation, inflammation, and cancer. Cell. 2010;140:883-99. doi:10.1016/j.cell.2010.01.025. PMID:20303878

4. Diederichsen AC, Hjelmborg J, Christensen PB, Zeuthen J, Fenger C. Prognostic value of the CD4+/-CD8+ ratio of tumour infiltrating lymphocytes in colorectal cancer and HLA-DR expression on tumour cells. Cancer Immunol Immunother. 2003;52:423-8. doi:10.1007/s00262-003-0388-5. PMID:12695859

5. Halama N, Michel S, Kloor M, Zoernig I, Pommerenke T, von Knebel Doeberitz M, Schirmacher P, Weitz J, Grabe N, Jager D. The localization and density of immune cells in primary tumors of human metastatic colorectal cancer shows an association with response to chemotherapy. Cancer Immun. 2009;9:1. PMID:19226101

6. Katz SC, Pillarissetty V, Bamboat ZM, Shia J, Hedvat C, Gonen M, Jar-nagin W, Fong Y, Blumgart L, D’Angelica M, DeMatteo RP. T cell infiltrate predicts long-term survival following resection of colorectal liver metastases. Ann Surg Oncol. 2009;16:2524-30. doi:10.1245/s10434-009-0585-3. PMID:19568816

7. Joyce JA, Fearon DT. T cell exclusion, immune privilege, and the tumor microenvironment. Science. 2015;348:74-80. doi:10.1126/science.aaa6204. PMID:25838376

8. O’Connell J, Bennett MW, O’Sullivan GC, O’Callaghan J, Collins JK, Shanahan F. Expression of Fas (CD95/APO-1) ligand by human breast cancers: significance for tumor immune privilege. Clin Diagn Lab Immunol. 1999;6:457-63. PMID:10391843

9. Saio M, Radioja S, Marino M, Frey AB. Tumor-infiltrating macrophages induce apoptosis in activated CD8+ T cells by a mechanism requiring cell contact and mediated by both the cell-associated form of TNF and nitric oxide. J Immunol. 2001;167:5583-93. doi:10.4049/jimmunol.167.10.5583. PMID:11698429

10. Liu Y, Van Ginderachter JA, Brys I, De Baetselier P, Raes G, Geldhof AB. Nitric oxide-independent CTL suppression during tumor progression: association with arginase-producing (M2) myeloid cells. J Immunol. 2003;170:5064-74. doi:10.4049/jimmunol.170.10.5064. PMID:12734351

11. Pelletier M, Micheletti A, Cassatella MA. Modulation of human neutrophil survival and antigen expression by activated CD4+ and CD8+ T cells. J Leukoc Biol. 2010;88:1163-70. doi:10.1189/jlb.0310172. PMID:20686115

12. Shen M, Hu P, Donskov F, Wang G, Liu Q, Du J. Tumor-associated neutrophils as a new prognostic factor in cancer: a systematic review and meta-analysis. PLoS One. 2014;9:e98259. doi:10.1371/journal.pone.0098259. PMID:24906014
13. Granot Z, Jablonska J. Distinct functions of neutrophil in cancer and its regulation. Mediators Inflamm. 2015;2015:701067. doi:10.1155/2015/701067. PMID:26648665

14. Cofellt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral or more. Nat Rev Cancer. 2016;16:431-46. doi:10.1038/nrc.2016.52. PMID:27282249

15. Sionov RV, Fridlender ZG, Granot Z. The multifaceted roles neutrophils play in the tumor microenvironment. Cancer Microenviro. 2015;8:125-58. doi:10.1007/s12307-014-0147-5. PMID:24895166

16. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, Stephen TL, Ranathan A, Deshpande C, Akimoto T, Vachani A, Litzky L, Hancock WW, et al. Tumor-associated neutrophil stimulate T cell responses in early-stage human lung cancer. J Clin Invest. 2014;124:5466-80. doi:10.1172/JCI77053. PMID:25384214

17. Mishalani I, Bayuh R, Eruslanov E, Michaeli J, Levy L, Zolotarov L, Singhal S, Albelda SM, Granot Z, Fridlender ZG. Neutrophils recruit regulatory T-cells into tumors via secretion of CCL17-a new mechanism of impaired antitumor immunity. Int J Cancer. 2014;135:1178-86. doi:10.1002/ijc.28770. PMID:25401019

18. Cofellt SB, Kersten K, Doorbal CW, Weiden J, Vrijeland K, Hau CS, Venteggen NJ, Giampicotti M, Hawinkels LJ, Jonkers J, et al. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. Nature. 2015;529:435-8. doi:10.1038/nature16336. PMID:26729288

19. Andzinskii L, Wu CF, Lienenklaus K, Kroger A, Weiss S, Jablonska J. Delayed apoptosis of tumor associated neutrophils in the absence of endogenous IFN-beta. Int J Cancer. 2015;136:572-83. PMID:24806531

20. Andzinskii L, Kasnitz N, Stahnke S, Wu CF, Gereke M, von Kockritz-Blickwede M, Schilling B, Brandau S, Weiss S, Jablonska J. Type I IFNs induce anti-tumor polarization of tumor associated neutrophils in mice and human. Int J Cancer. 2016;138:1992-83. doi:10.1002/ijc.29945. PMID:26619320

21. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Ling L, Hancock WW, et al. Producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. Nature. 2015;529:435-8. doi:10.1038/nature16336. PMID:26619320

22. Suzuki E, Kim S, Cheung HK, Corbley MJ, Zhang X, Sun L, Shan F, Albelda SM. Polarization of tumor-associated neutrophil phenotype by TGF-beta: mechanisms for inhibiting antitumor immunity. Cancer Immunol Immunother. 2015;64:4499-506. doi:10.1007/s00262-014-1738-z. PMID:24501019

23. Torok-Storb B, D’Andrea AJ. Modulation of CD8(+) T-cell activation events by myeloid and granulocytic myeloid-derived suppressor cells. Immunobiology. 2013;218:1385-91. doi:10.1016/j.imbio.2013.07.003. PMID:23932436

24. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients. Cancer Res. 2001;61:4756-60. PMID:11406548

25. Moss E, Klein JC, Männ L, Klingenberg A, Gunzer M, Brandau S. Survival of residual neutrophils and accelerated myelopoiesis limit the efficacy of antibody-mediated depletion of Ly-6G+ cells in tumor-bearing mice. J Leukoc Biol. 2016;99:81-23. doi:10.1189/jlb.11H107-289R. PMID:26819319

26. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and infection. Cancer Immunol Immunother. 2009;58:451-66. doi:10.1007/s00262-008-0523-4. PMID:18446337

27. Srivastava MK, Zhu L, Harris-White M, Kar UK, Huang M, Johnson MF, Lee JM, Elashoff D, Strieter R, Dubinet S, Sharma S. Myeloid suppressor cell depletion augments antitumor activity in lung cancer. PLoS One. 2012;7:e40677. doi:10.1371/journal.pone.0040677. PMID:22815789

28. Nagaraj S, Youn JJ, Gabrilovich DI. Reciprocal relationship between myeloid-suppressor cells and T cells. J Immunol. 2013;191:17-23. doi:10.4049/jimmunol.1300654. PMID:1300654. doi:10.4049/jimmunol.1300654. PMID:23794702

29. Singhal S, Albelda SM, Granot Z, Fridlender ZG. Neutrophils recruit regulatory T-cells into tumors via secretion of CCL17-a new mechanism of impaired antitumor immunity. Int J Cancer. 2014;135:1178-86. doi:10.1002/ijc.28770. PMID:25401019

30. Das-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Meanwell A, Jordan C, Li, et al. Increased myeloid-suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother. 2009;58:49-59. doi:10.1007/s00262-008-0523-4. PMID:18446337

31. Srivastava MK, Zhu L, Harris-White M, Kar UK, Huang M, Johnson MF, Lee JM, Elashoff D, Strieter R, Dubinet S, Sharma S. Myeloid suppressor cell depletion augments antitumor activity in lung cancer. PLoS One. 2012;7:e40677. doi:10.1371/journal.pone.0040677. PMID:22815789

32. Singhal S, Albelda SM, Granot Z, Fridlender ZG. Neutrophils recruit regulatory T-cells into tumors via secretion of CCL17-a new mechanism of impaired antitumor immunity. Int J Cancer. 2014;135:1178-86. doi:10.1002/ijc.28770. PMID:25401019

33. Mazzoni A, Bronte V, Visinanti A, Spitzer JH, Apolloni E, Serafini P, Zanovello P, Segal DM. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. J Immunol. 2002;168:689-95. doi:10.4049/jimmunol.168.2.689. PMID:11779962

34. Ryan SO, Johnson JL, Cobb BA. Neutrophils confer T cell resistance to myeloid-suppressor-cell-mediated suppression to promote chronic inflammation. J Immunol. 2013;190:5037-47. doi:10.4049/jimmunol.1203404. PMID:23576679

35. Rodriguez PC, Quincon DG, Zabaleta J, Ortiz B, Zeh A, Piazzuelo MB, Delgado A, Correa P, Brayer J, Sotomayor EM, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res. 2004;64:5839-49. doi:10.1158/0008-5472.CAN-04-0465. PMID:15313928

36. Raber P, Ochoa AC, Rodriguez PC. Metabolism of L-arginine by myeloid-derived suppressor cells in cancer: mechanisms of T-cell suppression and therapeutic perspectives. Immuno Invest. 2012;41:614-34. doi:10.3109/0822461X.2012.680634. PMID:23017138

37. Friberg M, Jennings R, Alsarraj M, Dessureault S, Cantor A, Extermann M, Mellor AL, Munn DH, Antonia SJ. Indoleamine 2,3-dioxygenase contributes to tumor cell evasion of T cell-mediated rejection. Int J Cancer. 2010;127:151-5. doi:10.1002/ijc.25708. PMID:20100992

38. Vuutenhove C, Pilotte I, Theate I, Stroobant V, Colau D, Parmentier N, Boon T, Van den Eynde BJ. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med. 2003;9:1269-74. doi:10.1038/nm834. PMID:12924672

39. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients. Cancer Res. 2001;61:4756-60. PMID:11406548

40. N1*
tumor growth through interleukin-17. Cancer Res. 2008;68:3915-23. doi:10.1158/0008-5472.CAN-08-0206. PMID:18483277

48. Burkholder B, Huang RY, Burgess R, Luo S, Jones VS, Zhang W, Lv ZQ, Gao CY, Wang BL, Zhang YM, et al. Tumor-induced perturbations of cytokines and immune cell networks. Biochim Biophys Acta. 2014;1845:182-201. PMID:24440852

49. Molon B, Ugel S, Del Pozzo C, Zilio S, Avella D, De Palma A, Mauri P, Monegal A, Rescigno M, et al. Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. J Exp Med. 2011;208:1949-62. doi:10.1084/jem.20101956. PMID:21930770

50. Angulo I, Rullas J, Campillo JA, Obregon E, Heath A, Howard M, Munoz-Fernandez MA, Subiza JL. Early myeloid cells are high producers of nitric oxide upon CD40 plus IFN-gamma stimulation through a mechanism dependent on endogenous TNF-alpha and IL-1alpha. Eur J Immunol. 2000;30:1263-71. doi:10.1002/(SICI)1521-4141(200005)30:5%3c1263::AID-IMMU1263%3e3.0.CO;2-5. PMID:10820371

51. Bingisser RM, Tilbrook PA, Holt PG, Kees UR. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. J Immunol. 1998;160:5729-34. PMID:9637481

52. Zinocker S, Vaage JT. Rat mesenchymal stromal cells inhibit T cell proliferation but not cytokine production through inducible nitric oxide synthase. Front Immunol. 2012;3:62. doi:10.3389/fimmu.2012.00062. PMID:22566943

53. Alderson MR, Tough TW, Davis-Smith T, Braddy S, Falk B, Schooley KA, Goodwin RG, Smith CA, Ramsdell F, Lynch DH. Fas ligand mediates activation-induced cell death in human T lymphocytes. J Exp Med. 1995;181:71-7. doi:10.1084/jem.181.1.71. PMID:7528780

54. Ju ST, Panka DJ, Cui H, Ettinger R, el-Khatib M, Sherr DH, Stanger BZ, Marshak-Rothstein A. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature. 1995;373:444-8. doi:10.1038/373444a0. PMID:7530337

55. Bronte V, Serafini P, Mazzoni A, Segal DM, Zanovello P. L-arginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol. 2003;24:302-6. doi:10.1016/S1471-4906(03)00132-7. PMID:12810105

56. Yu J, Du W, Yan F, Wang Y, Li H, Cao S, Yu W, Shen C, Liu J, Ren X. Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer. J Immunol. 2013;190:3783-97. doi:10.4049/jimmunol.1201449. PMID:23440412

57. Rotondo R, Barisone G, Mastracci L, Grossi F, Orengo AM, Costa R, Truini M, Fabbri M, Ferrini S, Barbieri O. IL-8 induces exocytosis of arginase 1 by neutrophil polymorphonuclears in nonsmall cell lung cancer. Int J Cancer. 2009;125:887-93. doi:10.1002/ijc.24448. PMID:19431148

58. Wang TT, Zhao YL, Peng LS, Chen N, Chen W, Lv YP, Mao FY, Zhang JY, Cheng P, Teng YS, et al. Tumour-activated neutrophils in gastric cancer foster immune suppression and disease progression through GM-CSF-PD-L1 pathway. Gut. 2017. doi:10.1136/gutjnl-2016-313075