

Tumor-associated neutrophils (TAN) develop pro-tumorigenic properties during tumor progression

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Abstract The role and characteristics of tumor-associated neutrophils (TAN) in cancer are poorly defined. We have recently shown that TAN can have anti-tumorigenic (N1) or pro-tumorigenic (N2) functions. An interesting unanswered question is how the phenotype of TAN is influenced by the ongoing evolution of tumor microenvironment. We therefore studied the phenotype and effects of TAN at different time points during tumor progression. We used two models of murine tumor cancer cell lines—Lewis lung carcinoma (LLC) and AB12 (mesothelioma). Neutrophils were studied at early and late stages and compared to each other and to neutrophils from bone marrow/periphery of naïve mice. Although there was no difference in the number of neutrophils entering the tumor, we found that at early stages of tumor development, neutrophils were almost exclusively at the periphery of the tumor. Only at later stages, neutrophils were also found scattered among the tumor cells. We further found that TAN from early tumors are more cytotoxic toward tumor cells and produce higher levels of TNF- α , NO and H₂O₂. In established tumors, these functions are down-regulated and TAN acquire a more pro-tumorigenic phenotype. In line with this phenotype, only depletion of

neutrophils at later stages of tumor development inhibited tumor growth, possibly due to their central location in the tumor. Our work adds another important layer to the understanding of neutrophils in cancer by further characterizing the changes in TAN during time. Additional research on the functional role of TAN and differences between subsets of TAN is currently underway.

Keywords Tumor immunology · Tumor-associated neutrophils · Lung cancer · Mesothelioma · Tumor microenvironment

Introduction

Tumors evade the immune system despite the presence of non-self antigen, by developing a complex immunosuppressive network that paralyzes the effector arms of the immune system [1]. Tumor immunosuppression is directed by inhibitory cytokines [2, 3] and more importantly, by myeloid cells, including myeloid derived suppressor cells (MDSC), tumor-associated macrophages (TAM) and tumor-associated neutrophils (TAN) [4–6]. These myeloid cells have been shown to promote tumor progression by direct immune suppression and by production of angiogenic factors, matrix-degrading enzymes and growth factors [7, 8].

Macrophages are known to be plastic cells that can adopt different phenotypes depending on the immune context. Microenvironmental stimuli can drive macrophages either toward a “classic” (M1) anti-tumorigenic state or an “alternative” (M2) pro-tumorigenic phenotype [9–11]. Macrophages present in malignant tumors (TAM), exhibit predominantly an M2-like phenotype [12–14]. However, there is growing evidence that phenotype is depended on

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the stage of tumor development, with M1-like phenotype in sites where tumors develop and an M2-like phenotype in established tumors [15].

In contrast to TAM, there is only limited work on the role and characteristics of TAN in tumor development. Neutrophils have been linked with angiogenesis, tumor development and metastasis [16–18], while others have shown that neutrophils can counteract the progression of malignancies through direct tumor cytotoxicity [19, 20] and enhancement of anti-tumoral mediators [21]. We have recently shown that like TAM, TAN can have anti-tumorigenic (N1) or pro-tumorigenic (N2) functions, and we demonstrated in the established tumor models studied that the predominant N2 phenotype was driven by the presence of TGF- β [22]. In other studies, Jablonska et al. [23] suggested that interferon-beta (IFN- β) can polarize TAN toward N1 phenotype. Granot et al. [24] have recently shown that the neutrophils residing in areas at which metastases later develop can inhibit tumor growth both directly and by secretion of immune modifiers.

Since it is known that the tumor microenvironment evolves as tumors progresses [25], an interesting unanswered question in these studies was how the phenotype of TAN was influenced by the stage of tumor development. We therefore analyzed the phenotype of TAN at different time points during tumor development with a focus on their ability to affect tumor growth. We then assessed the effect of depleting TAN at different time points during tumor progression. Our results show that TAN at early stages of tumor growth are more cytotoxic to tumor cells both directly and indirectly, whereas later in tumor growth they acquire a more supportive phenotype for tumor growth. Understanding the effect of tumor on neutrophils and the mechanisms tumor microenvironment modifies TAN, as well as the ways these cells can support or fight cancer, could help us developing strategies to direct the immune system against the tumor.

Materials and methods

Animals

C57BL/6 and BALB/c mice were purchased from Harlan Laboratories (Jerusalem, Israel). Mice were housed under specific pathogen-free conditions at the Hebrew University School of Medicine Animal Resource Center. The protocols were approved by the Animal Research Committee of the Hebrew University School of Medicine. In all experiments, animals were euthanized prior to surgery.

Cell lines

AB12, a murine malignant mesothelioma cell line, derived from an asbestos-induced tumor in a BALB/c mouse has

been previously described in detail [26]. Lewis lung carcinoma (LLC) cell line (syngeneic to C57BL/6 mice) was purchased from the American Type Culture Collection (Manassas, VA). Tumor cells were cultured and maintained in DMEM supplemented with 10 % heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 12.5 units/ml nystatin (all from Biological Industries, Beit Haemek, Israel). The cultures were maintained at 37 °C in an atmosphere containing 5 % CO₂. All cell lines were regularly tested and maintained negative for mycoplasma contamination.

Animal model

Mice were injected on the right flank with 2×10^6 AB12 or LLC tumor cells in the appropriate syngeneic host. Tumor growth was measured every 2–3 days, using the accepted formula for tumor volume of length \times width² \times 3.14/6. At indicated times, flank tumors were harvested from the mice, minced and digested with 1.5 mg/ml DNase I (Roche applied science) and 3 mg/ml collagenase type IV (Sigma-Aldrich) at 37 °C for 1 h. TAN were evaluated using the different methods described below, at 7 (early) and 14 (late) days after cells inoculation. The mean size \pm SD of tumors at 7 days was 164 ± 65 mm³ for AB12 tumors ($n = 38$) and 107 ± 43 mm³ for LLC tumors ($n = 49$). At 14 days, the average size of tumors was 368 ± 226 mm³ ($n = 32$) for AB12 tumors and 275 ± 97 mm³ ($n = 36$) for LLC tumors.

FACS analysis of tumors

Tumor cells were suspended in flow cytometry buffer (PBS supplemented with 2 % FCS and 0.01 % sodium azide), blocked with “Fc blocker” (CD16/CD32) and stained with FITC-conjugated anti-Mac-1 (CD11b), PE-conjugated anti-Ly6G (1A8), allophycocyanin-conjugated anti-ICAM-1 or matched isotype controls (all from BioLegend, San Diego, CA). For intracellular cytokine analysis, tumor cells were stimulated with 1 μ g/ml LPS (Sigma-Aldrich) for 3 h, treated with GolgiPlug™ (BD Biosciences, San Jose, CA) and stained with Abs to CD11b and Ly6G. After fixation and permeabilization, cells were stained with allophycocyanin-conjugated anti-TNF alpha (BioLegend). Immunostained cells were analyzed with LSRII flow cytometry (BD Biosciences) using FlowJo software (Ashland, OR).

Neutrophil isolation

Tumors were harvested and digested as mentioned above. Ly6G⁺ cells were isolated using the EasySep PE Selection Kit (STEMCELL technologies, Vancouver, Canada) 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. RBCs were lysed using lysis buffer containing 0.15 M NH_4Cl , 10 mM KHCO_3 and 0.1 mM $\text{Na}_2\text{-EDTA}$, and Ly6G⁺ cells were purified using the EasySep PE selection kit. Purification of neutrophils was confirmed with FACS analysis with PE-conjugated anti-Ly6G abs, showing a purity of above 85 % for TAN and above 98 % for BMN. For isolation of blood neutrophils, whole blood was collected by cardiac puncture using heparinized syringe. The blood was diluted with PBS-BSA (0.5 %) and subjected to a discontinuous Histopaque gradient (1.077 and 1.119, Sigma). Neutrophils were collected from the 1.077–1.119 interface, and RBCs were eliminated by hypotonic lysis. Neutrophils purity was >98 %.

Immunohistochemistry (IHC) staining of tumor neutrophils

Formalin-fixed, paraffin-embedded tumor sections were deparaffinized with xylene and rehydrated through gradient ethanol immersions (100 and 96 %, respectively). Endogenous peroxidase activity was quenched by 3 % H_2O_2 (DAKO, Glostrup, Denmark). Ag retrieval of Ly6G was performed by microwaving the section for 20 min in citric acid buffer (pH 6) (Dako Cytomation, DAKO). The slides were washed twice in distilled water and treated again with 3 % H_2O_2 . The sections were washed with PBS for 5 min and then incubated with a 1/900 dilution of anti-Ly6G (BD Biosciences). Sections were treated with anti-rat HRP (ImmPRESS Reagent, VECTOR laboratories, Burlingame, CA) for 30 min following three washes with PBS. Reaction product was visualized using the DAB substrate kit (VECTOR laboratories). Sections were counterstained with hematoxylin for 20 s and rinsed with tap water, immediately dehydrated by sequential immersion in gradient ethanol and xylene, and then mounted with mounting solution (VECTOR laboratories) on coverslips. Stained cells were evaluated under a light microscope.

Evaluation of tumor cytotoxicity by TAN

Tumor cytotoxicity was evaluated using an AB12 mesothelioma cell line transfected with a luciferase reporter (AB12-Luc), cocultured with TAN isolated from tumors at 7 and 14 days after AB12 inoculation or with neutrophils isolated from blood of naïve BALB/c mice (Blood Neutrophils, BN). Five-thousand AB12-Luc cells were plated in each well in 96-well plates and cocultured with TAN/BN cells at a ratio of 2.5, 5 or 10 TAN/BN to one AB12-Luc cell. After 24 h, non-adherent cells were washed away with PBS, the number of surviving cells was evaluated using Luciferase Assay Systems (Promega, Madison WI) and the percentage of killing was calculated.

Secretion of NO and H_2O_2 by TAN

TAN were isolated from tumors at 7 and 14 days after cells inoculation or isolated from blood of naïve mice. TAN were placed in a 96-well plate (2.5×10^5 cells per well) with culture media. After 24 h, the media were collected, and NO levels were evaluated using the Griess reagent system kit (Promega). H_2O_2 levels were evaluated in isolated TAN/BN, following activation with 1 mM PMA (Sigma-Aldrich) for 30 min, using a Hydrogen Peroxide Colorimetric Detection Kit (Abnova, Taipei, Taiwan).

RNA isolation and real-time RT-PCR

RNA was isolated from TAN using the PerfectPure™ RNA Cell & Tissue Kit (5 PRIME, Hamburg, Germany). In all samples used, the absorbance at 260/280 nm (for mRNA purity) was at a ratio of above 1.9. cDNA was made using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Carlsbad, California). Gene expression was assessed using relative quantification ($\Delta\Delta\text{Ct}$) RT-PCR and is shown as relative expression in TAN versus BM neutrophils, or comparing late TAN with early TAN. RNA level was normalized to GAPDH levels. Each sample was run in triplicate, and the experiment was repeated 3 times. The list of primers used is detailed in Supplementary Table 1.

Ly6G neutrophil depletion

Systemic Ly6G depletion was achieved by i.p. injection of 300 μg of purified monoclonal anti-Ly6G antibody 1A8 (BioXCell, West Lebanon, NH), in 100 μl PBS before or after tumor inoculation. Antibodies were injected every 2–3 days. As we have previously shown [22], neutrophil depletion lasted for 3 days following the last injection of anti-Ly6G. One day following 2–3 injections of antibody, mice were killed, and blood and tumor were taken for neutrophil depletion analysis. Depletion of neutrophils from the blood as well as intratumoral depletion of TAN was shown by flow cytometry using the anti-Ly6G antibody (Supplementary Figure 1). In order to confirm that the lack of Ly6G cells was not due to the fact that the Ly6G epitope was blocked by the in vivo administered antibody (and therefore not accessible to the FACS antibody), we used a secondary antibody directed to the Ly6G antibody that was given in vivo for depletion. Adding the secondary antibody did not change the results as evaluated by flow cytometry, confirming the depletion of neutrophils (Supplementary Figure 2). We further analyzed tumor sections of control and neutrophil-depleted mice by immunohistochemistry of Ly6G, using a secondary antibody capable of recognizing the depleting antibody as well. As can be seen in supplementary Figure 3, these IHC stainings further confirmed depletion of neutrophils in the tumor.

Statistical analysis

For studies comparing differences between two groups, we used unpaired Student's *t* test. For studies comparing more than two groups, we used analysis of variance (ANOVA) with appropriate post hoc testing. Differences were considered significant when $p < 0.05$. Data are presented as mean \pm SD.

Results

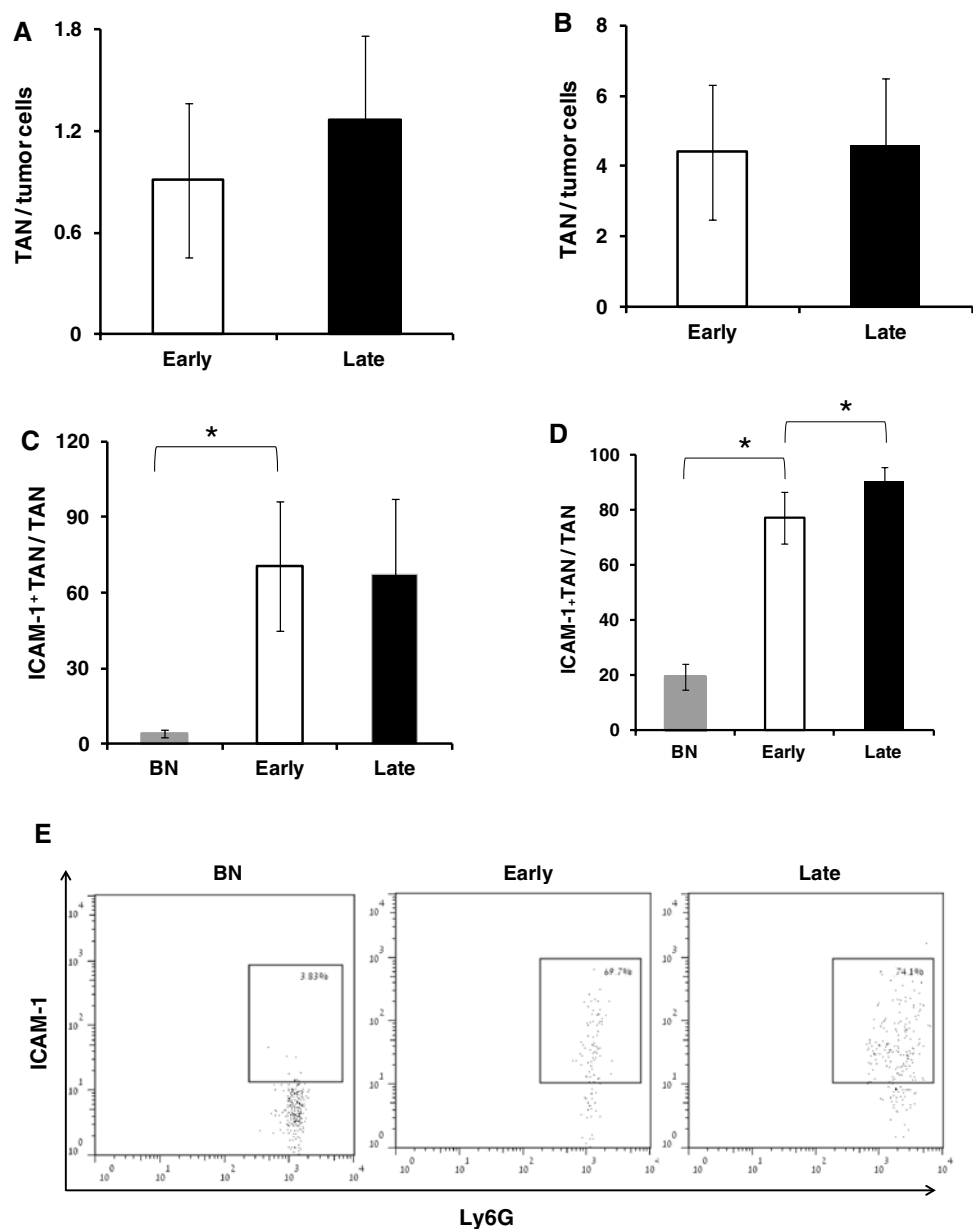
Neutrophils infiltration into the tumors

In order to characterize the phenotype of TAN during tumor development, we studied flank tumors isolated from

mice in which AB12 mesothelioma tumors or LLC lung tumors were growing. Early tumors (7 days after cell inoculation with a size of about 100–150 mm³) and established tumors (14 days after cell inoculation with a size of about 300 mm³) were harvested and subjected to FACS analysis of TAN cells. The percentage of TAN in the early tumors (defined as the percent of CD11b⁺/Ly6G⁺ cells out of the total number of tumor cells) was 0.9 ± 0.4 % in AB12 tumors (Fig. 1a) and 4.4 ± 1.8 % in LLC tumors (Fig. 1b). The percentage of TAN out of AB12 tumor cells was slightly increased at later stages (1.2 ± 0.5 %, NS). There was no difference in the percentage of TAN in early, compared to late, LLC tumors.

Adhesion molecules, such as ICAM-1 (CD54), are involved in the interaction between endothelial cells and

Fig. 1 TAN influx during tumor development. The percentage of CD11b⁺Ly6G⁺ (TAN) cells out of all tumor cells after AB12 (a) or LLC (b) cell inoculation. Each bar represents the mean \pm SD of 9 mice, combined from 2 separated experiments. Percentage of ICAM-1 in CD11b⁺LY6G⁺ cells as assessed using flow cytometry at early and late stages of AB12 (c) and LLC (d), compared to the expression in neutrophils from blood of syngeneic naïve mice (BN). Each bar represents the mean \pm SD of 9 mice, combined from 2 separate experiments. e Shows representative FACS tracing of ICAM-1 in CD11b⁺LY6G⁺ cells. The number in the gate is the percentage out of CD11b⁺LY6G⁺ cells, * $p < 0.05$



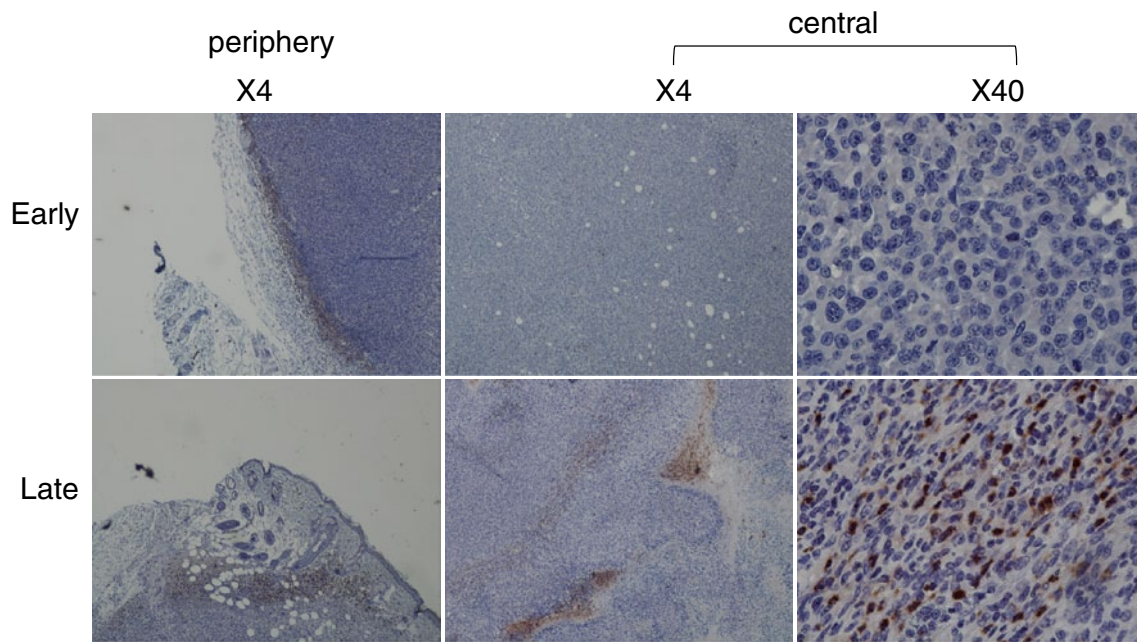


Fig. 2 TAN distribution within tumor. Representative photomicrographs of tumor sections labeled with anti-Ly6G mAb (immunoperoxidase method) at early (7 day—*top*) and late (14 day—*bottom*) stages after AB12 cells inoculation. The periphery of the tumor is

shown in the *left panel* and the *central part* of the tumor in the *central and right panel*. This staining was done in 3 separate mice of each group with similar results

neutrophils leading to the accumulation of neutrophils at the site of inflammation, and ICAM-1 has been considered a marker of activated neutrophils [27]. We therefore compared by flow cytometry the expression of ICAM-1 on TAN at different time points of tumor progression, to their expression in isolated neutrophils from naïve blood. We found that a markedly high percentage of both early and late TAN expressed ICAM-1 in both AB12 and LLC tumors, compared with the expression of ICAM-1 in naïve neutrophils (Fig. 1c, d and representative traces at Fig. 1e, $p < 0.05$ naïve neutrophils vs. each early and late, in both lines). There was no significant difference, however, in the expression of ICAM-1 between the early and late TAN (Fig. 1c–e).

We next evaluated whether the localization of TAN in the tumor changed with tumor progression. At early stages of tumor development, neutrophils were found almost exclusively at the periphery of the tumor, with almost no staining for neutrophils seen in the more central parts of the tumor (Fig. 2, top panel). TAN were stained in the periphery of the tumor at later stages as well, but at this point we also found many neutrophils scattered among the tumor cells, suggesting that only at later stages, are the TAN attracted to the center of the tumors (Fig. 2, bottom panel). We found no clear differences in the morphology of early versus late neutrophils (Supplementary Figure 4).

TAN tend to have more anti-tumor phenotype at early stages and become more tumorigenic at later stages of tumor development

To characterize the phenotype of TAN, we evaluated their cytotoxic potential, i.e., their ability to directly kill tumor cells. TAN (Ly6G⁺ cells) were isolated from early and late tumors and cocultured at different ratios with luciferase-labeled AB12 tumor cells for 24 h, after which the number of viable tumor cells was determined. The TAN showed a dose-dependent ability to kill tumor cells. However, at every ratio examined, the killing of tumor cells by TAN from early tumors was higher than the killing of tumor cells by TAN isolated from late, established tumors, with a statistical significant difference at a ratio of 10:1 ($p < 0.05$). Neutrophils isolated from naïve mice were completely non-cytotoxic to tumor cells (Fig. 3).

In order to elucidate possible mechanisms by which TAN can directly kill tumor cells, we next measured NO and H₂O₂ secretion from purified TAN. TAN from early AB12 tumors secreted $3.064 \pm 0.56 \mu\text{M NO}/2.5 \times 10^5$ cells compared with $1.7 \pm 0.19 \mu\text{M NO}/2.5 \times 10^5$ cells in TAN from late AB12 tumors (Fig. 4a, $p < 0.01$). In LLC, early TAN secreted $12.7 \pm 2.19 \mu\text{M NO}/2.5 \times 10^5$ cells compared with $3.8 \pm 2.16 \mu\text{M NO}/2.5 \times 10^5$ cells in late TAN (Fig. 4b, $p < 0.01$). The level of NO secreted by early and late TAN, however, was significantly lower than the

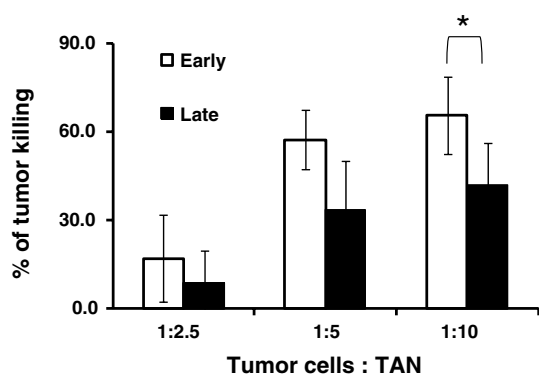


Fig. 3 TAN are capable of killing tumor cells ex vivo. TAN were purified from pooled tumors ($n = 5-6$) at early (7 day—white) and late (14 day—black) stages after AB12 cells inoculation. Isolated TAN were cocultured with AB12-Luciferase cells at different ratios for 24 h, and the percentage of tumor cells killing was calculated. Each bar represents the mean \pm SD at each ratio of 3 (early) and 6 (late) samples, combined from 2 separate experiments, * $p < 0.05$

level of secretion from naïve blood neutrophils (data not shown). The level of H_2O_2 secretion from PMA-activated cells was also higher in TAN isolated from early compared with late tumors, in both AB12 and LLC tumors (Figs. 4c, d, respectively, $p < 0.01$ for AB12 only). The secretion from early TAN was similar to that seen from naïve blood neutrophils (Fig. 4c).

We also analyzed the level of spontaneous intracellular TNF- α production in (CD11b⁺/Ly6G⁺) TAN at the single cell level using FACS analysis. The percentage of TAN from early AB12 tumors expressing TNF- α was 10.8 ± 4.6 %, compared with only 5.7 ± 2.95 % of TAN from established tumors ($p < 0.05$) (Fig. 4e, and representative FACS traces in Fig. 4f). In LLC tumors, however, the percentage of TAN producing TNF- α increased in late tumors compared with early tumors (Supplementary Figure 5), possibly reflecting differences in the rate of tumor growth and the appearance of necrosis.

Combined, these data could explain the lower cytotoxicity shown in TAN from established tumors compare to those from early tumor. Moreover, these data support the hypothesis that the phenotype of TAN is influenced by tumor development, becoming more tumor promoting during tumor progression.

mRNA evaluation of TAN at early and late stages

To study other phenotypic changes in the neutrophils during tumor progression, we performed real-time RT-PCR of selected receptors, chemokines and cytokines. We compared the fold changes in mRNA levels in TAN isolated from tumors at early and late time points to their expression in naïve neutrophils isolated from BM (Table 1). We

examined mainly mRNA levels of several molecules that were previously shown to be preferential in N1 or N2 TAN [4, 22, 28]. As expected, “N2-associated genes” such as CCL17, CCL2, ARG1, CCL5 and VEGF were all highly expressed in the TAN subset from both AB12 and LLC tumors (Table 1). For example, the expression of CCL17, CCL2 and Arg1 was up-regulated by a mean of 5,100-, 1,980-, and 1,800-fold, respectively, in TAN from AB12 tumor compared with BM neutrophils. N1-associated genes like CCL3, ICAM1 and iNOS were also up-regulated in TAN but with lower fold change compared to BM neutrophils.

We have previously shown that N1-like TAN up-regulate genes associated with CD8⁺ recruitment and activation [29], whereas N2-associated genes are involved in T-reg attraction and activation and in recruitment of macrophages, thus supporting tumor growth. Our data support the existence of “mixed” TAN phenotype in the tumor as shown previously for TAM [30–32]. These data suggest that TAN have a differential activation state, being more N2 oriented, and possibly implying that the percentage of N2-like TAN in the tumor increases as tumor progresses. However, as previously mentioned, some N1-markers were elevated as well during tumor progression. Comparisons of mRNA levels between established and early tumor showed also that other chemokines and cytokines, such as CXCL2, IL1 β , IL10 and IL12, were up-regulated in TAN isolated from established AB12 tumor, compared with early tumors (Table 1).

The functional importance of TAN at early versus late stages of tumor development in vivo

We next studied the functional significance of TAN in tumors at different stages of tumor development. This was done by depleting TAN using a specific anti-Ly6G monoclonal antibody. The anti-Ly6G, 1A8, antibody was injected intraperitoneally to mice following tumor inoculation. Neutrophil depletion was vigorously confirmed in the blood and intratumorally as described above (Supp. Fig. 1-3). Systemic neutrophil depletion at early stage of tumor development did not show any effect (Fig. 5a). In contrast, neutrophil depletion that started after the tumors were well established resulted in significantly reduced tumor growth ($p < 0.05$) (Fig. 5b). After neutrophil depletion was terminated, the growth inhibition stopped, and the tumors started to grow again. Together, these data indicate that TAN in established tumor contribute to tumor growth, whereas TAN in early stages of tumor development do not contribute to tumor growth, supporting the hypothesis that TAN develop a more pro-tumorigenic phenotype as tumor progresses, and possibly implying that TAN can enter and support tumor progression only at later stages of tumor growth.

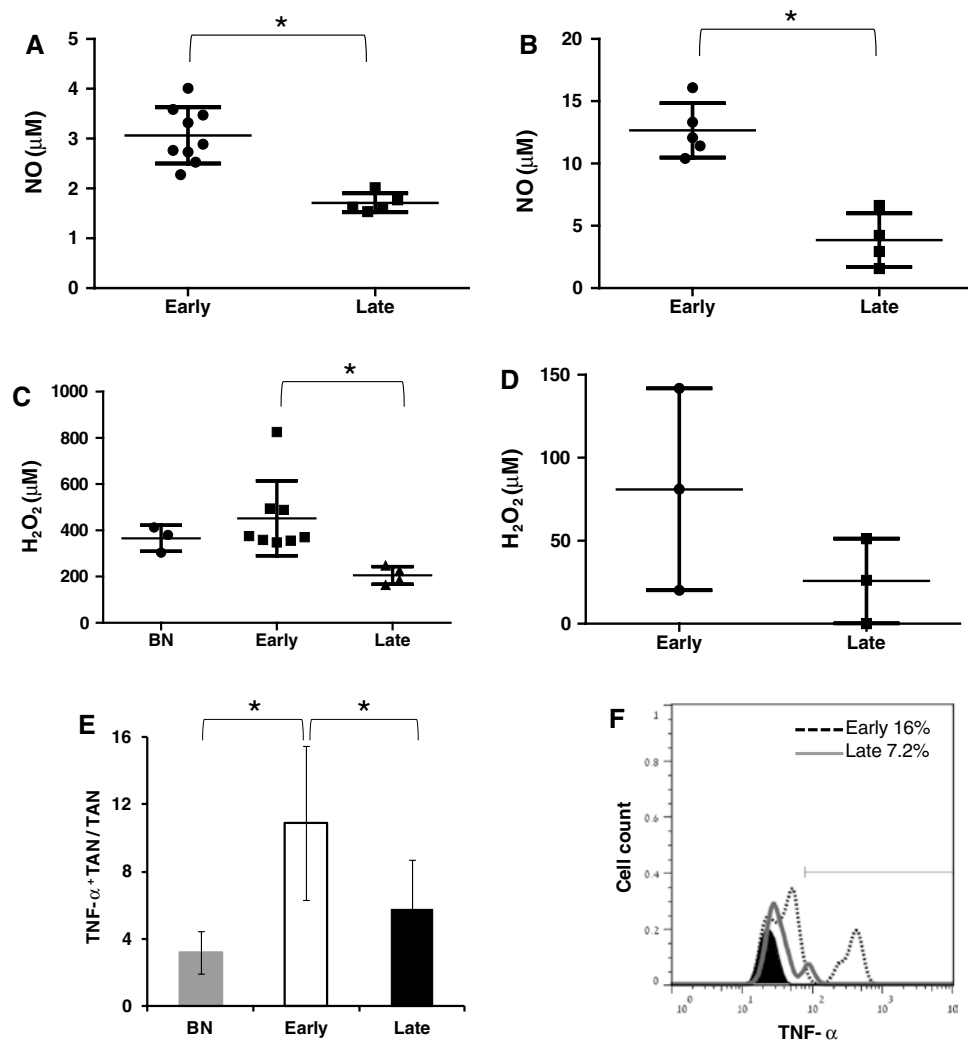


Fig. 4 Potential cytotoxic secretion of ROS and $\text{TNF-}\alpha$ expression in TAN increases with tumor progression. TAN were purified from pooled tumors ($n = 5\text{--}6$) at early (7 day—white) and late (14 day—black) stages after AB12 or LLC cells inoculation. Blood neutrophils (BN) were purified from blood of naïve BALB/c mice ($n = 5$). Detection of NO released from AB12 (a) and LLC (b) tumors, after culturing 2.5×10^5 cells in medium for 24 h. Each dot represents one sample. Samples were combined from 2 separate experiments. Mean \pm SD is shown, $*p < 0.01$. Experiment was repeated two times for each time point. Detection of H_2O_2 released from TAN purified from AB12 (c) and LLC (d) tumors or from naïve Balb/C mice (BN). 2.5×10^5 cells were activated in medium with PMA, prior to

the measurement. Each dot represents one sample. Samples were combined from 2 separate experiments (AB12, c) or taken from a single experiment (LLC, d). Mean \pm SD is shown, $*p < 0.01$. Flow cytometry analysis of naïve blood and digested tumor at early (7 day) and late (14 day) stages after AB12 cell inoculation. The percentage of intracellular $\text{TNF-}\alpha$ staining in isolated $\text{CD11b}^+\text{Ly6G}^+$ cells is shown. Each bar represents the mean \pm SD of 8 mice, combined from 2 separated experiments, $*p < 0.01$ (e). Representative FACS tracing of $\text{TNF-}\alpha$ in $\text{CD11b}^+\text{LY6G}^+$ cells is shown (f). The number in the bar is the percentage out of $\text{CD11b}^+\text{LY6G}^+$ cells. The black area represents proper isotype

Discussion

Neutrophils are the most abundant circulating leukocyte in humans, and they play a well-established role in host defense against invading pathogens [33]. Recently, it has become clear that tumor-associated neutrophils (TAN) play an important role in cancer biology. The literature has described a dual role for neutrophils in tumor biology [28, 34]. It has not been previously investigated whether

the differences in TAN phenotype are dependent on the stage of tumor development or only on tumor type and specific microenvironment. Previous studies suggested that TAN display different patterns of function under the influence of different microenvironments. $\text{TGF-}\beta$ induces neutrophils to acquire an N2 pro-tumoral phenotype [22], whereas $\text{IFN-}\beta$ induces neutrophils to acquire a more anti-tumoral N1 phenotype [23]. The activity of N1 neutrophils includes the enhanced expression of

Table 1 Gene expression profile of TAN

	BM	AB12			LLC			
		Early	Late	Late/Early	Early	Late	Late/Early	
N2-markers								
Comparison of mRNA expression from purified TAN of pooled early and late flank tumors and naïve bone-marrow-derived neutrophils. Gene expression was assessed using relative quantification ($\Delta\Delta\text{Ct}$) RT-PCR and is shown as relative expression in TAN versus BM neutrophils. Gene expression was normalized to GAPDH expression	CCL17	1	+++	+++	0	++	++	–
	CCL2	1	+++	+++	0	+++	+++	+
	ARG	1	+++	+++	0	+++	+++	+
	CCL5	1	++	+++	0	+	+	0
	VEGF	1	+	+	0	+	+	0
N1 markers								
Late/early—comparison of gene expression at 14 day to gene expression at 7 day	ICAM-1	1	++	++	+	++	++	+
	iNOS	1	++	++	0	+	++	+
	CCL3	1	+	+	0	+	+	+
	TNF- α	1	0	0	0	0	–	0
Other								
Change fold is expressed as follows: +++ Fold change > 100, $p < 0.05$; ++ Fold change > 100, $p < 0.05$; + Fold change > 2, $p < 0.05$; 0 0.5 > Fold change < 2, NS; –Fold change < 0.5, $p < 0.05$	CXCL1	1	+++	+++	0	+++	+++	0
	IL-6	1	++	+++	0	++	+	0
	IL-10	1	++	+++	+	++	++	–
	IL-12	1	+	++	+	+	+	0
	CXCL2	1	0	+	+	–	0	+
	CXCL10	1	++	++	0	+	+	–
IL-1 β	1	0	+	+	0	0	0	

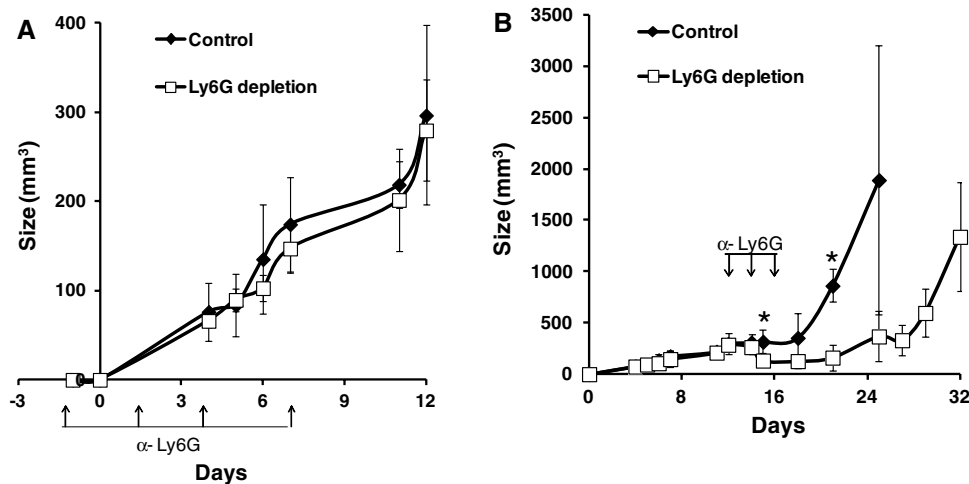


Fig. 5 Systemic depletion of neutrophils inhibits tumor growth only if done at late stages. Tumors were generated by s.c. inoculation of 2×10^6 AB12 cells into mice right flank. **a** Tumors were inoculated on day 0, and 300 μg of anti-Ly6G monoclonal antibody or control IgG was injected on days -1 , 1, 4 and 7. Tumor sizes were measured twice a week using calipers. Each dot represents the mean \pm SD of 5

mice, $*p < 0.05$. **b** Mice bearing relatively large tumors ($\sim 250 \text{ mm}^3$) were injected intraperitoneally with 300 μg of anti-Ly6G monoclonal antibody or control IgG on days 12, 14 and 16. Tumor sizes were measured twice a week using calipers. Each dot represents the mean \pm SD of 5 mice, $*p < 0.05$

immunoactivating cytokines and chemokines (e.g., TNF- α and CCL3), higher capacity to directly kill tumor cells and activation of CTL. N2 neutrophils express higher levels of CXCR4, VEGF, MMP-9 and arginase, supporting carcinogenesis, angiogenesis and immune suppression [22, 23, 28].

In the current study, we found that tumor-infiltrating neutrophils express higher mRNA levels of both N1 and N2 TAN. The expression of both N1 and N2 markers is markedly increased in TAN compared with naïve neutrophils, and the differences between early and late neutrophils are much less prominent, with later up-regulation of

both N1 and N2 markers, precluding the conclusion that TAN develop from being N1 at early stages to become N2 TAN at later stages of tumor development. We could suggest 2 main options to explain this observation: either tumor neutrophils are heterogeneous with the coexistence of distinct phenotypes, as supported for example by our TNF- α flow data (Fig. 4e, f), or they exhibit an intermediate phenotype that changes during tumor progression to be less anti-tumorigenic.

TNF- α is an important pro-inflammatory and immunostimulatory cytokine involved in immune regulation [35]. It has been demonstrated by several experimental models of cancer that TNF- α production by TAM provides pro-survival signals for premalignant cells, supporting tumorigenesis [36–38]. However, in the pro-tumorigenic M2 macrophages, there is down-regulation of TNF- α production, as well as impaired expression of the NF- κ B pathway, and other inflammatory functions [15]. In neutrophils, it has been demonstrated that TNF- α is a mediator of tumor cell killing [21]. We and others have shown that N1 TAN can promote CD8⁺ recruitment and activation by producing, among others, TNF- α , CCL3, CXCL9 and CXCL10 [22, 29]. There is also evidence that TAN can activate dendritic cells via cell–cell contact and through secretion of TNF- α [39]. These data, demonstrating the involvement of TNF- α in immune cell activation, are supported by our results showing that once tumor establishes, the percentage of TAN producing TNF- α decreases (Figs. 4e, f). Interestingly, there is no significant change in the level of TNF- α mRNA in TAN with tumor progression (Table 1). The exact role of TNF- α in tumor biology of N1 versus N2 remains to be determined.

In our studies, we found that the dynamics of TAN phenotype was dependent on the stage of tumor development, becoming more pro-tumorigenic as the tumor grew. The difference is so significant that whereas depletion of neutrophils at early stages does not affect tumor growth, their depletion when the tumors are well established slows further growth (Fig. 5). The marked cytotoxic effect of TAN *ex vivo* (Fig. 3) would suggest that early depletion of neutrophils can enhance tumor growth. However, we found no significant difference in tumor growth when TAN were depleted early. We believe that the answer for this alleged paradox lies in our findings on TAN localization in the tumor (Fig. 2). As seen in this figure, the early antitumor TAN do not enter the tumor; hence, their depletion does not affect markedly tumor growth. These data may suggest that the tumor could have two ways of defending itself from the neutrophils—preventing their entrance at early stages and inhibiting their activity later on.

We have previously shown that TGF- β receptor blockade increased the number of neutrophils in tumors and increased the expression of ICAM-1 on TAN. We therefore

suggested that the high levels of ICAM-1 are an N1 TAN marker [22]. The percentage of TAN expressing ICAM-1 was not higher in TAN from early tumors compared with TAN from established tumors. The majority of TAN from both early and late tumors expressed ICAM-1 (Fig. 1c, d), significantly more than in naïve neutrophils. Moreover, the mRNA level of ICAM-1 was up-regulated in TAN from established tumors (Table 1). It is therefore not clear whether changes in ICAM-1 are responsible for the increased levels of TAN seen following TGF- β receptor blockade [22]. It is possible that ICAM-1 is merely a marker of activation of TAN and not polarization.

Our data show that TAN from early tumors are directly cytotoxic toward tumor cells and suggest some possible mechanisms for this effect: enhanced production of H₂O₂, NO and possibly expression of TNF- α (Fig. 4). The capability of TAN to induce tumor cytotoxicity and inhibit tumor growth has been previously shown, with TAN being cytotoxic to various tumor cell types [40–42] through production of ROS [43–45] and proteases [46]. The decreased level of ROS expressed by TAN at later stages can be explained by effects mediated by the more advanced tumors. However, another possible explanation could be that late TAN have already released the content of their granules during their activation by the tumor, whereas earlier TAN were not activated enough to release their granules. The short living span of neutrophils, as well as the lack of change in morphology between early and late TAN (Supplementary Figure 4), argues against that possibility.

As mentioned above, we have previously found that TAN can assume tumor-cytotoxic N1 phenotype during TGF- β inhibition [22]. Recently, it was shown by Granot et al. [24] that TAN can actually directly inhibit metastatic seeding in the lungs. Although there is broad literature describing the ability of TAN to inhibit tumor growth, our results show that this ability is time dependent and is most effective at the initiation of tumor growth. Once the tumor succeeds in evading the immune system, its environment changes the TAN phenotype making them less harmful to the tumor. The description by Granot et al. [24] supports our findings in that the neutrophils in the metastatic lung can inhibit tumor growth up to the point that they “polarize” to become pro-tumorigenic. The strongest data showing that TAN become more pro-tumorigenic as tumor progress comes from the depletion experiments (Fig. 5). This result is also consistent with our previous data showing that depleting N2 TAN slows tumor growth whereas depletion of N1 TAN augments tumor expansion [22].

A major question arising from our results is related to the mechanisms involved in the changes seen in TAN toward being more pro-tumorigenic. It is possible that the location within the tumor microenvironment is crucial for driving tumor-promoting functions in TAN. However, we

did notice changes in TAN with time *ex vivo*. As suggested above, TGF- β could be a possible effector inducing these changes. We evaluated the level of TGF- β released from isolated tumor cells and found no difference in the amount secreted per a given number of cells (data not shown). However, it is possible that the total amount of TGF- β in the tumor microenvironment and its surroundings is high enough to affect TAN in a more prominent manner at later stages of tumor development. There are other potential mechanisms that could explain these changes, and we are currently investigating these options.

In contrast to our results, others have shown that early depletion of neutrophils using the anti-GR1 monoclonal antibody (RB6-8C5) reduced the number of T cells infiltrating the tumor and prevented tumor regression induced by different treatments [47, 48], suggesting that by augmenting T-cell proliferation, early tumor-infiltrating neutrophils play an essential role in the establishment of antitumor immunity. In an additional study, neutrophil depletion during the early stage of carcinogenesis suppressed angiogenesis [49]. In all of these studies, however, neutrophils depletion was achieved using an antibody against Gr-1 (RB6). This antibody recognizes both Ly6G on neutrophils and Ly6C, which is found on many cell types including monocytic myeloid derived suppressor cells (MDSC) and activated CD8⁺ cells [50]. It is therefore possible that the changes described were actually related to depletion of other cells depleted with anti-GR-1 mAb and not to the depletion of neutrophils. In our current and previous studies, we used a specific anti-Ly6G monoclonal antibody (1A8), depleting only neutrophils and elucidating their phenotype in tumor development, and their pro- and antitumor mechanisms. Interestingly, in our hands, neutrophils both at early and late time inhibited significantly the activation of CD8⁺ T-cells, as demonstrated by the level of the activation marker CD25 (data not shown). This effect could be one of the mechanisms by which neutrophils support tumor growth and partly explain the inhibition of tumor growth in later stages. It is possible that at earlier stages, the neutrophils could not enter the tumor and affect the CD8⁺ CTLs, or that these CTLs were not potent enough yet. Currently, we are further investigating these interesting effects of neutrophils on the activation and proliferation of CTLs.

In some of the experiments, we noted differences in TAN characteristics between the 2 cell lines evaluated—the lung cancer cell line LLC and the mesothelioma cell line—AB12. These differences represent a difference between different tumor types and specific lines. However, most of the results tested in both cell lines are similar, supporting generalization of our results. Another limitation of our study is the fact that TAN were evaluated in flank tumors and not in tumors developing *in situ*. The need to know the exact moment of tumor initiation prevents the possibility of

using more sophisticated *in situ* models. We believe, however, based on our previous work on TAN [22], that these results can be suggested to be a general phenomenon in tumor progression.

The overall data presented support the hypothesis that TAN from early tumors have a less tumor-supportive phenotype. They are more cytotoxic toward tumor cells and produce higher levels of TNF- α , NO and H₂O₂. In established tumors, these functions are down-regulated and TAN acquire a more pro-tumorigenic N2 phenotype. Our combined results suggest therefore that a “mixed” TAN phenotype is found within tumors, as previously described for TAM [30–32], possibly with the percentage of N2 TAN gradually increasing. This is especially demonstrated in the depletion studies, which show no effect of TAN depletion at early stages, but clear arrest in tumor growth later on, when the percentage of N2 in the mixture, appears to be more dominant. This is also supported by the RT-PCR data, showing that even at the early points of tumor development, the expression of clear N2 TAN genes is highly up-regulated compared with BM Neutrophils, suggesting that at least part of TAN have acquired N2 markers. As previously mentioned, an additional explanation for the lack of effect with early depletion of neutrophils could be due to the fact that TAN at early stages are not able to enter the tumor. We are currently investigating the mechanism of this interesting observation, *i.e.*, what prevents the neutrophils from entering the tumor at early stages and allows them entering later on.

Significant research has recently been done elucidating the important role of myeloid cells in the cancerous process. Our work adds another important layer to the understanding of neutrophils in cancer by further characterizing the changes in TAN during time. Further research on the functional role of different pathways and genes up-regulated in TAN and differences between the different subsets of TAN is currently underway.

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