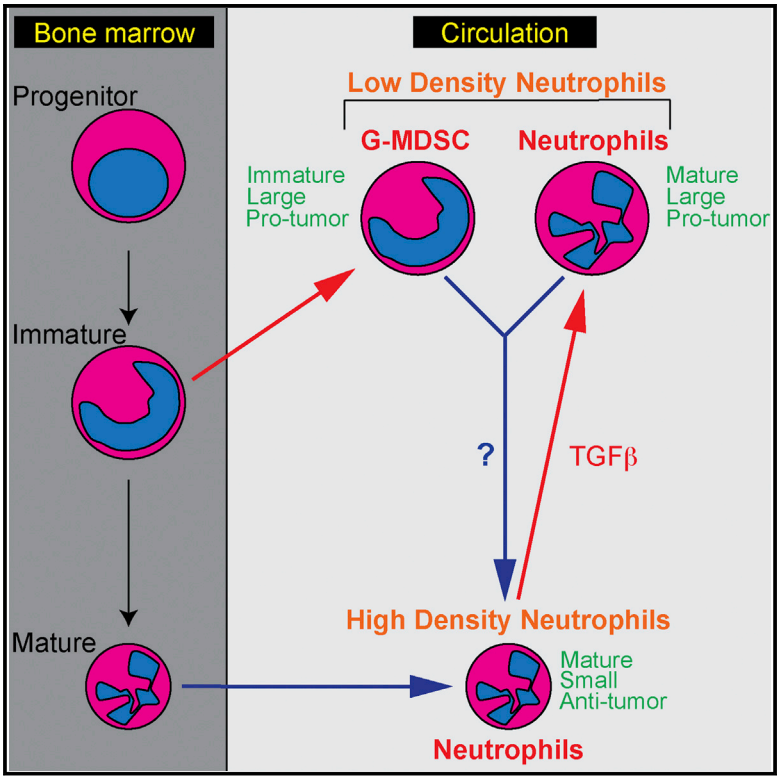
Article

Cell Reports

Phenotypic Diversity and Plasticity in Circulating Neutrophil Subpopulations in Cancer

Graphical Abstract



Highlights

- Low-density neutrophils are preferentially propagated in cancer
- LDNs consist of both mature and immature neutrophils
- The mature subtype of LDNs acquires suppressive functions
- Neutrophil contribution switches from anti- to protumor with tumor progression

Authors

Jitka Y. Sagiv, Janna Michaeli, ..., Zvi G. Fridlender, Zvi Granot

Correspondence

fridlender@hadassah.org.il (Z.G.F.), zvikag@ekmd.huji.ac.il (Z.G.)

In Brief

Controversy surrounds neutrophil function in cancer because neutrophils were shown to possess both pro- and antitumor properties. Sagiv et al. identify distinct circulating neutrophil subpopulations with conflicting cancer-related properties. With tumor progression, dynamic changes in neutrophil composition result in a switch from an overall anti- to protumor neutrophil contribution.

Sagiv et al., 2015, Cell Reports *10*, 562–573
February 3, 2015
http://dx.doi.org/10.1016/j.celrep.2014.12.039

CellPress

OPEN ACCESS
CellPress

Cell Reports
Article

Phenotypic Diversity and Plasticity in Circulating Neutrophil Subpopulations in Cancer

Jitka Y. Sagiv,[1] Janna Michaeli,[2] Simaan Assi,[1] Inbal Mishalian,[2] Hen Kisos,[1] Liran Levy,[2] Pazzit Damti,[2] Delphine Lumbroso,[3] Lola Polyansky,[1] Ronit V. Sionov,[1] Amiram Ariel,[3] Avi-Hai Hovav,[4] Erik Henke,[5] Zvi G. Fridlender,[2,6,*] and Zvi Granot[1,6,*]

[1]Department of Developmental Biology and Cancer Research, Institute for Medical Research Israel Canada, Hebrew University Medical School, 91120 Jerusalem, Israel
[2]Institute of Pulmonary Medicine, Hadassah-Hebrew University Medical Center, 91120 Jerusalem, Israel
[3]Department of Biology, Faculty of Natural Sciences, University of Haifa, 31905 Haifa, Israel
[4]Institute of Dental Sciences, Hebrew University-Hadassah School of Dental Medicine, 91120 Jerusalem, Israel
[5]Institut for Anatomy and Cell Biology, Tumour Angiogenesis and Experimental Therapeutics, Universität Würzburg, 97070 Würzburg, Germany
[6]Co-senior author
*Correspondence: fridlender@hadassah.org.il (Z.G.F.), zvikag@ekmd.huji.ac.il (Z.G.)
http://dx.doi.org/10.1016/j.celrep.2014.12.039

SUMMARY

Controversy surrounds neutrophil function in cancer because neutrophils were shown to provide both pro- and antitumor functions. We identified a heterogeneous subset of low-density neutrophils (LDNs) that appear transiently in self-resolving inflammation but accumulate continuously with cancer progression. LDNs display impaired neutrophil function and immunosuppressive properties, characteristics that are in stark contrast to those of mature, high-density neutrophils (HDNs). LDNs consist of both immature myeloid-derived suppressor cells (MDSCs) and mature cells that are derived from HDNs in a TGF-β-dependent mechanism. Our findings identify three distinct populations of circulating neutrophils and challenge the concept that mature neutrophils have limited plasticity. Furthermore, our findings provide a mechanistic explanation to mitigate the controversy surrounding neutrophil function in cancer.

INTRODUCTION

Neutrophils are the predominant circulating leukocyte population in humans, accounting for 50%–70% of circulating leukocytes (Welch et al., 1989). Neutrophils play a well-established role in host defense, where they phagocytose and kill invading microorganisms by releasing activating cytokines, defensins, and reactive oxygen species (Heifets, 1982; Mayadas et al., 2014). In recent years, interest in these cells in the context of cancer has increased because accumulating data suggest important and significant roles for neutrophils in tumor biology (Mantovani et al., 2011; Piccard et al., 2012).

The nonmalignant stroma surrounding the tumor plays a crucial role in tumor initiation, growth, and metastatic progression. The function of cells in the tumor surroundings is modified by the growing tumor to generate a supportive microenvironment (Joyce and Pollard, 2009). During the last years, the notion that neutrophils are terminally differentiated cells with effects limited to antimicrobial protection and inflammation is being gradually revised. Recent evidence questions this limited point of view, indicating that neutrophils can substantially affect tumor growth (Galdiero et al., 2013; Galli et al., 2011). Several studies have provided compelling evidence for protumor neutrophil functions (Pekarek et al., 1995), and they were shown to promote tumor angiogenesis, (Nozawa et al., 2006), enhance tumor cell dissemination (De Larco et al., 2004), and to promote metastatic seeding of tumor cells in distant organs (Kowanetz et al., 2010). In contrast, other studies, including our own, have provided evidence for antitumor and antimetastatic neutrophil functions. Neutrophils were shown to limit malignant progression through direct tumor cytotoxicity (Colombo et al., 1992; Hicks et al., 2006) and enhancement of antitumoral mediators (Di Carlo et al., 2001). It has also been demonstrated that neutrophils can acquire a cytotoxic phenotype, accumulate in the premetastatic organ and limit metastatic seeding (Granot et al., 2011; López-Lago et al., 2013). Our understanding of neutrophil function in human cancers is even more vague. Many patients with advanced cancer show high levels of blood neutrophils (Schmidt et al., 2005). The mechanisms by which tumors induce neutrophilia are not fully understood, although granulocyte macrophage colony-stimulating factor production has been implicated in several tumor types (McGary et al., 1995). Interestingly, neutrophilia and high neutrophil to lymphocyte ratio were found to be associated with poorer prognosis in many cancers (Fridlender and Albelda, 2012).

These conflicting reports fuel a debate regarding the role neutrophils play in cancer and bring forth the hypothesis that neutrophil function may be dictated in a context-dependent fashion. We were able to demonstrate that this is in fact the case at the primary tumor microenvironment where transforming growth factor-β (TGF-β) induces an N2 protumor neutrophil phenotype, rather than the N1 antitumor phenotype (Fridlender et al., 2009).

In the current work, we further explore the functional and phenotypic plasticity neutrophils display in cancer, and in self-resolving inflammation. We discovered and characterized a distinct low-density neutrophil (LDN) subpopulation that appears transiently in self-resolving inflammation and accumulates in tumor-bearing mice and cancer patients. This LDN subpopulation consists of at least two morphologically distinct neutrophil subsets that are regulated via discrete mechanisms. We further show that the mature, "normal," high-density neutrophils (HDNs) are capable of switching to become LDNs in a TGF-β-dependent fashion, a switch accompanied by loss of antitumor properties and the gain of immunosuppressive properties, similar to what has been described for immature granulocytic myeloid-derived suppressor cells (G-MDSCs) (Waight et al., 2011; Youn et al., 2008). With this, the overall circulating neutrophil phenotype switches from mostly antitumor early on to become more protumor with tumor progression. Taken together, our data reveal the existence of neutrophil subpopulations with conflicting functions and provides evidence for functional and physical neutrophil plasticity. These insights offer a mechanistic explanation to mitigate the controversy that surrounds neutrophil function in cancer and highlight unexplored aspects of neutrophil biology.

RESULTS

Distinct Circulating Neutrophil Subpopulations in Cancer

Neutrophils' contribution to tumor growth and metastatic progression has been a matter of debate where compelling evidence show both pro- and antitumor functions. This has led us to speculate that rather than being a homogenous population, circulating neutrophils consist of several subpopulations whose function in the context of cancer might differ considerably. To test this hypothesis, we used the 4T1 mouse mammary tumor model where circulating neutrophil numbers continuously increased with tumor progression (Figure 1A). When purifying neutrophils on a density gradient, we noticed that a significant proportion of neutrophils copurifies with the low-density (LD) mononuclear cells layer (Figure 1B, M) rather than the expected high-density (HD) granulocytic fraction (Figure 1B, G). In tumor-free mice (Figures 1C and 1D), over 95% of the neutrophils were high-density neutrophils (HDNs). However, with tumor progression the proportion of low-density neutrophils (LDNs) increased dramatically to the extent that LDNs often became the dominant circulating neutrophil population (Figure 1C). This phenomenon was also seen in other mouse models of breast cancer (E0771 and AT-3), mesothelioma (AB12), and orthotopic K-ras-driven lung cancer (K-ras) (Figure 1D). Interestingly, whereas increase in LDN prevalence was greater than 5-fold in all models tested (Figure 1E), there was significant variability in the extent of LDN propagation between different models. We noted that propagation of LDNs correlates well with the extent of neutrophil mobilization (data not shown); however, HDNs are still abundant in the bone marrow when LDNs are deployed, suggesting that LDN release from the bone marrow may not be due to the mere depletion of HDNs (Figure S1). Furthermore, LDNs had higher forward scatter (FSC) than HDNs with similar side scatter (SSC) (Figure 1F), suggesting that they are larger than HDNs while maintaining similar granularity. Interestingly, LDNs had reduced CD11b expression, which may indicate a lower state of activation (Borregaard et al., 1994) (Figure 1F). We also found that a subset of LDNs (~5%) had increased expression of TRAIL (Figure S2) raising the possibility that this LDN subpopulation may play a role in antitumor immunity (Cretney et al., 2002). Extensive fluorescence-activated cell sorting (FACS) analyses showed no other significant differences between HDNs and LDNs in commonly used neutrophils-related surface markers (Figure S2). In addition, morphological analyses showed that, whereas HDNs appeared to be a homogenous population of mature, segmented neutrophils (Figure 1G), LDNs were heterogeneous and contained segmented (~35%) as well as morphologically immature (banded and ring-shaped; Tsuda et al., 2004; Zhu et al., 2007) neutrophils (Figures 1H and 1I).

LDNs in Human Cancer Patients

Next, we wanted to test whether LDNs are also generated during the natural course of the disease in cancer patients. Although LDNs were rare in healthy volunteers (n = 9), their prevalence in the LD fraction was significantly increased in cancer patients (n = 15; Figures 2A and 2B). Furthermore, resembling the mouse phenotype, LDNs had increased FSC and similar SSC in patients (Figure 2C), but increased surface expression of CD11b (Figure 2D). In addition, human LDNs had increased surface expression of CD66b (Figure 2E), another marker of neutrophil activation. The similarity between human and mouse HDNs and LDNs was further manifested in their cellular composition in patients; HDNs were a homogeneous population of mature (segmented) neutrophils, whereas LDNs were heterogeneous and consisted of both immature (banded) and mature neutrophils (Figure 2F). This finding was also observed by FACS analyses where two subpopulations distinct in size (FSC) were noted within the LDNs (Figure 2C, left panel). Of note, there was no significant difference in the age distribution between healthy volunteers (57.4 ± 4.6 years, range 38–87) and cancer patients (64.4 ± 2.5 years, range 40–79). Most patients had advanced disease (stage IV), and we found no correlation between the tumor type, tumor site, or treatment history and the extent of LDN propagation (Table S1).

Taken together, the observations made in patients and in mouse models of cancer suggest that LDNs consisting of both mature and immature neutrophils are generated during the natural course of the disease.

LDNs Consist of Neutrophils Derived from Two Distinct Cellular Origins

Because LDNs consist of both immature and mature cells, we wanted to gain insight into their cellular origin. Do they represent different stages of differentiation of the same cell or are they distinct neutrophil subpopulations? To answer this question, we performed a bromodeoxyuridine (BrdU) pulse-chase experiment and followed the rate by which newly generated neutrophils populate the LD and HD fractions. Within 24 hr of BrdU injection, the LDN fraction was populated by ~50% $BrdU^+$ cells, whereas only ~3% of the HDNs were $BrdU^+$ (Figure 3A). Overall,

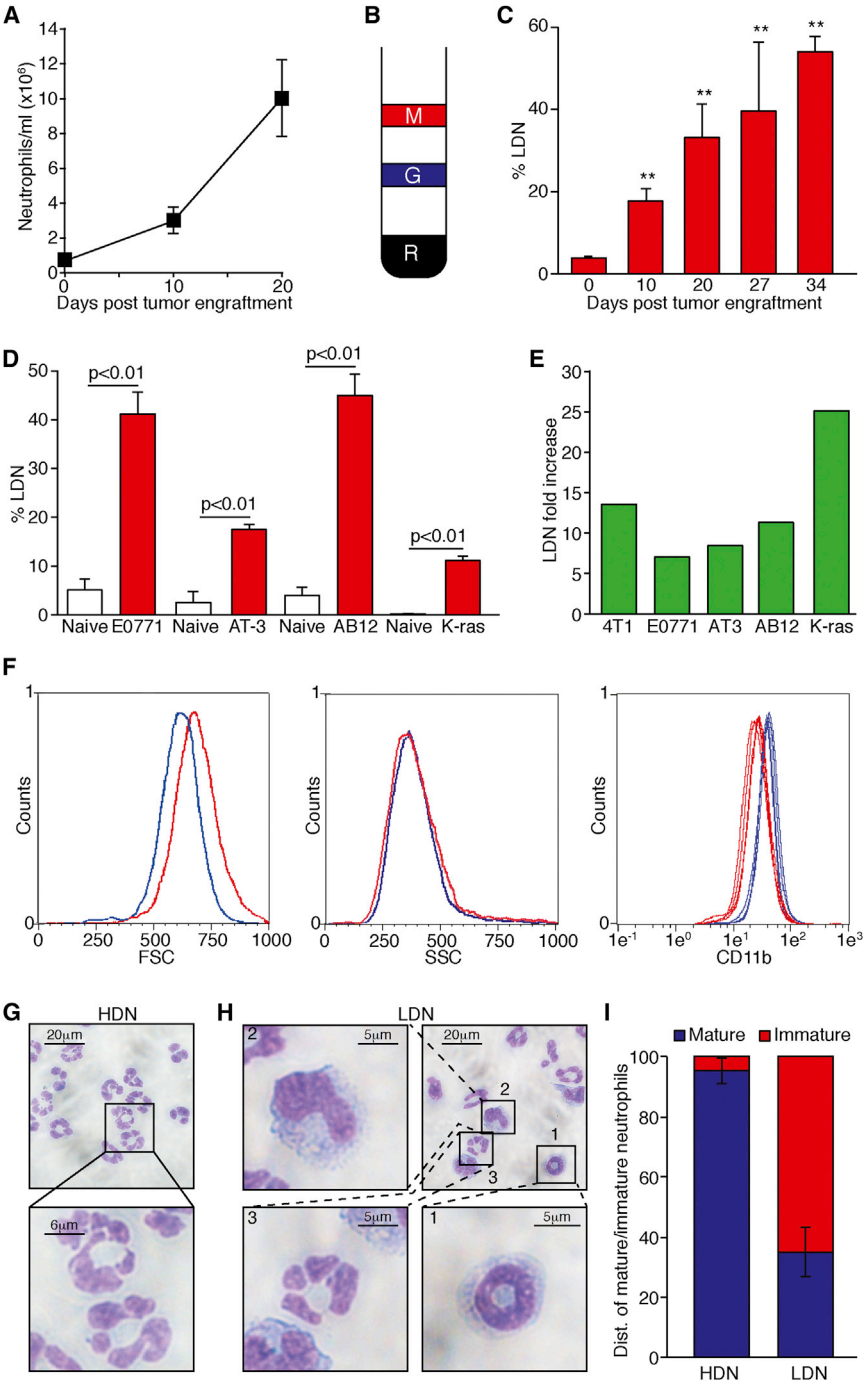


Figure 1. Dynamic Changes in Neutrophil Subpopulations with Cancer Progression

(A) Circulating $CD11b^+Ly6G^+$ neutrophil numbers continuously increase with 4T1 tumor progression in Balb/c mice.

(B) Separation of whole blood retrieved by cardiac puncture on a Histopaque density separating granulocytes (G), mononuclear cells (M), and RBCs (R).

(C) The percentage of LDNs out of all $CD11b^+Ly6G^+$ cells increases dramatically with 4T1 tumor progression in Balb/c mice.

(D) The proportion of LDNs (of total neutrophils) increases dramatically in mice bearing E0771, AT-3, AB12, and K-ras-driven orthotopic lung tumors (all in red) compared to naive mice (white).

(E) Fold increase in LDN prevalence in the different tumor models.

(F) FACS analysis showing similar SSC but increased FSC in LDNs (red) compared to HDNs (blue).

(G) Cytospin images of the HD fraction showing that HDNs are a homogenous population of segmented neutrophils.

(H) Cytospin images of the LD fraction showing that LDNs are morphologically heterogeneous consisting of immature neutrophils with ring-shaped (1) and banded (2) nuclei as well as mature segmented neutrophils (3).

(I) Ratio of immature (ring-shaped and banded) to mature neutrophils in the HDN versus LDN fractions.

Error bars represent ±SEM. $^{**}p < 0.01$.

See also Figures S1 and S2.

95% of newly generated $Ly6G^+$ cells at this time were found in the LDN fraction, whereas only 5% were found to be HDNs (Figure 3B). By 48 hr, the situation changed dramatically where ~50% of the HDNs were $BrdU^+$ (Figure 3A), down to 30% at 72 hr. These results suggest that immature $CD11b^+Ly6G^+$ cells, possibly G-MDSCs, rapidly leave the bone marrow. An additional day is required to reach full maturation from dividing precursor cells, and only by then the HDN layer is populated. Interestingly, the $BrdU^+Ly6G^+$ population in the HD fraction increases dramatically from 24 to 48 hr postlabeling; however, the proportion of $BrdU^+$ LDNs does not change much during this time (Figure 3A). The different dynamics of LDNs and HDNs may be better illustrated by presenting the distribution of $BrdU^+Ly6G^+$ cells between these fractions, where 95% of $BrdU^+Ly6G^+$ cells are LDN after 24 hr, whereas 70% of the labeled cells are HDNs after 72 hr (Figure 3B). Importantly, the HDN/LDN ratio remains similar throughout the experiment (Figure 3C), suggesting that, although there are significant dynamic changes in the composition of the neutrophil subpopulations, these changes do not affect the HDN/LDN ratio, or the total neutrophil number. In addition, at 48 hr there was still a significant amount of BrdU labeling in bone marrow neutrophils (data not shown) and a very low rate of apoptosis in freshly isolated circulating HDNs and LDNs (Figure 3D).

These findings suggested that the LDN fraction may also be populated by $BrdU^-Ly6G^+$ cells coming from another source and raised the striking possibility that LDNs are derived, at least in part, from neutrophils in the HD fraction. To test this hypothesis, we followed the fate of labeled HDNs and LDNs adoptively

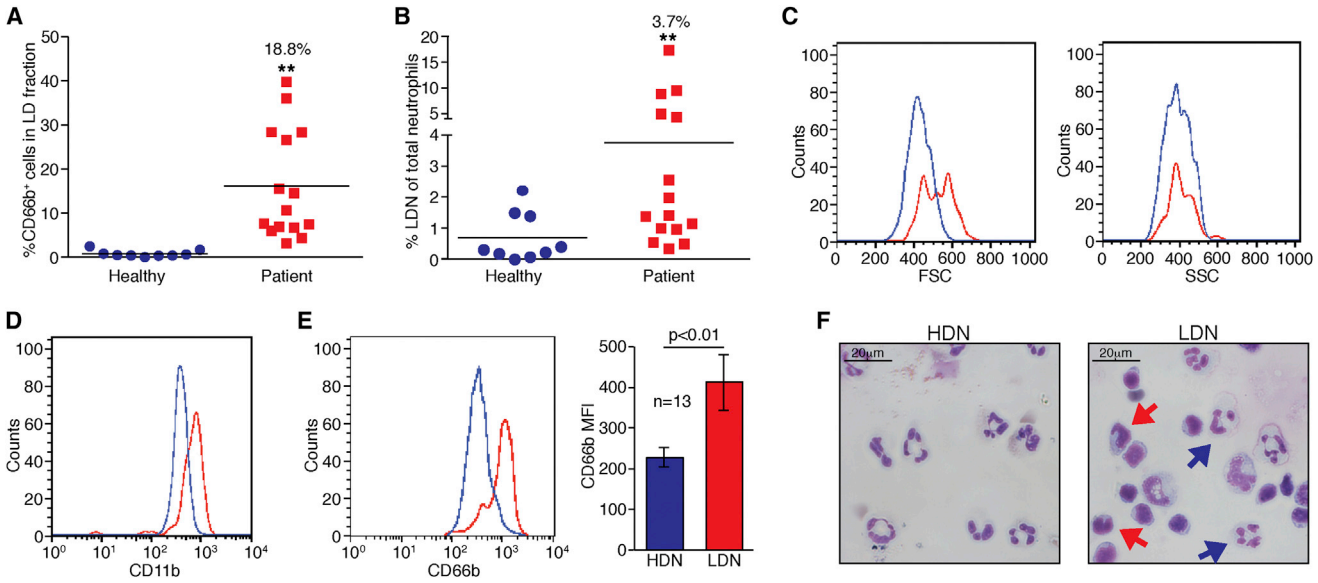


Figure 2. Heterogeneous LDNs Are Also Found in Cancer Patients

(A) FACS analysis of the percentage of LDNs ($CD66^+$ cells) out of the entire LD fraction in healthy volunteers (blue) and in cancer patients (red).

(B) FACS analysis of the percentage of LDNs out of the entire neutrophil ($CD66^+$ cells) population in healthy volunteers (blue) and in cancer patients (red).

(C) FACS analysis of LDNs (red) and HDNs (blue) isolated from blood of a lung cancer patient showing that LDNs have increased FSC with no change in SSC.

(D and E) Patient LDNs show increased surface expression of CD11b (D) and CD66b (E) compared to HDNs.

(F) Cytospin images of the HD and LD fractions in a lung cancer showing homogeneous HDNs and heterogeneous LDNs, consisting of both segmented (blue arrows) and banded neutrophils (red arrows), in the LD fraction.

Error bars represent ±SEM. **$p < 0.01$. See also Table S1.

transferred into label-free tumor-bearing mice (Figure 3E). The recipient mice were sacrificed 3 hr after neutrophil transfer and their neutrophils were refractionated (Figure 3E). Using this strategy, we found an impressive phenotypic switch: ~40% of transferred labeled HDNs shifted to the LD fraction and ~10% of transferred labeled LDNs shifted to the HD fraction (Figure 3F), demonstrating the plasticity of these circulating neutrophils in vivo.

LDNs Increase Transiently during Self-Resolving Inflammation

A major question arising from our results was whether similar transitions between HDN and LDN are relevant not only to the persistent inflammation associated with cancer, but also to other states of inflammation. To this end, we studied zymosan-induced peritonitis, a widely used model for acute and self-resolving inflammation that peaks within a few hours with an influx of neutrophils to the peritoneum and is mostly resolved by 72 hr (Bannenberg et al., 2005). In this context of limited inflammation, circulating and peritoneal LDNs increased transiently, peaking at 48 hr (Figure 3G). With striking resemblance to the observations made in tumor-bearing mice, peritoneal HDNs are a homogeneous population of mature neutrophils, whereas LDNs consist of both mature and immature neutrophils (Figure 3H) suggesting that circulating LDNs are also associated with self-resolving inflammation. Adoptive transfer experiments showed that within 3 hr of their transfer into the peritoneum, ~90% of labeled HDNs switched to the LD fraction, whereas only 16% of labeled LDNs switched to the HD fraction (Figure 3I). Importantly, significant numbers of BrdU-labeled HDNs could be detected in the circulation and peritoneum only 48 hr following BrdU administration, suggesting that this represents the normal timing of neutrophil maturation and release to the circulation under inflammatory conditions (Figure 3J). Together, the observations made in tumor-bearing mice and in mice with acute peritoneal inflammation suggest that $BrdU^+$ HDNs cannot be detected in the circulation earlier than 48 hr following BrdU administration. This means that HDNs require 48 hr to fully mature, supporting our hypothesis that the $BrdU^+$ neutrophils seen in the LD layer at 24 hr are indeed immature cells (Figure 3A).

During the resolution of inflammation peritoneal macrophages undergo a conversion from a $CD11b^{high}$ to a $CD11b^{low}$ phenotype that is accompanied by an increase in the expression of 12-/15-lipoxygenase (LO) and a decrease in arginase-1 (Schif-Zuck et al., 2011). Hence, we tested the consequences of introducing LDNs from 4T1 tumor-bearing mice into the peritoneum of mice with zymosan-A-induced peritonitis. LDNs were transferred at 24 hr following zymosan induction, and the mice were sacrificed at 48 hr. Our data show that LDN transfer induced a switch in the WBC composition in the peritoneum and a reduction in the overall number of peritoneal WBC compared with vehicle-treated controls (Figure S3). These changes were accompanied with a reduction in CD11b expression and an increase in 12/15-LO and arginase-1.

Together, our findings in both cancer and inflammation suggest the existence of at least three distinct neutrophil subpopulations in the circulation classified into immature LDNs, mature LDNs, and mature HDNs. The dynamic transition between these

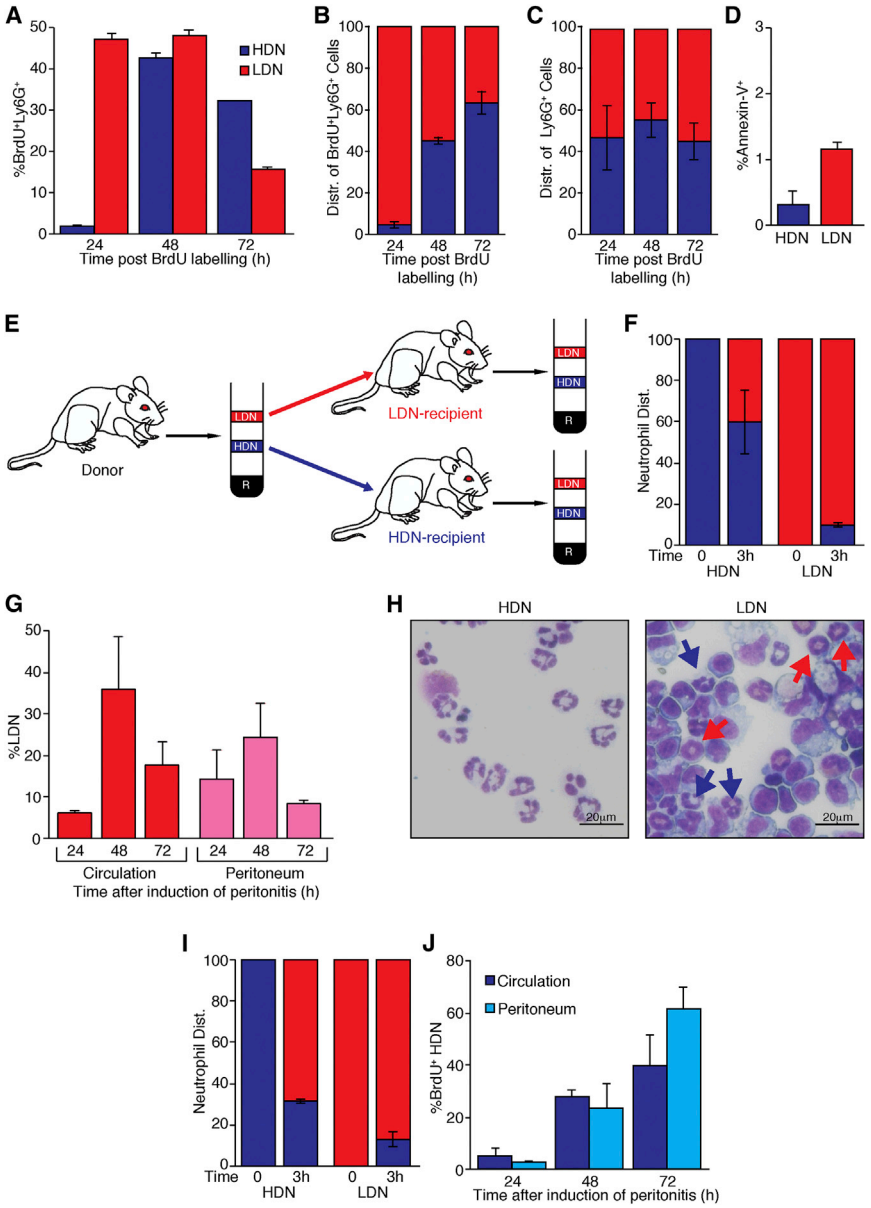


Figure 3. The Origins and Plasticity of Neutrophil Subpopulations

(A) The percentage of Ly6G$^+$BrdU$^+$ neutrophils in the HD (blue) and LD (red) fractions as determined at 24, 48, and 72 hr after BrdU labeling.

(B) Distribution of BrdU$^+$ neutrophils between the HD and LD fractions at 24, 48, and 72 hr after BrdU labeling.

(C) HDN/LDN ratio does not change during the time course of the experiment.

(D) FACS analysis of Annexin-V$^+$ cells in freshly isolated HDNs (blue) and LDNs (red) shows very low levels of cells undergoing apoptosis.

(E) Labeled HDNs and LDNs were purified and introduced via intracardiac injection to tumor-bearing mice (LDN recipients and HDN recipients). Three hours later, neutrophils were refractionated and the distribution of labeled neutrophils in the low- and high-density fractions was determined by flow cytometry.

(F) Sixty percent of the labeled HDNs were detected in the LD fraction, whereas 10% of the labeled LDNs were detected in the HD fraction, suggesting for dynamic transition between these neutrophil subpopulations.

(G) Appearance of LDNs in the circulation (red) and peritoneum (pink) in the zymosan-induced model of peritonitis at 24, 48, and 72 hr after zymosan administration.

(H) Cytospin imaging of peritoneal LD and HD fractions taken at 48 hr after zymosan injection. HDNs exclusively consist of mature neutrophils, whereas LDNs consist of both mature (blue arrows) and immature (red arrows) neutrophils.

(I) Approximately 70% of the labeled HDNs introduced into the inflamed peritoneum (blue) switch to the LD fraction (red), whereas 16% of the labeled LDNs were detected in the HD fraction.

(J) Time course of BrdU$^+$ HDN accumulation in the circulation (blue) and peritoneum (light blue) in zymosan-induced peritonitis.

Error bars represent ±SEM. See also Figure S3.

subpopulations may account for the significant numbers of mature neutrophils in the LD fraction (Figures 1G and 1H).

HDN to LDN Transition Occurs Spontaneously in Late-Stage Tumor-Bearing Mice, Outweighs the Opposite Transition, and Is Not Merely Degranulation

Although these findings highlight an aspect of neutrophil biology, the low retrieval rate (~5%) of adoptively transferred neutrophils precludes a clear indication of whether the transition from HDN to LDN predominates, or vice versa. To overcome this difficulty, we tested whether the transition between HDN and LDN can be mimicked in confined conditions, ex vivo. We compared the HDN/LDN ratio in freshly drawn blood and in whole blood incubated for 4 hr ex vivo. Under these conditions, the rate of apoptosis increases significantly with time in both HDNs and LDNs. LDNs consistently had a reduced rate of apoptosis compared with HDNs (Figure 4A). That said, we were able to retrieve close to 100% of neutrophils following 4 hr of incubation ex vivo. Although there was no apparent change in the HDN/LDN ratio after incubation in blood taken from mice bearing early tumors (Figure 4B, day 13 postengraftment), 90% of all neutrophils in blood taken from mice bearing late tumors were located in the LD fraction after 3 hr of incubation (Figure 4B, day 25 postengraftment). These results corroborated the experiments made in vivo and show that the HDN to LDN transition occurs spontaneously in blood from late-stage tumor-bearing mice. Given the fact that we were able to retrieve close to 100% of neutrophils, this result demonstrates that the transition from HDN to LDN significantly outweighs the transition in the opposite direction. Transition is evident only in blood drawn from mice bearing late-stage tumors, suggesting that it may be mediated via a change in the cytokine/chemokine milieu that is associated with advanced disease.

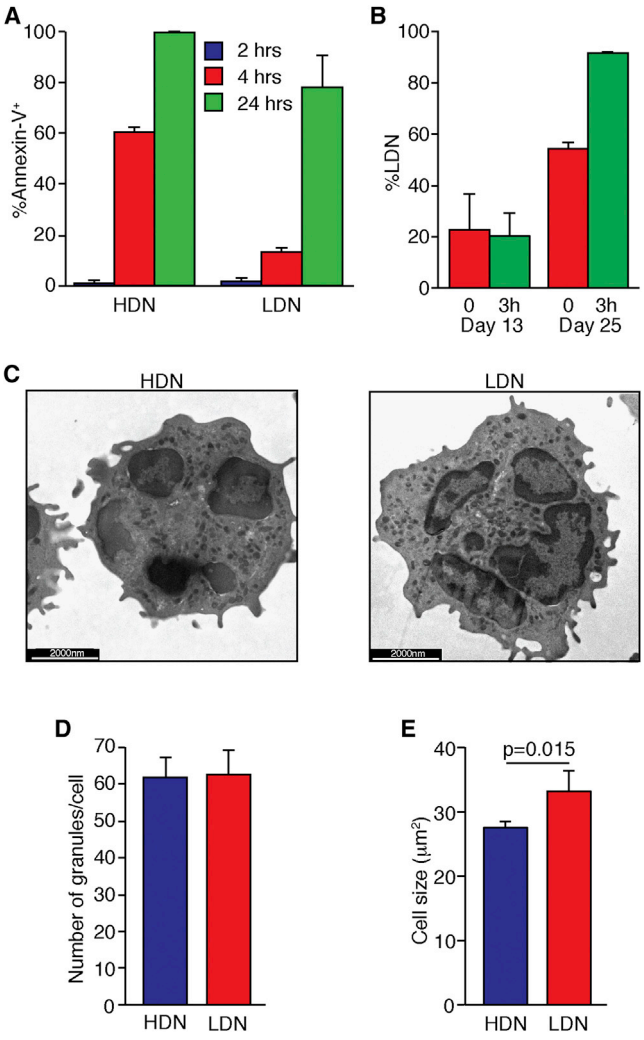


Figure 4. HDNs from Late-Stage Tumor-Bearing Mice Spontaneously Switch to LDNs In Vitro

(A) Extent of apoptosis (Annexin-V$^+$) in purified HDNs (blue) or LDNs (red) at 2, 4, and 24 hr.

(B) The percentage of LDNs out of all Ly6G$^+$ neutrophils in freshly drawn whole blood (red) and in blood incubated ex vivo for 3 hr (green) in mice bearing early- (day 13) and late- (day 25) stage tumors.

(C) Representative TEM images of segmented neutrophils taken from the HD or LD fractions.

(D) The number of granules per cell (<20 per group) in HDNs (blue) and segmented LDNs (red).

(E) Cell size measurements show that LDNs (red) are significantly larger than HDNs (blue).

Error bars represent ±SEM.

What regulates the transition from HDN to LDN? Neutrophils have the capacity to respond to a wide range of stimuli. This response may lead to changes in density as a result of a dramatic decrease in cellular content (i.e., degranulation), a change in cell volume (i.e., increase in size) or a combination of both processes (Jan et al., 2006). To understand which of these processes mediates the change in neutrophil density, we used transmission electron microscopy (TEM) focusing on segmented LDNs and HDNs. This analysis showed that, whereas LDNs were indeed larger than HDNs, both neutrophil subpopulations contained a similar number of granules (Figures 4C–4E). This finding was in line with the FACS analyses showing increased FSC with unchanged SSC (Figures 1E and 2C) and suggests that, in this context, LDNs are not derived from degranulated HDNs.

TGF-β Mediates the Transition from HDN to LDN

We have previously demonstrated that TGF-β has a dramatic effect on neutrophils function as it can efficiently block neutrophil cytotoxicity (Granot et al., 2011) and mediate the polarization of tumor-associated neutrophils from antitumor N1 neutrophils to protumor N2 neutrophils (Fridlender et al., 2009). These observations were supported by the fact that TGF-β inhibited neutrophil H_2O_2 production in response to phorbol myristate acetate (PMA), a pan-PKC receptor-independent neutrophil activator (Figure 5A) and degranulation induced by lipopolysaccharide (Shen et al., 2007). Because H_2O_2 was shown to mediate antitumor neutrophil cytotoxicity (Granot et al., 2011) and PMA was found to be sufficient for inducing antitumor neutrophil cytotoxicity (Ackermann et al., 1989), its inhibitory effect on PMA-induced H_2O_2 secretion highlights TGF-β as a major regulator of neutrophil cytotoxicity. Furthermore, the circulating levels of TGF-β were shown to correlate with tumor progression (Barcellos-Hoff and Akhurst, 2009), making it a prime candidate for promoting the transition from HDN to LDN. To examine if TGF-β is sufficient for inducing the transition from HDN to LDN, we tested whether incubating whole blood ex vivo in the presence of increasing concentrations of TGF-β can modify the LDN/HDN ratio. Indeed, we observed a dose-dependent increase in the proportion of LDNs with the addition of TGF-β (Figure 5B). We next incubated GFP$^+$ HDNs in whole blood taken from tumor-bearing GFP$^-$ mice, in the presence or absence of SB431542, an inhibitor of TGF-β signaling. We could therefore distinguish the donor GFP$^+$ HDNs from the unlabeled neutrophils present in the recipient blood. SB431542 induced a moderate but significant inhibition of HDN to LDN transition in the unlabeled neutrophil population (Figure 5C). This effect of SB431542 was far more pronounced when looking specifically at the GFP$^+$ HDN population where the transition from HDN to LDN was dramatically inhibited (Figure 5D, left). Interestingly, GFP$^+$ HDNs efficiently switched to LDNs not only in blood taken from tumor-bearing mice, but also in blood drawn from tumor-free mice. In contrast, HDNs from tumor-free mice incubated in blood drawn from either tumor-free or tumor-bearing mice did not show significant transition to LDN (Figure 5D, right), demonstrating that the mere transfer of HDNs from tumor-free mice to blood drawn from tumor-bearing mice is not sufficient for inducing the HDN to LDN transition. Moreover, it suggests that neutrophils in tumor-bearing mice have undergone preconditioning that directs them toward the transition to LDN even in blood from tumor-free mice. To further provide insight into the role TGF-β plays in regulating the LDN/HDN ratio in vivo, we treated tumor-bearing mice with the TGF-β-depleting antibody 1D11. Administration of 1D11 to tumor-bearing mice results in a reduced LDN/HDN ratio (Figure 5E), suggesting that TGF-β is involved in regulating this ratio in vivo. However, because TGF-β alone had no effect on the number of LDNs (Figure 5F) or the proportion LDNs

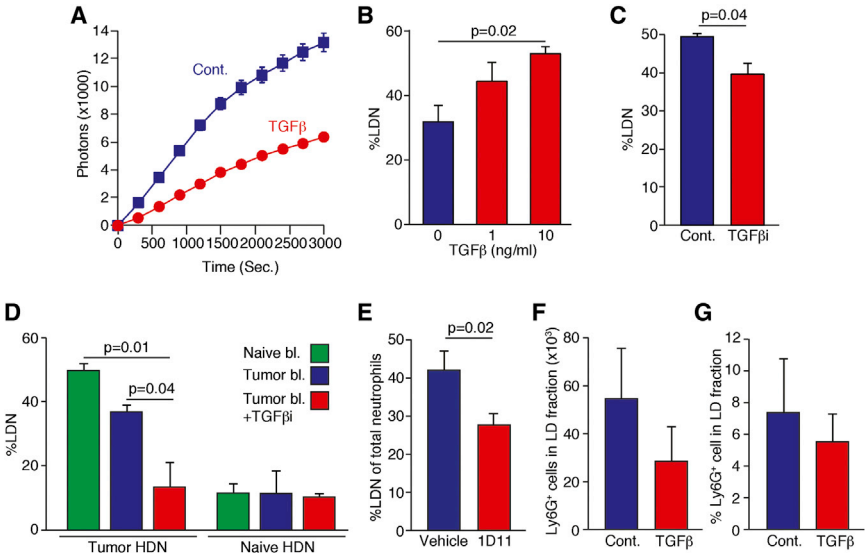


Figure 5. TGF-β Signaling Is Both Required and Sufficient for Driving the HDN to LDN Transition in Tumor-Bearing Mice

(A) TGF-β is a potent inhibitor of H_2O_2 production by PMA-stimulated neutrophils.

(B) Ex vivo incubation of whole blood from tumor-bearing mice with increasing TGF-β concentrations.

(C) Ex vivo incubation (3 hr) of whole blood drawn from tumor-bearing mice with SB431542 (TGF-βi).

(D) HDNs purified from naive or tumor-bearing GFP^+ mice incubated ex vivo for 3 hr in whole blood from GFP^- naive (Naive bl., green) or tumor-bearing mice in the absence (Tumor bl., blue) or presence of SB431542 (Tumor bl.+TGF-βi, red). Data show the percentage of GFP^+ LDNs make of all GFP^+ neutrophils.

(E) Administration of 1D11 to tumor-bearing mice results in a significant reduction in the proportion of LDNs compared to vehicle-treated controls (vehicle).

(F and G) Ex vivo incubation of whole blood from naive mice with TGF-β (10 ng/ml) has no significant effect on the number of LDNs (F) or the fraction they make of the entire neutrophil population (G). Error bars represent ±SEM.

make of the entire neutrophil population (Figure 5G), we conclude that, whereas TGF-β is required and sufficient for driving the HDN to LDN transition in tumor-bearing animals, naive neutrophils require further activation for this transition.

HDNs and LDNs Show Remarkably Opposing Cancer-Related Functions

We evaluated several basic neutrophil functions and found that LDNs show impaired activity compared to HDNs: (1) LDNs showed reduced chemotaxis toward 4T1-conditioned media compared with HDNs (Figures 6A and 6B); (2) LDNs were able to phagocytose fewer FITC-labeled microbeads than did HDNs (Figure 6C); and (3) LDNs had impaired oxidative burst, compared with HDNs, in response to PMA (Figure 6D). The reduced H_2O_2 release following PMA induction suggested that under maximal stimulation, using this receptor-independent activator, LDNs might have flawed antitumor activity compared with HDNs. To test this hypothesis, we analyzed cancer-related properties of these neutrophil subsets. Cancer-related HDNs, previously referred to as tumor-entrained neutrophils (TENs) (Granot et al., 2011), showed high cytotoxicity toward tumor cells in culture (Figure 6E), whereas LDNs showed no significant cytotoxicity. We next tested whether the reduced LDN cytotoxicity stems from the interaction with other LD suppressive cells. For this purpose, we added a 1:1 mixture of HDNs and cells of the LD fraction to the coculture and saw no significant difference in cytotoxicity, suggesting that the cells in the LD mononuclear fraction have no significant effect on the cytotoxic capacity of HDNs (Figure 6F). Tumor cell killing by HDNs from mice with early-stage tumors was similar to HDNs from late-stage tumors (Figure 6G), showing that, although there is a dramatic increase in LDNs with tumor progression, the generation of tumor-cytotoxic HDNs (TENs) persists.

We next performed a modified Winn assay to test the consequences of coinjecting tumor cells with either HDNs or LDNs on tumor growth. Although LDNs had no significant effect on initial tumor growth, HDNs dramatically retarded tumor growth (Figure 6H), demonstrating that HDNs play an antitumor role, whereas LDNs are tumor permissive. Because several studies have suggested a protumor role for neutrophils (De Larco et al., 2004; Fridlender and Albelda, 2012; Kowanetz et al., 2010; Nozawa et al., 2006; Pekarek et al., 1995; Piccard et al., 2012), we hypothesized that LDNs may represent the protumor neutrophil subset. We found that LDNs have a gene expression signature associated with a reduced inflammatory state including reduced expression of various chemokines (CXCL1, CXCL2, CXCL10, CCL2, and CCL3), chemokine receptors (CXCR2 and CCR5), and proinflammatory molecules, but with increased expression of CCR7 (Figure S4), that is known to be rapidly expressed at the membrane upon stimulation (Beauvillain et al., 2011). Most importantly, LDNs spontaneously generated from HDNs acquired a previously nonexisting suppressive capacity and markedly limited the proliferation of αCD3-stimulated $CD8^+$ T cells (Figures 6I–6J). This observation demonstrates the striking extent of neutrophil plasticity and challenges the concept that only immature myeloid cells contribute to immune suppression in cancer. This effect was shown to have impact in vivo, and, using another modification of the Winn assay, we found that the inhibition of tumor growth induced by mixing tumor cells with $CD8^+$ cytotoxic T lymphocytes (CTLs) was abrogated when LDNs were added to the mixture (Figure 6K). Altogether, our data suggest the existence of three distinct circulating neutrophil subpopulations in cancer, namely, HDNs that possess antitumor functions and LDNs that possess protumor functions and consist of MDSCs and mature neutrophils.

DISCUSSION

Neutrophils, the predominant circulating leukocyte population (Welch et al., 1989), play a well-established role in host defense

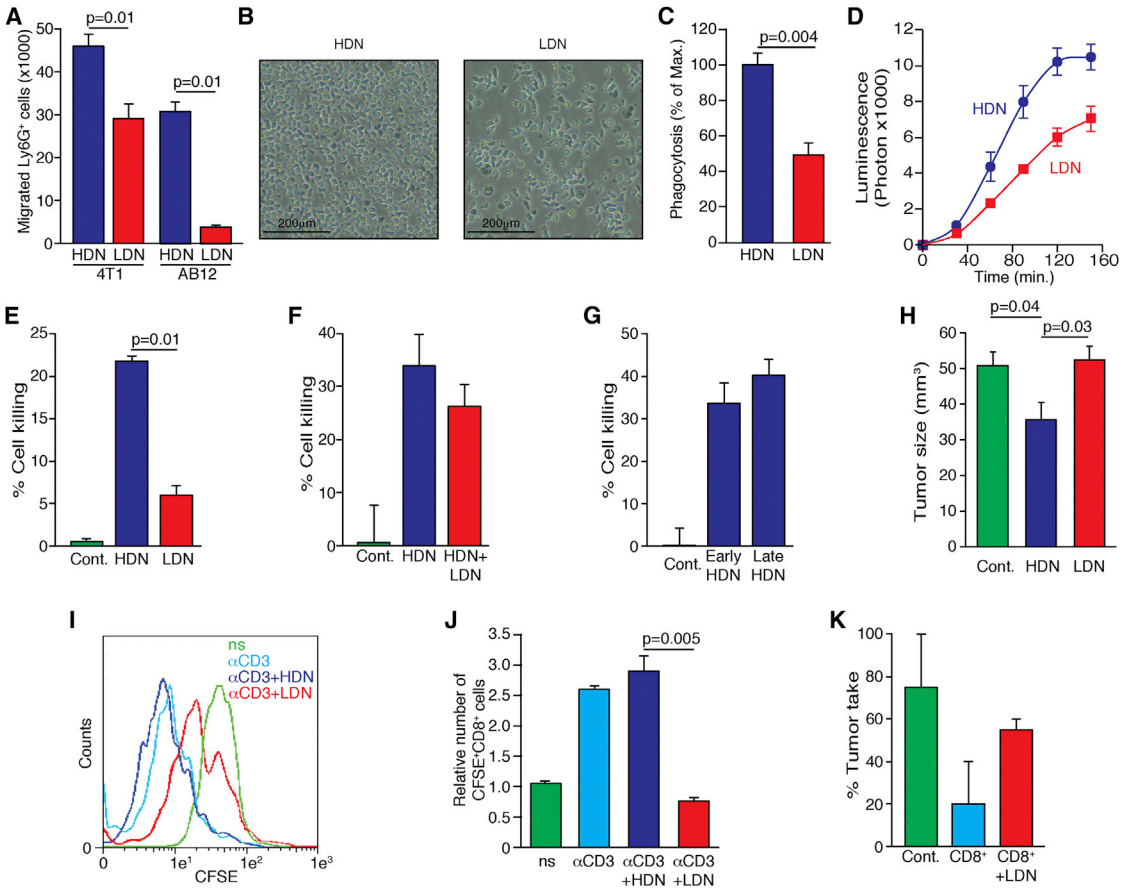


Figure 6. HDNs and LDNs Differ in General and Cancer-Related Functions

(A) Reduced migration of LDNs (red), purified from either 4T1 or AB12 tumor-bearing mice, compared to HDNs (blue). The number of migrating cells was counted.

(B) Representative images of light microscopy showing the extent of HDN versus LDN migration in a Boyden chamber (bottom well).

(C) LDNs (blue) have reduced phagocytic capacity of labeled beads compared with HDNs (red).

(D) LDNs (blue) show reduced PMA induced oxidative burst compared with HDNs (red).

(E) LDNs show reduced cytotoxicity toward tumor cells compared with HDNs (blue).

(F) Coculture of tumor cells with HDNs alone or together with LDN (1:1 ratio) shows that LDNs have no significant inhibitory effect on HDN cytotoxicity.

(G) HDNs purified from early- and late-stage tumors show similar cytotoxicity.

(H) HDNs (blue) but not LDNs (red) reduce tumor size compared with control (green) in a modified Winn assay.

(I and J) In vitro $CD8^+$ T cell proliferation, as measured by reduction in carboxyfluorescein succinimidyl ester (CFSE) labeling assay. Stimulation with αCD3 induces proliferation (αCD3, light blue) compared to nonstimulated (ns, green). HDNs (blue) have no impact on $CD8^+$ T cell proliferation (αCD3+HDN), whereas LDNs (red) significantly inhibit T cell proliferation (I). LDNs, but not HDNs, significantly reduce the number of $CD8^+$ T cells in a splenocyte culture stimulated with αCD3 (J).

(K) LDNs (red) abrogate the tumor limiting effect of $CD8^+$ CTLs (light blue) in a modified Winn assay. Control mice were injected with tumor cells alone (green). Error bars represent ±SEM. See also Figure S6.

(Heifets, 1982; Mayadas et al., 2014). Interest in these cells in the context of cancer has increased during the last decade, because accumulating data suggest important and significant roles for neutrophils in tumor biology (Mantovani et al., 2011; Piccard et al., 2012). Controversy, however, surrounds the role these cells play in tumor growth and metastatic progression. Neutrophils have been shown to contribute to tumor progression by secreting chemokines, cytokines, growth factors, and ECM remodeling enzymes and through promotion of angiogenesis and metastatic seeding. In addition, neutrophils may play an important part in mediating tumor immune evasion both as immature neutrophils (G-MDSCs) (Peranzoni et al., 2010) and as mature cells (e.g., by attracting T-regulatory cells to the tumor bed, recently reviewed in Sionov et al., 2014). In contrast, other reports have shown that neutrophils also possess antitumor properties where they acquire the capacity to kill tumor cells either directly or through antibody-dependent cellular cytotoxicity (ADCC) (Kushner and Cheung, 1992) and limit tumor growth and metastatic seeding (Granot et al., 2011; López-Lago et al., 2013).

Dynamic Changes in Neutrophil Subpopulations with Tumor Progression

In our current work, we show that circulating neutrophils, both in cancer patients and in mouse models of cancer, are heterogeneous and consist of distinct subpopulations. These

subpopulations are dynamic, have distinct cellular origins, and may possess different and even conflicting functions in the context of cancer. The ratio between these subpopulations and the potency of their activity should determine the overall effects of neutrophils. This notion is supported by our previous studies showing that within the tumor microenvironment, neutrophils acquire a tumor-promoting "N2" phenotype in a TGF-β-dependent manner (Fridlender et al., 2009). We further show that subpopulations of circulating neutrophils in cancer can be distinguished according to their densities, one with "normal," HD characteristics (HDNs) having antitumor properties (Figures 1 and 2), which we have previously described as TENs (Granot et al., 2011), whereas the other (LDNs) being of lower density with features associated with protumor activity. The functional differences between HDNs and LDNs together with the continuous increase in the proportion of LDNs with tumor progression provide a solid explanation for the controversy that has surrounded neutrophil function in cancer. Namely, the ratio between the functionally opposing neutrophil subtypes dictates the overall pro- or antitumor contribution of neutrophils. During early tumor development, HDNs predominate leading to an overall antitumor response. With tumor progression, LDNs become dominant resulting in an overall neutrophil phenotype switch toward tumor-permissive functions. These results mirror our previous findings that tumor neutrophils enter the tumor and become more protumor with tumor progression (Mishalian et al., 2013). Importantly, because our data show that significant expansion of LDNs is a relatively late event, we propose that, whereas LDNs may contribute to tumor growth and progression, their contribution to tumor initiation is less likely.

Generation of LDNs in Cancer: A Futile Attempt at Resolving Tumor-Associated Inflammation?

Our findings raise a major question regarding the biological significance of the large numbers of LDNs in cancer. A possible explanation may be deduced from the observation that LDNs are also generated in a model of self-resolving inflammation. The transient increase in LDNs toward the onset of resolution suggests that they may play a role in this process. Indeed, we found that the increase in LDNs in this context has functional consequences as the adoptive transfer of LDNs into the inflamed peritoneum induced changes associated with earlier onset of resolution (Figure S3). In addition, our findings indicate that LDNs are generated by TGF-β (Figure 5), a major effector cytokine in the resolution of inflammation (Henson and Bratton, 2013). These findings suggest that the accumulation of LDNs may be an integral part of the inflammatory process where neutrophils with a reduced inflammatory profile take part in its resolution. By analogy, the tumor microenvironment is a site of chronic inflammation, and we propose that in this scenario LDNs are generated toward resolution of tumor-associated inflammation. However, because tumor-associated inflammation is never resolved, this process is skewed and LDNs continue to accumulate, leading to an overall protumor neutrophil phenotype.

LDNs were previously implicated in several pathological scenarios including autoimmune diseases (Denny et al., 2010), HIV (Cloke et al., 2012), severe infection (Morisaki et al., 1992), and cancer (Brandau et al., 2011). In the context of cancer, LDNs were usually thought to be G-MDSCs. However, MDSCs are characterized as immature cells, and, whereas immature neutrophils in the LD fraction could be indeed G-MDSCs (Youn et al., 2008), the presence of mature neutrophils, in patients and in tumor-bearing mice, in this fraction was not previously shown. With the ability to physically separate pro- and antitumor neutrophils, we were able to gain insight into the cellular origins of the different neutrophil subpopulations and their dynamics with tumor progression. Although HDNs and LDNs may be derived from a common progenitor, their release into the circulation is differentially regulated (Figure 4). LDNs rapidly accumulate in the circulation, whereas HDNs appear in the circulation much later. These data are compatible with the appearance of immature neutrophils in the LD fraction and the time needed for HDNs to complete their maturation in the bone marrow. Importantly, the release of immature neutrophils to the circulation is not the result of bone-marrow depletion of HDNs because it occurs while there are still significant numbers of bone marrow residing HDNs.

Neutrophil Heterogeneity and Plasticity

Through adoptive transfer experiments, we were able to show that in tumor-bearing mice mature HDNs can switch to the low-density fraction. This is accompanied by an increase in size and the gain of an immunosuppressive protumor function. Whereas these experiments demonstrate that a switch in neutrophil phenotype occurs in vivo, the full extent of this transition is difficult to evaluate in vivo due to the low degree of neutrophil retrieval. However, when we followed the transition from HDN to LDN ex vivo, we found that it occurs spontaneously only in late-stage tumor-bearing mice (Figure 4). We further demonstrate that this functional change does not represent degranulation and is driven by TGF-β (Figure 5). Interestingly, we have previously shown that TGF-β blocks the antitumor function of neutrophils (Granot et al., 2011) and induces an N2 protumor phenotype (Fridlender et al., 2009). Finally, in vivo depletion of TGF-β results in a significant decrease in LDNs providing an indication for the role TGF-β plays in regulating the LDN/HDN ratio in vivo. However, although TGF-β is both required and sufficient for driving the transition of HDNs in tumor-bearing mice to LDNs, it had no significant effect on HDNs from naive mice suggesting that further activation of HDNs is required for TGF-β to have an effect. This activation is most likely a direct consequence of the significantly different chemokine/cytokine milieu neutrophils encounter in the context of cancer (Sionov et al., 2014). Taken together, these findings demonstrate again the major importance of TGF-β in the innate immune responses to tumor progression and highlight this signaling pathway as a possible target for anticancer therapy. This notion is further supported by observations showing that myeloid cell-specific ablation of TGF-β signaling enhances myeloid cell antitumor properties and inhibits tumor growth (Novitskiy et al., 2012). Our observations suggest that, on top of blocking neutrophil cytotoxicity, TGF-β mediates a shift in the equilibrium between neutrophil subtypes with conflicting roles. We further show that not only can HDNs switch to become LDNs, but also a fraction of the circulating LDNs have

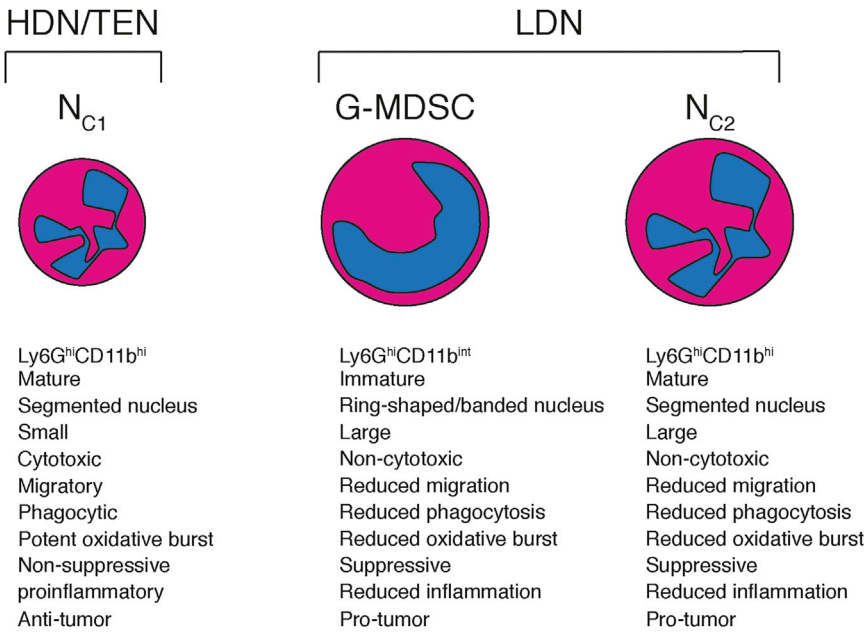


Figure 7. Distinct Circulating Neutrophil Subtypes in Cancer

Circulating neutrophils, characterized as Ly6G$^+$CD11b$^+$, may be distinguished according to their density. Circulating high-density neutrophils (HDNs) previously described as TENs (Granot et al., 2011) are N1-like, provide antitumor functions, and are designated N_{C1}. Circulating low-density neutrophils consist of mature and immature cells. The immature cells were previously described as G-MDSCs (Youn et al., 2008) and provide immunosuppressive functions associated with protumor contribution. The mature low-density neutrophils are derived from N_{C1} but have reduced neutrophil functions, are less inflammatory, are not cytotoxic, acquire immunosuppressive functions, have an overall N2-like phenotype, and are designated N_{C2}.

the capacity to switch to the HDN fraction, both in vivo and ex vivo (Figure 3). In light of the immature state of a subset of LDNs and the mature state of all HDNs, the low- to high-density transition raises the possibility of neutrophil maturation in the circulation, challenging the current paradigm (Galli et al., 2011) and requiring further exploration.

Collectively, our data show that circulating neutrophils may be roughly divided into mature and immature cells. Although immature neutrophils are commonly referred to as G-MDSCs, there is clear need for a nomenclature to account for the distinction between mature HDN (TENs) and our newly described mature LDNs. Therefore, we propose that the two subtypes of mature circulating neutrophils (N_C) be designated N_{C1} (N1-like N_C) for mature HDNs and N_{C2} for mature LDNs (Figure 7).

It is generally accepted that identifying human MDSCs is complicated by the lack of a specific known marker and due to the absence of a human homolog of murine GR1 (Solito et al., 2014). Different markers have been suggested to describe G-MDSCs, but no specific marker has been agreed upon (Dumitru et al., 2012; Solito et al., 2014). Our data may suggest a previously unevaluated way to isolate and further characterize human G-MDSCs, namely, by their density and appearance. Further research to explore this possibility is warranted.

Our study provides a perspective of neutrophil function in cancer where dynamic changes within multiple neutrophil subtypes are integrated toward overall pro- or antitumor neutrophil contribution. These observations challenge the current concept of circulating neutrophils as a fully differentiated homogenous cell population that has limited plasticity. They also provide a mechanistic explanation for the controversy that surrounds neutrophil function in cancer. It is possible that our findings also open an avenue for the identification of additional neutrophil subtypes. Although neutrophils are short lived, their remarkable plasticity is manifested in acquisition of different, or even opposing, phenotypes in response to environmental cues. Understanding these cues and their consequences on neutrophil function may serve to enhance the proportion of favorable neutrophil subpopulations at the expense of the harmful subpopulations. This, in turn, may serve as an unexplored strategy for treating cancer and possibly immune-mediated diseases.

EXPERIMENTAL PROCEDURES

Animals

Five- to 7-week-old Balb/c, C57/Bl6, and C57/Bl6-CAG-EGFP mice were purchased from the Jackson Laboratory and Harlan (Israel). LSL KrasG12D mice were kindly provided by Prof. Yuval Dor of the Hebrew University, Jerusalem, Israel. All experiments involving animals were approved by the Hebrew University's Institutional Animal Care and Use Committee (IACUC).

Cell Lines

4T1 cells were purchased from the ATCC. E0771 cells were kindly provided by Dr. Ross L. Levine, Memorial Sloan-Kettering Cancer Center. AB12 cells were kindly provided by Dr. Steven Albelda from the University of Pennsylvania. AT-3 cells were kindly provided by Dr. Scott Abrams, Roswell Park Cancer Institute, Buffalo, NY. Cells were transduced with a lentiviral vector (pLVX-Luc) to stably express firefly luciferase. 4T1, E0771, and AT-3 cells were orthotopically injected into the mammary fat pad. AB12 cells were introduced subcutaneously.

Mutated K-ras Orthotopic Lung Cancer Model

The orthotopic lung cancer model using intratracheal Ad.Cre in transgenic K-ras mice has been previously described in detail (Wilderman et al., 2005). Briefly, to activate the conditional oncogene and induce tumors, 100 ml of saline with 3×10^{10} particles of adenovirus containing Cre recombinase (Ad.Cre) were administered intranasally to LSL KrasG12D mice. Circulating neutrophils were evaluated 2 months after instillation of Ad.Cre.

Mouse Neutrophil Purification

Whole blood was collected by cardiac puncture using heparinized (Sigma) syringe. The blood was diluted with 5 vol of PBS containing 0.5% BSA and subjected to a discontinuous Histopaque (Sigma) gradient (1.077 and 1.119). HDNs were collected from the 1.077-1.119 interface. LDNs were collected from the plasma-1.077 interface. Red blood cells (RBCs) were eliminated by hypotonic lysis. Neutrophil purity and viability were determined visually and were consistently >98% for HDNs. LDN purity depended on tumor stage and was up to 82%. For functional assays, where greater neutrophil purity

was required, Ly6G$^+$ cells were further purified using the anti-Ly6G MicroBeads kit (Miltenyi) or EasySep kit (STEMCELL Technologies).

Human Neutrophil Purification

Collection of blood from patients was approved by Hadassah Medical Center institutional review board. Following informed consent, blood samples (~10 ml) were collected from healthy volunteers or from lung or breast cancer patients. Blood samples were transferred to the lab for analysis no more than 15 min after blood draw. Heparinzed blood (20 U/ml) was mixed with an equal volume of Dextran 500 (3% in saline) and incubated 30 min at room temperature. The leukocyte-rich supernatant was layered on top of Histopaque 1077 (Sigma) and centrifuged. High-density neutrophils were collected in the pellet fraction. LDNs were collected from the 1077-plasma interface. Neutrophils were resuspended in 10 ml 0.2% NaCl for 30 s to remove contaminating erythrocytes. Isotonicity was restored by the addition of 10 ml 1.6% NaCl. Neutrophils were than washed three times in Hanks's balanced salt solution. The percentage of neutrophils out of the LD fraction was measured with flow cytometry, and the percentage of LDNs out of all neutrophil was then calculated.

Statistical Analysis

For studies comparing differences between two groups, we used unpaired Student's t tests. For studies comparing more than two groups, we used ANOVA with appropriate post hoc testing. Differences were considered significant when $p < 0.05$. Data are presented as mean ± SEM. Differences were considered significant when $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.12.039.

AUTHOR CONTRIBUTIONS

J.Y.S., J.M., S.A., I.M., H.K., L.L., P.D., D.L., L.P, R.V.S., and E.H. conducted the experiments. J.Y.S., A.A, A.H.H., E.H., Z.G.F., and Z.G. planned the experiments. Z.G.F. and Z.G. wrote the manuscript.

ACKNOWLEDGMENTS

Z.G. is supported by grants from the I-CORE Gene Regulation in Complex Human Disease, Center no. 41/11, the Abisch-Frenkel Foundation, the Rosetrees Trust, the Israel Cancer Research Foundation (RCDA grant), and the CONCERN foundation. Z.G.F. is supported by grants from the Israel Cancer Research Foundation (RCDA grant), Chief Scientist of the Israel Ministry of Health, and the Israel Lung Association. We thank Dr. Myriam Grunewald for critical reading of this manuscript. D.L. is supported by the Azrieli Foundation Fellowship for which she is grateful.

Received: July 14, 2014
Revised: November 4, 2014
Accepted: December 17, 2014
Published: January 22, 2015

REFERENCES

Ackermann, M.F., Lamm, K.R., Wiegand, G.W., and Luster, M.I. (1989). Antitumor activity of murine neutrophils demonstrated by cytometric analysis. Cancer Res. *49*, 528–532.

Bannenberg, G.L., Chiang, N., Ariel, A., Arita, M., Tjonahen, E., Gotlinger, K.H., Hong, S., and Serhan, C.N. (2005). Molecular circuits of resolution: formation and actions of resolvins and protectins. J. Immunol. *174*, 4345–4355.

Barcellos-Hoff, M.H., and Akhurst, R.J. (2009). Transforming growth factor-beta in breast cancer: too much, too late. Breast Cancer Res. *11*, 202.

Beauvillain, C., Cunin, P., Doni, A., Scotet, M., Jaillon, S., Loiry, M.L., Magistrelli, G., Masternak, K., Chevailler, A., Delneste, Y., and Jeannin, P. (2011). CCR7 is involved in the migration of neutrophils to lymph nodes. Blood *117*, 1196–1204.

Borregaard, N., Kjeldsen, L., Sengeløv, H., Diamond, M.S., Springer, T.A., Anderson, H.C., Kishimoto, T.K., and Bainton, D.F. (1994). Changes in subcellular localization and surface expression of L-selectin, alkaline phosphatase, and Mac-1 in human neutrophils during stimulation with inflammatory mediators. J. Leukoc. Biol. *56*, 80–87.

Brandau, S., Trellakis, S., Bruderek, K., Schmaltz, D., Steller, G., Elian, M., Suttmann, H., Schenck, M., Welling, J., Zabel, P., and Lang, S. (2011). Myeloid-derived suppressor cells in the peripheral blood of cancer patients contain a subset of immature neutrophils with impaired migratory properties. J. Leukoc. Biol. *89*, 311–317.

Cloke, T., Munder, M., Taylor, G., Müller, I., and Kropf, P. (2012). Characterization of a novel population of low-density granulocytes associated with disease severity in HIV-1 infection. PLoS ONE *7*, e48939.

Colombo, M.P., Lombardi, L., Stoppacciaro, A., Melani, C., Parenza, M., Bottazzi, B., and Parmiani, G. (1992). Granulocyte colony-stimulating factor (G-CSF) gene transduction in murine adenocarcinoma drives neutrophil-mediated tumor inhibition in vivo. Neutrophils discriminate between G-CSF-producing and G-CSF-nonproducing tumor cells. J. Immunol. *149*, 113–119.

Cretney, E., Takeda, K., Yagita, H., Glaccum, M., Peschon, J.J., and Smyth, M.J. (2002). Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. J. Immunol. *168*, 1356–1361.

De Larco, J.E., Wuertz, B.R., and Furcht, L.T. (2004). The potential role of neutrophils in promoting the metastatic phenotype of tumors releasing interleukin-8. Clin. Cancer Res. *10*, 4895–4900.

Denny, M.F., Yalavarthi, S., Zhao, W., Thacker, S.G., Anderson, M., Sandy, A.R., McCune, W.J., and Kaplan, M.J. (2010). A distinct subset of proinflammatory neutrophils isolated from patients with systemic lupus erythematosus induces vascular damage and synthesizes type I IFNs. J. Immunol. *184*, 3284–3297.

Di Carlo, E., Forni, G., Lollini, P., Colombo, M.P., Modesti, A., and Musiani, P. (2001). The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood *97*, 339–345.

Dumitru, C.A., Moses, K., Trellakis, S., Lang, S., and Brandau, S. (2012). Neutrophils and granulocytic myeloid-derived suppressor cells: immunophenotyping, cell biology and clinical relevance in human oncology. Cancer Immunol. Immunother. *61*, 1155–1167.

Fridlender, Z.G., and Albelda, S.M. (2012). Tumor-associated neutrophils: friend or foe? Carcinogenesis *33*, 949–955.

Fridlender, Z.G., Sun, J., Kim, S., Kapoor, V., Cheng, G., Ling, L., Worthen, G.S., and Albelda, S.M. (2009). Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN. Cancer Cell *16*, 183–194.

Galdiero, M.R., Bonavita, E., Barajon, I., Garlanda, C., Mantovani, A., and Jaillon, S. (2013). Tumor associated macrophages and neutrophils in cancer. Immunobiology *218*, 1402–1410.

Galli, S.J., Borregaard, N., and Wynn, T.A. (2011). Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. Nat. Immunol. *12*, 1035–1044.

Granot, Z., Henke, E., Comen, E.A., King, T.A., Norton, L., and Benezra, R. (2011). Tumor entrained neutrophils inhibit seeding in the premetastatic lung. Cancer Cell *20*, 300–314.

Heifets, L. (1982). Centennial of Metchnikoff's discovery. J. Reticuloendothel. Soc. *31*, 381–391.

Henson, P.M., and Bratton, D.L. (2013). Antiinflammatory effects of apoptotic cells. J. Clin. Invest. *123*, 2773–2774.

Hicks, A.M., Riedlinger, G., Willingham, M.C., Alexander-Miller, M.A., Von Kap-Herr, C., Pettenati, M.J., Sanders, A.M., Weir, H.M., Du, W., Kim, J., et al. (2006). Transferable anticancer innate immunity in spontaneous regression/complete resistance mice. Proc. Natl. Acad. Sci. USA *103*, 7753–7758.

Jan, M.S., Huang, Y.H., Shieh, B., Teng, R.H., Yan, Y.P., Lee, Y.T., Liao, K.K., and Li, C. (2006). CC chemokines induce neutrophils to chemotaxis, degranulation, and alpha-defensin release. J. Acquir. Immune Defic. Syndr. *41*, 6–16.

Joyce, J.A., and Pollard, J.W. (2009). Microenvironmental regulation of metastasis. Nat. Rev. Cancer *9*, 239–252.

Kowanetz, M., Wu, X., Lee, J., Tan, M., Hagenbeek, T., Qu, X., Yu, L., Ross, J., Korsisaari, N., Cao, T., et al. (2010). Granulocyte-colony stimulating factor promotes lung metastasis through mobilization of Ly6G+Ly6C+ granulocytes. Proc. Natl. Acad. Sci. USA *107*, 21248–21255.

Kushner, B.H., and Cheung, N.K. (1992). Absolute requirement of CD11/CD18 adhesion molecules, FcRII and the phosphatidylinositol-linked FcRIII for monoclonal antibody-mediated neutrophil antihuman tumor cytotoxicity. Blood *79*, 1484–1490.

López-Lago, M.A., Posner, S., Thodima, V.J., Molina, A.M., Motzer, R.J., and Chaganti, R.S. (2013). Neutrophil chemokines secreted by tumor cells mount a lung antimetastatic response during renal cell carcinoma progression. Oncogene *32*, 1752–1760.

Mantovani, A., Cassatella, M.A., Costantini, C., and Jaillon, S. (2011). Neutrophils in the activation and regulation of innate and adaptive immunity. Nat. Rev. Immunol. *11*, 519–531.

Mayadas, T.N., Cullere, X., and Lowell, C.A. (2014). The multifaceted functions of neutrophils. Annu. Rev. Pathol. *9*, 181–218.

McGary, C.T., Miele, M.E., and Welch, D.R. (1995). Highly metastatic 13762NF rat mammary adenocarcinoma cell clones stimulate bone marrow by secretion of granulocyte-macrophage colony-stimulating factor/interleukin-3 activity. Am. J. Pathol. *147*, 1668–1681.

Mishalian, I., Bayuh, R., Levy, L., Zolotarov, L., Michaeli, J., and Fridlender, Z.G. (2013). Tumor-associated neutrophils (TAN) develop pro-tumorigenic properties during tumor progression. Cancer Immunol. Immunother. *62*, 1745–1756.

Morisaki, T., Goya, T., Ishimitsu, T., and Torisu, M. (1992). The increase of low density subpopulations and CD10 (CALLA) negative neutrophils in severely infected patients. Surg. Today *22*, 322–327.

Novitskiy, S.V., Pickup, M.W., Chytil, A., Polosukhina, D., Owens, P., and Moses, H.L. (2012). Deletion of TGF-β signaling in myeloid cells enhances their anti-tumorigenic properties. J. Leukoc. Biol. *92*, 641–651.

Nozawa, H., Chiu, C., and Hanahan, D. (2006). Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proc. Natl. Acad. Sci. USA *103*, 12493–12498.

Pekarek, L.A., Starr, B.A., Toledano, A.Y., and Schreiber, H. (1995). Inhibition of tumor growth by elimination of granulocytes. J. Exp. Med. *181*, 435–440.

Peranzoni, E., Zilio, S., Marigo, I., Dolcetti, L., Zanovello, P., Mandruzzato, S., and Bronte, V. (2010). Myeloid-derived suppressor cell heterogeneity and subset definition. Curr. Opin. Immunol. *22*, 238–244.

Piccard, H., Muschel, R.J., and Opdenakker, G. (2012). On the dual roles and polarized phenotypes of neutrophils in tumor development and progression. Crit. Rev. Oncol. Hematol. *82*, 296–309.

Schif-Zuck, S., Gross, N., Assi, S., Rostoker, R., Serhan, C.N., and Ariel, A. (2011). Saturated-efferocytosis generates pro-resolving CD11b low macrophages: modulation by resolvins and glucocorticoids. Eur. J. Immunol. *41*, 366–379.

Schmidt, H., Bastholt, L., Geertsen, P., Christensen, I.J., Larsen, S., Gehl, J., and von der Maase, H. (2005). Elevated neutrophil and monocyte counts in peripheral blood are associated with poor survival in patients with metastatic melanoma: a prognostic model. Br. J. Cancer *93*, 273–278.

Shen, L., Smith, J.M., Shen, Z., Eriksson, M., Sentman, C., and Wira, C.R. (2007). Inhibition of human neutrophil degranulation by transforming growth factor-beta1. Clin. Exp. Immunol. *149*, 155–161.

Sionov, R.V., Fridlender, Z.G., and Granot, Z. (2014). The multifaceted roles neutrophils play in the tumor microenvironment. Cancer Microenviron., Published online June 4, 2014. http://dx.doi.org/10.1007/s12307-014-0147-5.

Solito, S., Marigo, I., Pinton, L., Damuzzo, V., Mandruzzato, S., and Bronte, V. (2014). Myeloid-derived suppressor cell heterogeneity in human cancers. Ann. N Y Acad. Sci. *1319*, 47–65.

Tsuda, Y., Takahashi, H., Kobayashi, M., Hanafusa, T., Herndon, D.N., and Suzuki, F. (2004). Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant Staphylococcus aureus. Immunity *21*, 215–226.

Waight, J.D., Hu, Q., Miller, A., Liu, S., and Abrams, S.I. (2011). Tumor-derived G-CSF facilitates neoplastic growth through a granulocytic myeloid-derived suppressor cell-dependent mechanism. PLoS ONE *6*, e27690.

Welch, D.R., Schissel, D.J., Howrey, R.P., and Aeed, P.A. (1989). Tumor-elicited polymorphonuclear cells, in contrast to "normal" circulating polymorphonuclear cells, stimulate invasive and metastatic potentials of rat mammary adenocarcinoma cells. Proc. Natl. Acad. Sci. USA *86*, 5859–5863.

Wilderman, M.J., Sun, J., Jassar, A.S., Kapoor, V., Khan, M., Vachani, A., Suzuki, E., Kinniry, P.A., Sterman, D.H., Kaiser, L.R., and Albelda, S.M. (2005). Intrapulmonary IFN-beta gene therapy using an adenoviral vector is highly effective in a murine orthotopic model of bronchogenic adenocarcinoma of the lung. Cancer Res. *65*, 8379–8387.

Youn, J.I., Nagaraj, S., Collazo, M., and Gabrilovich, D.I. (2008). Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J. Immunol. *181*, 5791–5802.

Zhu, B., Bando, Y., Xiao, S., Yang, K., Anderson, A.C., Kuchroo, V.K., and Khoury, S.J. (2007). CD11b+Ly-6C(hi) suppressive monocytes in experimental autoimmune encephalomyelitis. J. Immunol. *179*, 5228–5237.