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Quantification of Early-Stage Myeloid-Derived Suppressor Cells in Cancer Requires Excluding Basophils

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Abstract

Myeloid derived suppressor cells (MDSCs) are a heterogeneous group of immature cells that accumulate in the peripheral blood and tumor microenvironment and are barriers to cancer therapy. MDSCs serve as prognostic biomarkers and are targets for therapy. Based on surface markers, three subsets of MDSCs have been defined in humans: granulocytic, monocytic and early-stage (e-MDSCs). The markers attributed to e-MDSCs overlap with those of basophils, which are rare circulating myeloid cells with unrecognized roles in cancer. Thus, we asked whether e-MDSCs in circulation and the tumor microenvironment include basophils. On average 58% of cells with e-MDSC surface markers in blood and 36% in ascites from patients with ovarian cancer (OC) were basophils based on CD123^{high} expression and cytology, whereas cells with immature features were

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Author contributions

ANHK and BHS developed the study and wrote the manuscript. ANHK, TRE, EA, BS, and BHS designed and performed experiments and analyzed data. JTW performed morphological analysis and JM performed clinical data analysis. KHE oversaw the statistical analysis. KLS, TRE, JM, and JTW contributed to the manuscript preparation. JDT, KBM, KO, SIA, and BHS provided technical support and conceptual advice. All authors discussed the results and implications and commented on the manuscript at all stages.

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rare. Circulating and ascites basophils did not suppress proliferation of stimulated T cells, a key feature of MDSCs. Increased accumulation of basophils and basogranulin, a marker of basophil degranulation, were observed in ascites compared to serum in patients with newly diagnosed OC. Basophils recruited to the tumor microenvironment may exacerbate fluid accumulation by their release of pro-inflammatory granular constituents that promote vascular leakage. No significant correlation was observed between peripheral basophil counts and survival in patients with OC. Our results suggest that studies in which e-MDSCs were defined solely by surface markers should be re-revaluated to exclude basophils. Both immaturity and suppression are criteria to define e-MDSCs in future studies.

Keywords

MDSC; e-MDSC; basophil; cancer; immunosuppression

Introduction

Myeloid-derived suppressor cells (MDSCs) are an immature, heterogeneous group of myeloid cells that suppress T-cell responses (1, 2). In cancer, disordered myelopoiesis driven by tumor-derived factors (e.g., G-CSF, GM-CSF, and IL6) can result in an expansion of immature suppressive myeloid cells that impairs antitumor immunity. In addition, MDSCs can secrete pro-angiogenic factors that facilitate tumor progression.

Two MDSC subpopulations have been described in tumor-bearing mice: granulocytic ($CD11b^{+}Ly6G^{+}Ly6C^{low}$) and monocytic ($CD11b^{+}Ly6G^{-}Ly6C^{high}$) cells. These myeloid cells expand in various organs, including bone marrow, blood, secondary lymphoid organs, and within tumor (3–5). Due to difficulties in obtaining and purifying MDSC populations from the tumor microenvironment, most human studies focus on circulating MDSCs. Consensus guidelines define granulocytic MDSCs (PMN-MDSCs) as $CD11b^{+}CD14^{-}CD115^{+}$ or $CD11b^{+}CD14^{-}CD66b^{+}$ and monocytic MDSCs (M-MDSCs) as $CD11b^{+}CD14^{+}HLA\text{-}DR^{-/low}CD15^{-}$ (6). Although M-MDSCs are $CD33^{high}$, PMN-MDSCs are $CD33^{dim}$. Early-stage MDSCs (e-MDSCs) likely a mixed group of immature progenitor cells, are defined as Lin^{-} (including CD3, CD14, CD15, CD19, CD56) and $HLA\text{-}DR^{-}CD33^{+}$ (6, 7). In contrast to conventional mature normal-density neutrophils, MDSCs, including various maturation stages of PMN-MDSCs, co-sediment with peripheral blood mononuclear cells (PBMC) following density gradient separation. Since expression of surface markers does not uniquely define MDSCs, functional assays can further identify MDSCs through their ability to inhibit T-cell activity. However, these functional assays are often not performed because of the paucity of MDSCs in human samples and the practical limitations of performing such labor-intensive studies when large numbers of patients' samples are used for epidemiological or biomarker studies. Thus, the identification of putative MDSCs based on surface marker expression of myeloid cells within the PBMC fraction is commonly used as a practical standard for MDSC quantification. Although MDSCs are typically defined as immature, microscopic analysis of putative MDSCs to demonstrate immaturity is not performed routinely and not a part of consensus criteria for defining MDSCs. MDSC status as immature underlies the rationale for therapeutic

approaches intended to drive differentiation of immature suppressive cells into mature nonsuppressive myeloid cells (8–10).

Almand et al. (11) described the expansion of circulating myeloid cells in patients with cancer that were defined by morphologic immaturity, $CD33^{+}CD15^{-}CD14^{-}HLA\text{-}DR^{-}$ expression, and suppression of antigen-specific T-cell responses. These findings established the foundation for the subsequent definition of MDSCs. Of the three MDSC populations, e-MDSCs are considered to be the least mature (hence the description “Early-stage”) based on lack of expression of granulocytic or monocytic markers. e-MDSCs have been identified based on surface markers in circulating myeloid cells, and, to a lesser extent, in myeloid cells in the tumor microenvironment of various cancers (12–16) without well-defined morphologic criteria. Additional MDSC subsets have been defined based on variation in surface markers (16). The fraction of e-MDSCs in PBMCs is similar between healthy donors (about 1%) and patients with head and neck cancer and patients with ovarian cancer (OC), and does not correlate with survival (13, 16). Although higher amounts of circulating e-MDSCs are evident in patients with OC (0.06%) than healthy donors (0.01%), the fraction of circulating e-MDSCs does not correlate with survival (15). Putative circulating e-MDSCs from patients with head and neck cancer (13) and from patients with OC (16) show little to no T-cell suppression *ex vivo* depending on the mode of T-cell stimulation. These findings suggest that the population of cells defined in the field as e-MDSCs shows inconsistent immunosuppressive properties. Improved definition of e-MDSCs will aid study of their functional roles in the tumor microenvironment.

Basophils are a subset of myeloid cells that make up only about 0.5% of circulating leukocytes, mediate anti-parasitic host defense, and drive allergic and anaphylactic disorders (17). IL3 promotes development of progenitor cells. Its receptor CD123 (IL3R-α) is expressed on common myeloid progenitors (18, 19). CD123 expression is lost in most mature myeloid cells, though expression is retained in plasmacytoid DCs and mature basophils. Surface markers of basophils ($CD33^{+}CD14^{-}CD15^{-}HLA\text{-}DR^{-}CD3^{-}CD19^{-}CD56^{-}$) (20) and e-MDSCs overlap. Indeed, basophils may contaminate samplies identified as circulating e-MDSCs (21). We therefore evaluated the putative e-MDSC population in circulation and in the tumor microenvironment of patients with newly diagnosed metastatic OC. We found that, in healthy donors and in patients with newly diagnosed metastatic OC, an average of 58% of what has been defined as circulating e-MDSCs were actually basophils. In addition, basophils constituted an average of 36% of the putative e-MDSC population in the ascites of patients with OC, whereas cytologically immature myeloid cells that could be e-MDSCs were rare. Purified basophils from blood and ascites of patients with OC did not suppress stimulated T-cell proliferation. Peripheral basophils from healthy donors did not suppress stimulated T-cell proliferation nor did they acquire a suppressor phenotype when cultured with OC ascites supernatants. The peripheral basophil concentration at diagnosis did not correlate with subsequent progression-free survival (PFS) or overall survival (OS) of patients with OC. Together our results demonstrate the need to re-evaluate prior studies of e-MDSCs based on the potential for basophil contamination and to exclude basophils from future studies of e-MDSCs. We propose criteria for defining e-MDSCs that include morphologic evidence of immaturity and suppressive function.

Materials and Methods

Patients and specimens

Participants included healthy donors (HD), patients with benign adnexal mass undergoing resection surgery, and patients with advanced OC. Blood and ascites samples were prospectively collected from patients with newly diagnosed advanced OC during 2015 – 2019 at Roswell Park Comprehensive Cancer Center (Roswell Park), Buffalo, NY, as previously described (22). Blood samples were collected prior to primary surgeries, and ascites were collected either by diagnostic paracentesis or in the operating room prior to surgery. Serum was collected from peripheral blood of healthy donors (n=3) and patients with OC (n=17). Ascites were filtered through 300 μm filters and then centrifuged (500 g, 10 min). Serum and ascites supernatants were stored at −80°C until further use and ascites cells were preserved (in RPMI-1640 + 5% DMSO + 20% FBS) in liquid nitrogen. When available, post-operative fluid from an abdominal drainage tube was collected the day after primary surgery for OC. Ascites and post-operative fluid were evaluated for CBC with differential prior to processing. The medical records of these patients were retrospectively reviewed for demographics, tumor stage and grade, and peripheral blood CBC with differential. The electronic medical records of 325 patients with OC who underwent debulking surgery followed by standard adjuvant chemotherapy at Roswell Park from 1995 – 2015, were also retrospectively reviewed for pre-treatment basophil concentrations, demographics, FIGO stage, histology, debulking status, PFS and OS.

Study approval

This study was approved by the Institutional Review Board of Roswell Park and followed federal and state requirements. All participants gave written informed consent prior to inclusion in the study (protocols I-215512 and I-188310). All studies were conducted in compliance with the Declaration of Helsinki.

Isolation and characterization of e-MDSCs in peripheral blood and ascites

PBMCs were isolated from peripheral blood of patients (n=18) and healthy donors (n=5) using lymphocyte separation media (Mediatech, 25–072-CV) and SepMate tubes (Stemcell, 85450) following manufacturer's protocol. PBMCs were used fresh or cryopreserved. Cell count and viability were assessed by Trypan blue staining. $CD3^+$ cells were depleted from PBMCs and ascites cells (n=5) using CD3 microbeads (Miltenyi, 130–050-101) and autoMACS. CD3-depleted PBMCs were analyzed by flow cytometry after staining with anti-human CD45, CD11b, CD33, CD14, CD15, HLA-DR, CD123, CD11c, CD303 mAb (Supplementary Table S1) and L/D-Aqua (Thermo Fisher, L34959). Non-aggregate and viable $CD45^+CD11b^+CD33^+$ cells were gated to get $CD15^-CD14^-$ fractions and those cells were gated to obtain $HLA\text{-}DR^-CD123^+$ and $HLA\text{-}DR^-CD123^-$ cells. $CD45^+CD11b^+CD33^+CD14^-CD15^-HLA\text{-}DR^-CD123^{high}$ (putative basophils), $CD45^+CD11b^+CD33^+CD14^-CD15^-HLA\text{-}DR^+CD123^{mid}$ (putative DCs) and $CD45^+CD11b^+CD33^+CD14^-CD15^-HLA\text{-}DR^-CD123^-$ ($HLA\text{-}DR^-CD123^-$) L/D-aqua-negative cells from PBMCs and ascites cells were flow sort-purified and assessed by cytology. Purified ascites-basophils were also assessed for T-cell suppression.

Cytology slide preparation, staining, and review

Cytospins were prepared using sort-purified cells from PBMCs and ascites cells, micro slides (VWR, 48311–703) and a Shandon Cytospin2 Centrifuge. A Wright-Giemsa staining protocol was used. Slides were fixed with methanol (1 min), residual methanol was decanted, and slides were stained with a modified-Wright stain (Sigma-WS-128, 0.3%, buffered at pH 6.9 in methanol, contains stabilizer and surfactant) (5 min). Three milliliter layers of freshly prepared modified-Giemsa stain (Sigma-GS-128, 0.4% in buffered methanol solution pH 6.8 with stabilizers) were placed onto the slides for 30 minutes. Afterwards, slides were rinsed with tap water, air dried, rinsed with methanol and washed under running tap water, dried and then cover slips were placed. Toluidine blue staining was performed to confirm magenta-colored staining of basophil granules. Morphologic evaluation was performed by a hematopathologist (JTW).

ELISA

The levels of basogranulin in frozen serum samples, supernatants collected from ascites and day-1 post-operative drainage fluid samples of patients with OC were plated in duplicate wells and analyzed by human basogranulin ELISA kit (MyBiosource, MBS2602918), following the manufacturer's protocol.

Assessment of suppressive function of basophils

We previously observed that circulating neutrophils acquire a suppressor phenotype when incubated with OC ascites and other malignant fluid supernatants (23, 24). In the current study, neutrophils were used as a comparator with basophils in T-cell suppression assays. Neutrophils and T cells were isolated from peripheral blood (HD) <1h post-collection using the MACSxpress Neutrophil Isolation Kit (Miltenyi, 130–104-434) and the Pan T-cell Isolation Kit (Miltenyi, 130–098-193), respectively. The purity of neutrophils was >90% based on cytology and $CD33^{mid}CD15^{+}$ expression. The purity of T cells was >90% based on $CD45^{+}CD3^{+}$, $CD45^{+}CD3^{+}CD4^{+}$ and $CD45^{+}CD3^{+}CD8^{+}$ expression. Circulating basophils were isolated from PBMCs of HD (fresh) and patients with OC (cryopreserved) using the basophil isolation kit II (Miltenyi, 130–092-662) and autoMACS. The purity of basophils was >90% based on cytology and viability was >95% based on trypan blue exclusion. Freshly isolated T cells (1×10^5) were stimulated with anti-CD3/CD28 Dynabeads (2.5 μl; Thermo Fisher Scientific, 11132D) and cocultured with basophils or neutrophils (from HD in 1:1 ratio) and ascites supernatants (50% final well volume) in a 5% CO_2 incubator at 37°C. Similarly, stimulated T cells (1×10^5) were used in co-culture with circulating basophils from patients with OC (1:1 ratio) in media. Because of limited recovery of sort-purified basophils from cryopreserved OC ascites cells, a lower number of basophils (0.5×10^5) and T cells (1:1 ratio) were used in co-culture. After 72h, [^{3}H] thymidine (1 μCi per well, PerkinElmer, NET027X001MC) was added and allowed to incorporate for 16–18h. Cells were harvested onto a filter mat and counted on a beta counter. Results are expressed as net CPM = average CPM of stimulated T – average CPM of unstimulated T cells.

Statistical analysis

Comparisons between two groups were assessed by the Mann-Whitney test (two tailed) using Graph Pad Prism 8.3.0 (538). A nominal significance threshold of 0.05 was used for P values. The association between basophil concentration prior to surgery and PFS/OS was assessed using a Cox regression model adjusting for standard prognostic factors: age, FIGO stage, histology and surgical debulking (R0 versus other) using R 3.4.0 statistical language.

Results

Putative circulating e-MDSCs, with and without cancer, contain basophils

Since putative e-MDSCs and basophils are present at low frequencies in peripheral blood and express overlapping surface markers, we asked to what extent basophils are components of the e-MDSC fraction. Based on consensus recommendations (6), we defined putative e-MDSCs as $CD3^{-}CD45^{+}CD33^{+}CD11b^{+}CD14^{-}CD15^{-}HLA\text{-}DR^{-}$. Basophils were defined based on the same surface expression criteria, but with the addition of $CD123^{high}$ (20, 25). CD123 is highly expressed on basophils, but its expression can be reduced with basophil activation (26).

CD3-depleted frozen or fresh PBMCs from patients with OC and healthy donors were analyzed for CD123-expressing cells. Representative plots show that $CD3^{-}CD45^{+}CD33^{+}CD11b^{+}CD14^{-}CD15^{-}$ fractions contain three populations based on CD123 and HLA-DR expression: (i) $HLA\text{-}DR^{-}CD123^{high}$, (ii) $HLA\text{-}DR^{-}CD123^{-}$, and (iii) $HLA\text{-}DR^{+}CD123^{mid}$ (occasionally, distinct $HLA\text{-}DR^{+}CD123^{mid}$ and $HLA\text{-}DR^{+}CD123^{low}$ populations were observed) (Fig. 1A–D). Since e-MDSCs are $HLA\text{-}DR^{-}$, we sort-purified $HLA\text{-}DR^{-}CD123^{high}$ and $HLA\text{-}DR^{-}CD123^{-}$ populations for morphologic evaluation. We found that the $CD123^{high}$ cells were basophils (Fig. 1E). Basophils are round to ovoid in shape, range in size from 10 to 15 μm in diameter, and have basophilic cytoplasmic granules that are coarse and blue-black or purple-red. The presence of coarse granules in basophils was confirmed by toluidine blue staining (Fig. 1E, middle). Mature basophils have segmented nuclei and clumped chromatin. The segmented nuclei usually have 2–3 lobes but can range from 1–4 lobes. Cryopreserved basophils showed variable degranulation, which might be a freeze-thaw artifact (Fig. 1E, right). The $CD45^{+}CD33^{+}CD11b^{+}CD14^{-}CD15^{-}$ cell fraction in PBMCs also contains a variable percentage (6 – 74%) of $HLA\text{-}DR^{+}CD123^{mid}$ cells, that were in some samples divided into $CD123^{mid}$ and $CD123^{low}$ populations (Fig. 1D).

The $CD123^{-}$ cells population contained agranular mononuclear cells, including lymphocytes and monocytes. Lymphocytes are generally 7–15 μm in diameter with round to ovoid nuclei, diffusely dense to clumped chromatin and scant agranular cytoplasm (Fig. 1F). Monocytes are usually larger than lymphocytes (12–20 μm in diameter) with variably shaped nuclei, (including indented, band-shaped, folded and nonspecifically irregular), mature-appearing chromatin, and moderate amounts of cytoplasm (Fig. 1F left). Agranular mononuclear cells with more round nuclei, higher nuclear-cytoplasmic ratio, and nucleoli were also present among $CD123^{-}$ cells and may represent immature myeloid cells.

Additional frozen samples of circulating PBMC from patients with OC were analyzed for $CD123^+$ basophils in putative e-MDSC fractions. Without exclusion of $CD123^{high}$ cells, the proportion of putative e-MDSCs in the PBMCs fraction ranged between 0 – 1.77%; after exclusion of $CD123^+$ cells, the proportion ranged between 0 – 0.38% when gated on $CD45^+$ cells (Fig. 2A). Fig. 2B shows a range in the proportion of basophils within the putative e-MDSC population and demonstrates that basophils comprised a substantial proportion (mean = 58%) of putative e-MDSCs in patients with OC. Similarly processed circulating PBMCs from healthy donors showed a proportion of basophils ranging from 81 to 97%, whereas a patient with benign adnexal mass had 72% basophils in the putative e-MDSC fraction (Fig. 2B). Thus, in patients with OC, basophils make up 58% of the cells in the putative circulating e-MDSC population defined by existing criteria of surface antigen expression and morphology.

Variable proportions of basophils accumulate in the ovarian cancer microenvironment

Previously, we observed variable numbers of $CD33^+CD14^-CD15^-HLA\text{-}DR^-$ cells in the ascites of patients with OC (12) and have now found basophils in the putative peripheral e-MDSC population from patients with OC. We next evaluated phenotypical and morphological characteristics of the putative e-MDSCs in the ascites from patients with newly diagnosed metastatic OC. Ascites was drained prior to debulking surgery and cells in ascites were cryopreserved. After thawing and $CD3^+$ T-cell depletion, cells in the putative e-MDSC fraction were sort-purified into $CD123^{high}$ and $CD123^-$ populations and analyzed by cytology. Similar to circulating PBMCs, the putative e-MDSCs fraction in ascites contains variable proportions of $CD123^+$ and $CD123^-$ cells (Fig. 3A to D and Supplementary Fig. S1A–D). $HLA\text{-}DR^-CD123^{high}$ cells comprised 36% (range 15% - 71%) of the putative e-MDSC population (n = 5). Cytologic review of $HLA\text{-}DR^-CD123^{high}$ cells shows variably degranulated granulocytic cells that are morphologically most consistent with basophils (Fig. 3E), whereas $HLA\text{-}DR^-CD123^-$ cells were mostly lymphocytes and monocytes (Fig. 3F). The $HLA\text{-}DR^-CD123^-$ fractions of ascites cells also contained presumptive immature myeloid cells based on high nuclear to cytoplasmic ratio, rounder nuclei, finer chromatin, and presence of nucleoli (Fig. 3F, box). Differential counts performed on representative slides of circulating and ascites cells showed that immature cells comprised ~20–30% of the $HLA\text{-}DR^-CD123^-$ fraction. Based on surface expression and morphology, this may be the population of immature myeloid cells expanded in patients with cancer (11) and defined as MDSCs. However, these cells were too few to assess for T-cell suppression, and therefore we are unable to determine whether they are actual MDSCs. Thus, the putative e-MDSC fraction in OC ascites is composed of a variety of cell types, including basophils; morphologically immature myeloid cells are about 20–30% of the $HLA\text{-}DR^-CD123^-$ fraction.

Similar to circulating PBMCs (Fig. 1D), a variable percentage (19 – 90%) of $HLA\text{-}DR^+CD123^{mid}$ cells was observed in $CD45^+CD33^+CD11b^+CD14^-CD15^-$ cell fraction of OC ascites (Fig. 3D and Supplementary Fig. S1A–D). From surface expression ($CD11c^+$ and $CD11b^+$) and morphology, these $HLA\text{-}DR^+CD123^{mid}$ cells resemble myeloid DCs, although they are also $CD303^+$, which is expressed on plasmacytoid DCs. In some of the ascites samples, $HLA\text{-}DR^+CD123^{mid}$ cells had plasmacytoid DC morphology (Supplementary Fig.

S1E and F). These results suggest that both HLA-DR and CD123 should be used to identify putative e-MDSCs by flow cytometry and, by extension, immunostaining of tumor samples.

Basophils feature in ascites of ovarian cancer and increase following surgery

Since the microenvironment in advanced OC is inflammatory and injurious (24, 27, 28) and basophils play a role in inflammation and in wound healing (17, 29), we compared basophil concentrations in blood, ascites and post-operative peritoneal drainage fluids. We found that the percentage of basophils in blood from patients with OC are within normal range (<2%), whereas the percentage of basophils in paired ascites are variable (0 – 7%) and significantly ($p = 0.008$) higher than the amounts in blood (Fig. 4A). In a cohort of 5 patients with OC with banked post-operative fluid supernatants 1-day after debulking surgery, the percentage of basophils was higher in post-operative drainage fluid than pre-operative blood (Fig. 4B). Basogranulin is located in basophil granules, (30) and its release is a marker of basophil activation (30, 31). Elevated plasma basogranulin is observed in patients with allergic asthma (32). Since basophil concentrations were elevated in the ascites and post-operative fluid versus blood, we evaluated basogranulin amounts in serum, ascites, and post-operative drainage fluid samples from patients with OC. Like healthy donor serum, basogranulin was generally not detectable in the serum of patients with newly diagnosed OC. In contrast, patients with OC had significantly higher basogranulin amounts in ascites and post-operative drainage fluid than in serum (Fig. 4C). These results point to increased basophil accumulation and degranulation at sites of injury, both in the tumor microenvironment and following surgery.

Basophils are not immunosuppressive in the ovarian cancer microenvironment

Although circulating neutrophils in healthy donors and in patients with newly diagnosed metastatic OC are not suppressive, circulating neutrophils acquire a suppressor phenotype, defined as abrogation of stimulated T-cell proliferation, following exposure to OC ascites supernatants (23, 24). Since the putative e-MDSCs fraction in peripheral circulation and OC microenvironment contains basophils, we asked whether OC ascites can induce peripheral basophils to become T-cell suppressive. Basophils incubated in media or in ascites supernatants from patients with OC did not suppress anti-CD3/CD28-stimulated proliferation of healthy donor T cells, whereas neutrophils exposed to ascites acquired a suppressor phenotype (Fig. 4D). Next, we evaluated the T-cell suppressive capacity of basophils isolated from peripheral blood or ascites of patients with OC. Viable basophils magnetically isolated from PBMCs did not suppress stimulated T-cell proliferation (Fig. 4E). Similarly, sort-purified basophils from OC ascites cells were not T-cell suppressive (Fig. 4F). Together, these data show that basophils in circulation and in ascites of OC are not T-cell suppressive and that normal basophils do not acquire a T-cell suppressive phenotype in OC ascites.

Basophil concentrations in blood are not independent prognostic factors in OC

We next performed a retrospective analysis of pre-treatment basophil concentrations from complete blood count (CBC) and differential recordings and PFS and OS in 325 patients with newly diagnosed OC who underwent debulking surgery followed by standard adjuvant chemotherapy at Roswell Park. Patient demographics, clinicopathological features, and

summary of basophil concentrations are shown in Tables 1 and 2. Baseline peripheral basophil concentrations ($0.01 – 0.17 \times 10^3$/microliter) were not associated with PFS (median 20 months) or OS (median 48.8 months) when analyzed using a Cox regression model adjusting for standard prognostic factors: age, FIGO stage, histology and surgical de-bulking (Table 3). These data show that basophil concentrations in blood are not independent prognostic factors in OC.

Discussion

Since MDSCs are considered potential prognostic biomarkers and various therapeutic studies aim to deplete MDSCs or alter their function to enhance antitumor immunity, it is essential to define MDSC subsets accurately in a standardized and reproducible fashion. We found that mature basophils, based on surface markers and cytology make up an average of 58% of the putative e-MDSC fraction in peripheral blood and 36% in ascites from patients with OC. Basophils from circulation and tumor microenvironment of patients with OC are not immunosuppressive and peripheral basophils from healthy donors do not acquire a suppressor phenotype in the OC microenvironment.

Uhel et al. (21) showed that $CD123^+$ basophils comprised 68% of the putative e-MDSC fraction of circulating PBMC from patients with B-cell lymphoma, whereas the $CD123^–$ fraction included immature myeloid cells with agranular cytoplasm-containing vacuoles morphologically consistent with e-MDSCs. These results and our studies of basophils in circulation and in the tumor microenvironment raise a cautionary note in defining e-MDSCs based on standard surface markers. Because our study was small and restricted to patients with newly diagnosed OC, we cannot make generalizations about e-MDSCs in patients with different cancer types or other chronic inflammatory diseases or disorders. Nonetheless, at a minimum, we recommend that studies of e-MDSCs include the following criteria: (i) demonstration of immaturity based on standard morphologic criteria; (ii) exclusion of basophils; and (iii) evidence of suppression of T cells.

In addition to basophils ($CD123^{high}HLA\text{-}DR^–$), there were $CD123^{dim}HLA\text{-}DR^+$ cells in purified $CD45^+CD33^+CD11b^+CD14^–CD15^–$ cell fraction of PBMC and ascites cells from patients with OC, which may be dendritic cells. The remaining populations included $CD123^–HLA\text{-}DR^–$ cells, which, based on cytology, consisted of monocytes, lymphocytes, and cells that may be immature myeloid cells. Monocytes and lymphocytes should have been excluded by sorting, which points to the potential for contamination even with purification by sorting when attempting to isolate rare cells from large populations. As the morphologically immature cells within the putative e-MDSC population were rare, we could not sort-purify them for functional studies; these cells should not be defined as e-MDSCs.

We observed increased accumulation of basophils in ascites and in post-operative drainage fluid compared to circulating basophils in patients with newly diagnosed OC. Basogranulin, a marker of activated basophils (33), was variably elevated in ascites and post-operative drainage fluid, but not in serum. Activated basophils can participate in the complex network of inflammation and angiogenesis by releasing various molecules such as histamine, platelet activation factor and vascular endothelial growth factor (VEGF), and by priming Th2

responses (34, 35). These findings suggest that basophils may be involved in the chronic inflammatory response in the OC microenvironment and also in the wound healing response after surgery. Basophil recruitment to the tumor microenvironment may aggravate fluid accumulation by the release of pro-inflammatory granular constituents that promote vascular leak.

Although the role of basophils in allergic disorders and anaphylaxis is established, the role of basophils in the tumor microenvironment is relatively unexplored. Basophils and mast cells express similar receptors and cytokines and infiltrate tissue in the presence of inflammation. Basophil-derived IL4 contributes to differentiation of monocytes into M2-macrophages in an allergic skin model (36). An increased percent of IL4-expressing basophils in tumor-draining lymph nodes correlated with increased tumor-infiltrating Th2 cells and worse disease-free survival after surgery in patients with pancreatic cancer (35). Mast cells can augment the activity of PMN-MDSCs through CD40L-CD40 interaction, resulting in impaired antitumor immunity and increased tumor growth in murine prostate cancer (37). These findings and our results point to the need for further investigation of the role of basophils in regulating immune responses in the tumor microenvironment.

Associations between circulating basophils with various hematologic and solid tumors and with clinical outcomes are variable. Pre-treatment circulating basophil counts were associated with recurrence in patients who received bacillus Calmette-Guerin after transurethral resection of the bladder tumor (38). Pre-treatment basophil counts did not correlate with survival in gastric cancer (39), whereas a higher basophil count was associated with improved outcome in patients with colorectal cancer (40) and in patients with melanoma who received checkpoint inhibitor therapy (41). With a clear distinction between basophils and e-MDSCs, the field will be better equipped to evaluate the roles of these cell populations in the context of newly diagnosed cancer and in response to therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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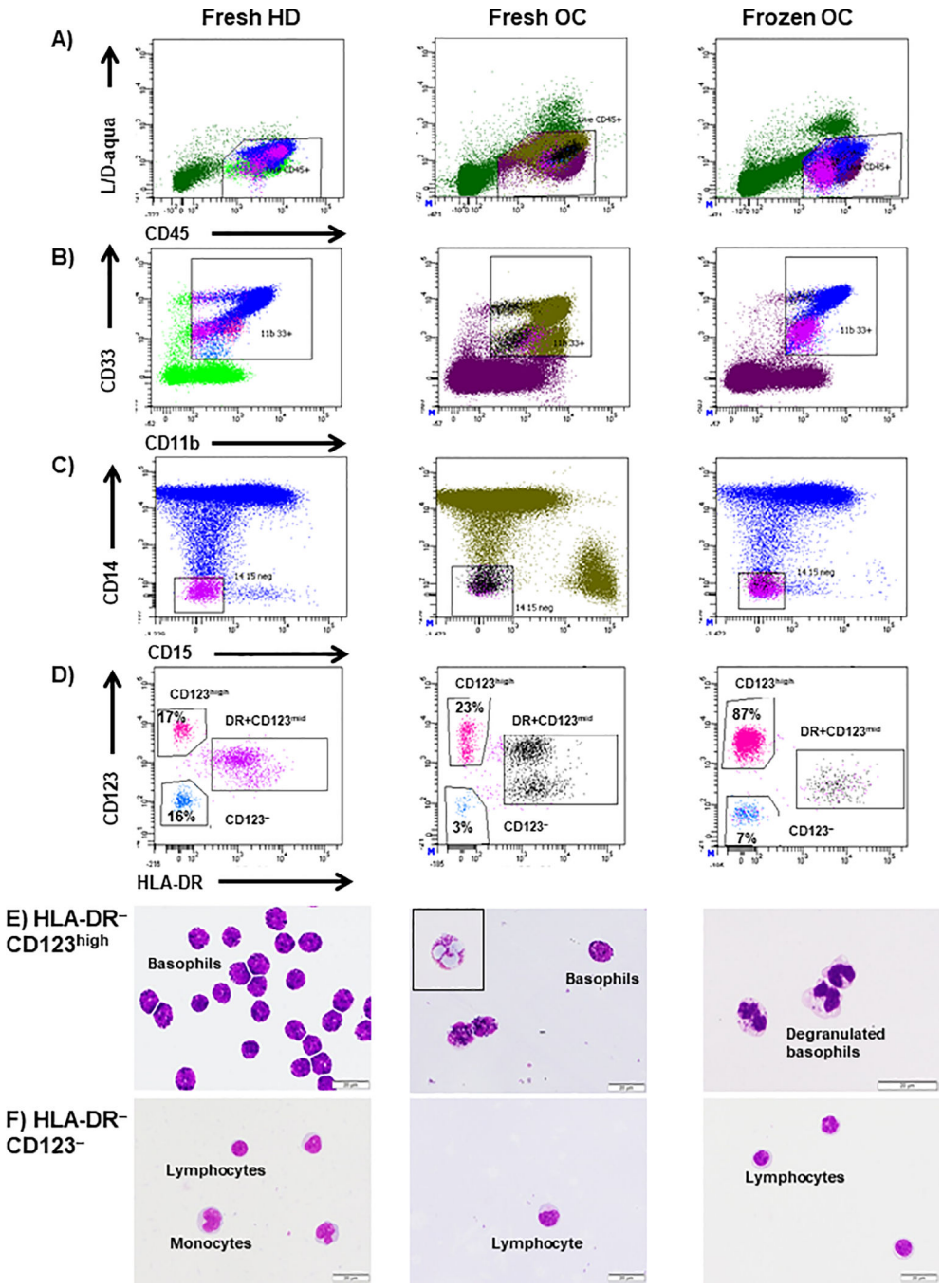

Figure 1: Putative e-MDSC fractions in PBMCs contain substantial proportions of basophils.

PBMCs from patients (n=3) with OC and healthy donor blood (HD, n=3) after $CD3^+$ cell depletion were analyzed for CD123-expressing cells in the putative e-MDSC fraction. Viable (L/D-aqua$^-$) $CD45^+$ cells (A) were gated on $CD11b^+CD33^+$ fractions (B), and those cells were gated on $CD15^-CD14^-$ fractions (**C**) and then on HLA-DR^-CD123^+ and HLA-DR^-CD123^- cells (**D**). $CD123^{high}$ (**E**) and $CD123^-$ (**F**) cells in $CD45^+CD33^+CD11b^+CD14^-CD15^-$HLA-$DR^-$ fraction of $CD3^-$ PBMCs were sort-purified and analyzed by cytology. **E and F (left)**: Based on surface markers and morphology, $CD123^{high}$ cells in fresh PBMCs

from HD are basophils, whereas the $CD123^{-}$ cells in e-MDSC fraction are comprised of lymphocytes and monocytes. **E and F (middle**): $CD123^{high}$ cells in the putative e-MDSC fraction of fresh PBMCs from a patient with OC are basophils. The box shows magenta-colored staining of the coarse secondary granules by the toluidine blue. The sparse $CD123^{-}$ cells are lymphocytes. **E and F (right)**: The $CD123^{high}$ cells in the putative e-MDSC fraction of frozen PBMCs from a patient with OC contain variably degranulated basophils. The $CD123^{-}$ fractions are mostly agranular mononuclear cells, including lymphocytes. The $CD45^{+}CD33^{+}CD11b^{+}CD14^{-}CD15^{-}$ cell fraction in PBMCs also contains HLA-DR $^{+}CD123^{mid}$ cells (D). The data presented are from 3 independent experiments.

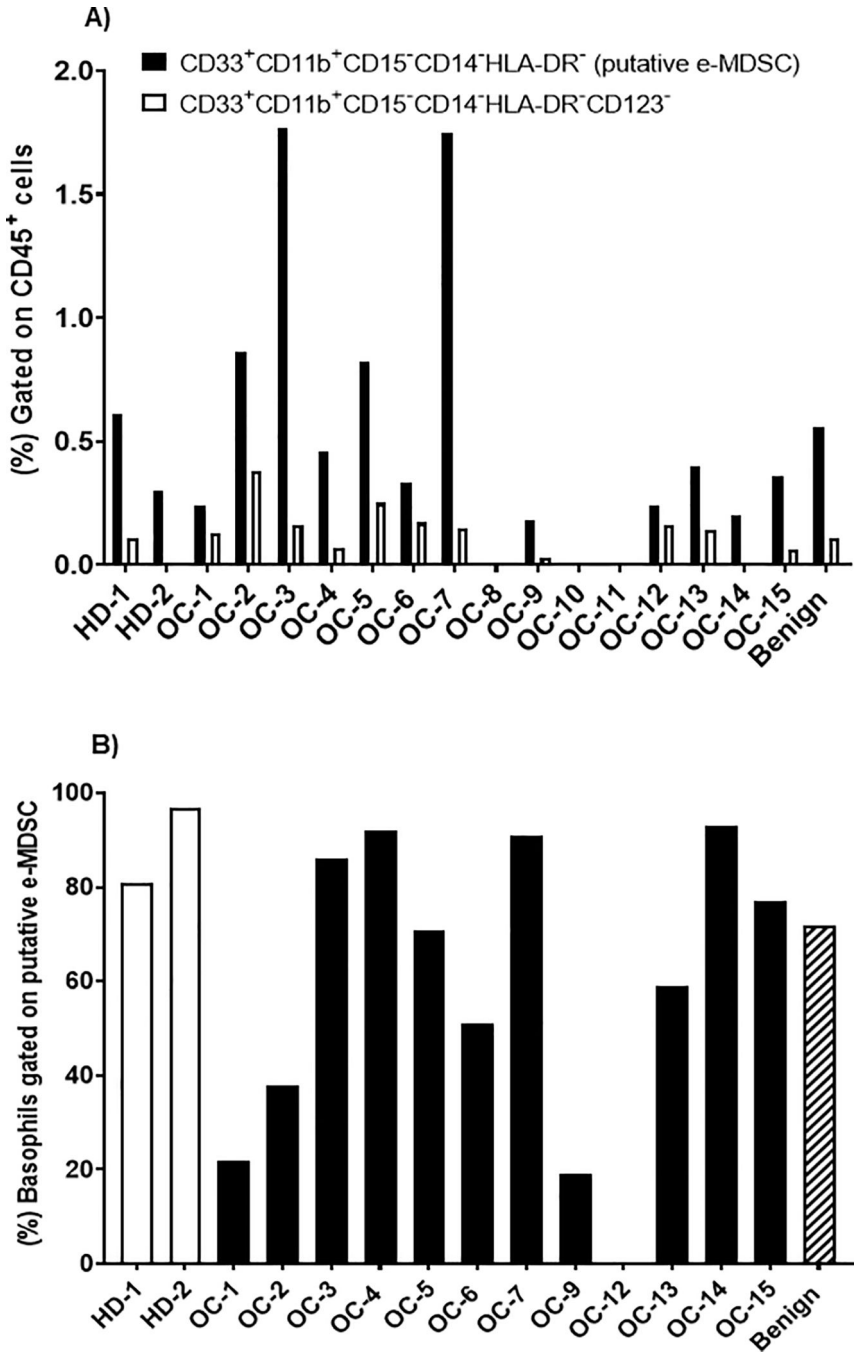

Figure 2: Basophils constitute a proportion of putative e-MDSCs as defined by surface markers in PBMCs from patients with OC.

Like Fig. 1, frozen PBMCs from patients with OC (n=15) were analyzed for basophils in the putative e-MDSC fraction. PBMC from two healthy donors (HD) and a patient with benign ovarian mass (Benign) were also included. **A**) $CD33^{+}CD11b^{+}CD14^{-}CD15^{-}HLA\text{-}DR^{-}$ (putative e-MDSCs) and $CD33^{+}CD11b^{+}CD14^{-}CD15^{-}HLA\text{-}DR^{-}CD123^{-}$ (basophil exclusion) cells are calculated from the $CD45^{+}$ cell region. Rare $HLA\text{-}DR^{-}CD123^{mid}$ cells were identified in putative e-MDSC population of some samples, and were excluded in this

analysis. The proportions of putative e-MDSCs are reduced when basophil exclusion is applied. **B**) Percent basophils ($CD45^{+}CD33^{+}CD11b^{+}CD14^{-}CD15^{-}HLA\text{-}DR^{-}CD123^{high}$) in the putative e-MDSC fraction of samples of patients with OC (N=12) and subjects without cancer. Data shown are restricted to samples in which putative e-MDSCs made up ≥ 0.1% of the total $CD45^{+}$ cells. The data presented are from 2 independent experiments.

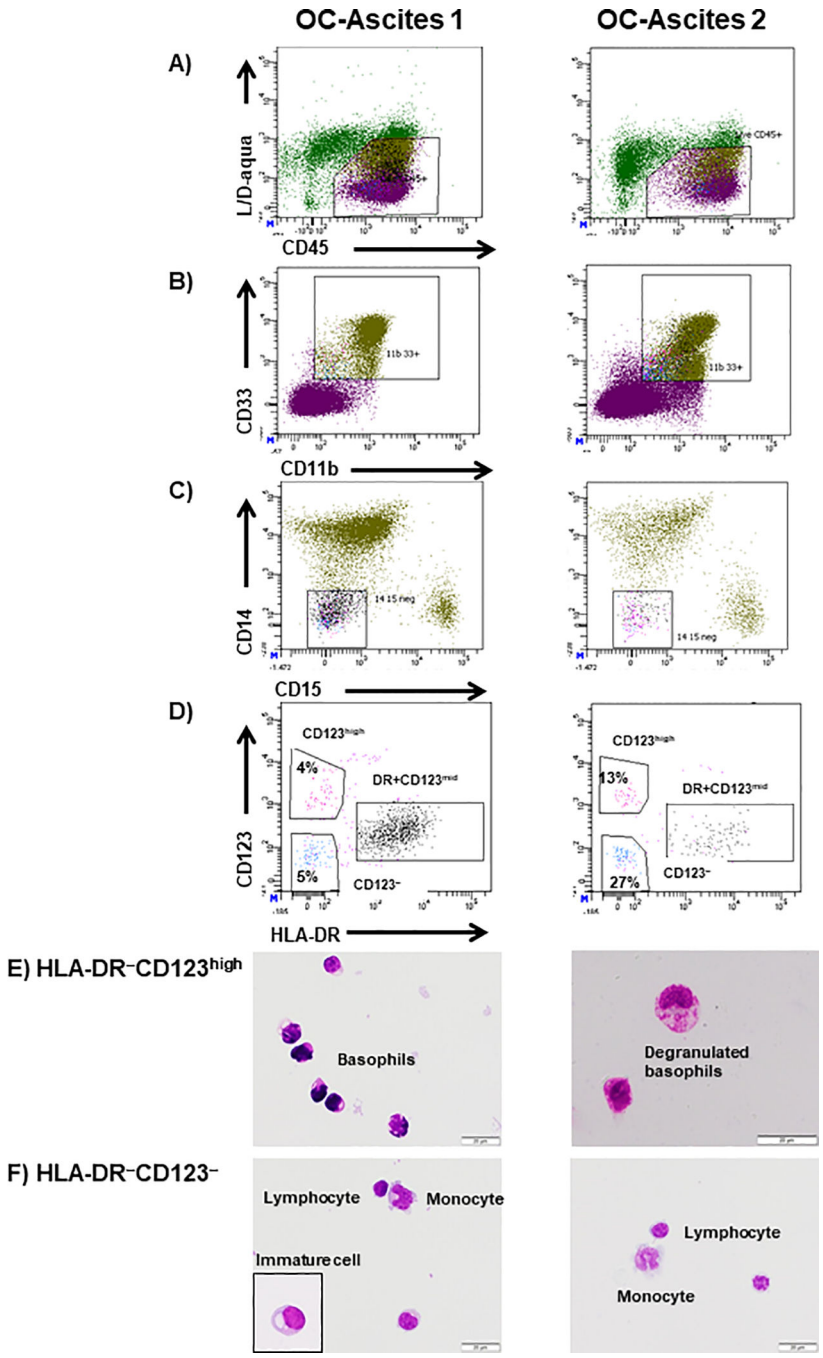

Figure 3: Putative e-MDSC fractions in OC ascites contain variable proportions of basophils.

Ascites cells collected from patients with OC (n=2) prior to surgery, after $CD3^+$ cells depletion, were analyzed for CD123-expressing cells in the putative e-MDSC fraction. **A – D**) Similar to Fig. 1, viable cells were gated to obtain $CD45^+CD33^+CD11b^+CD14^-CD15^-HLA\text{-}DR^-CD123^+$ and $CD45^+CD33^+CD11b^+CD14^-CD15^-HLA\text{-}DR^-CD123^-$ cells. $CD123^{high}$ (**E**) and $CD123^-$ (**F**) cells in $CD45^+CD33^+CD11b^+CD14^-CD15^-HLA\text{-}DR^-$ fraction were sort-purified and analyzed by cytology. **E and F (left)**: Based on surface markers and morphology, $CD123^{high}$ cells in e-MDSC fraction of frozen OC ascites cells

include variably degranulated basophils. Coarse basophilic granules are present in some of the basophils. Sparse agranular mononuclear cells, including lymphocytes, monocytes and cells with immature morphology comprise the $CD123^{-}$ fraction. The box (**F**) shows an agranular mononuclear cell with more round nuclei, higher nuclear-cytoplasmic ratio, and nucleoli. **E and F (right)**: Similar cytologic findings are seen from another frozen OC ascites cells. However, in this example, essentially all basophils have degranulated and only scant granules remain. The $CD45^{+}CD33^{+}CD11b^{+}CD14^{-}CD15^{-}$ cell fraction in ascites cells also contains a variable percentage of $HLA\text{-}DR^{+}CD123^{mid}$ cells (D). The data presented are from 2 independent experiments.

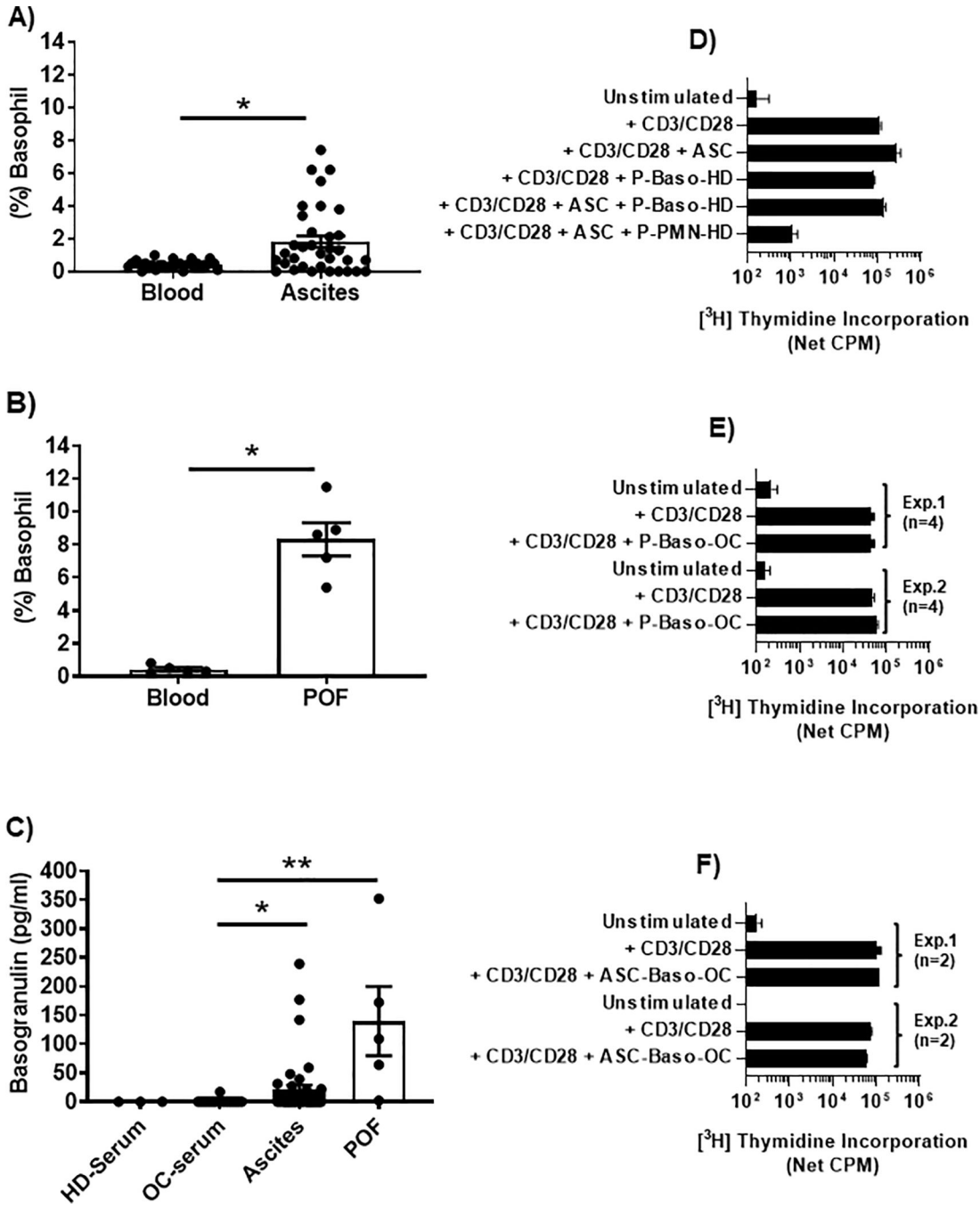

Figure 4: Circulating basophils from healthy donors and patients with OC and basophils from OC ascites do not suppress T cells.

A – B) Basophil counts were evaluated in paired blood and ascites samples from patients with newly diagnosed metastatic OC (n=34) prior to surgery and in blood, ascites and post-operative drainage fluid (POF) 1 day after surgery. Percent basophil counts are within normal range in blood and significantly increased in ascites (*p = 0.004) (**A**). Basophil counts (%) are significantly (*p = 0.0079) higher in POF than pre-operative blood (**B**). **C**) Basogranulin was evaluated by ELISA in serum from healthy donors (HD, n=3) and in

serum (n=17), ascites supernatants (n=40) and POF supernatants (n=5) from patients with OC. Basogranulin amounts were significantly higher in ascites (*p = 0.021) and POF (**p = <0.0001) samples compared to OC serum samples. Mean values from each sample with standard error are shown. **D**) Healthy donor basophils do not acquire a suppressive function when exposed to OC ascites supernatants (ASC). Peripheral basophils (P-Baso), neutrophils (P-PMN) and T cells ($CD3^+$) were isolated from HD blood. Anti-CD3/CD28-stimulated T cells were cocultured with autologous basophil or PMN and ASC. After 72h of coculture, T-cell proliferation was measured by [^{3}H] thymidine incorporation. Basophils in media or ASC did not suppress T-cell proliferation, whereas PMN in ASC suppressed T-cell proliferation by > 1 $\log_{10}$. Mean values with standard error (net CPM) from triplicate wells are presented. Stimulated T cells in media or ASC and unstimulated T cells in media were used as controls. Representative data from 2 separate experiments with ASC from 3 patients with OC are shown. **E**) Viable basophils from PBMCs of patients with OC (P-Baso-OC) were cocultured with stimulated T cells and T-cell proliferation was assessed. P-Baso-OC were pooled from 4 different patients per experiment, and 2 separate experiments (total of 8 patients) were performed. P-Baso-OC did not suppress stimulated T-cell proliferation. **F)** Basophils were sort-purified from ascites cells from a total of 4 patients with OC (ASC-Baso-OC). Pooling basophils from 2 ASC samples per experiment, two separate T-cell suppression assays were performed.

Table 1:

Data from patients with newly diagnosed ovarian cancer

Clinicopathological features	**N (%)**
Patients	325
Age, Mean (years)	61.3
Primary Site	
Ovary	253 (78%)
Fallopian Tube	14 (4%)
Primary Peritoneal	58 (18%)
Stage	
I/II	63 (19%)
IIIA/B	11 (3%)
IIIC	210 (65%)
IV	41 (13%)
Grade	
Well differentiated	30 (9%)
Moderately differentiated	37 (11%)
Poorly differentiated	214 (66%)
Undifferentiated	40 (12%)
Not available	4 (1%)
Histology	
Clear cell	15 (5%)
Endometrioid	16 (5%)
Mixed	28 (9%)
Mucinous	11 (3%)
Other	26 (8%)
Serous	229 (70%)
Debulking Status	
Optimal	247 (76%)
Suboptimal	61 (19%)
Not available	17 (5%)
Residual Tumor	
R0[a]	97 (30%)
Not R0	193 (59%)
Not available	35 (11%)
Platinum Status	
Resistant/Refractory	84 (26%)
Sensitive	160 (49%)
Not available	81 (25%)

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Clinicopathological features	N (%)
Survival	
Median PFS (95% CI)	20 months (18.2–23.9)
Median OS (95% CI)	48.8 months (42.8–61.1)

[a]No gross residual tumor after surgery

Table 2:

Basophil concentrations in pre-treatment peripheral blood from patients with ovarian cancer (N=325)

	Basophil concentrations (x1000/microliter)
Minimum - Maximum	0.01 – 0.17
Median	0.04
Mean	0.04743
Sd (+1)	0.0271181

Table 3:

Pre-treatment (baseline) peripheral basophil concentrations are not associated with PFS or OS in patients with OC (n=325) after debulking surgery and standard platinum-based adjuvant chemotherapy[b]

Period	Effect for +1 sd Basophil concentration on PFS	Effect for +1 sd Basophil concentration on OS
Baseline	0.93 (0.8–1.09), p=0.398	0.98 (0.82–1.17), p=0.805

[b] age, stage, histology and R0 adjusted Cox model hazard ratios are scaled for a 1 standard deviation change in concentration