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**Early-stage myeloid derived suppressor cell count: basophil exclusion matters**

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**Capsule summary:**

e-MDSCs, defined as Lin<sup>neg</sup> (CD3/CD14/CD15/CD19/CD56) HLA-DR<sup>neg</sup> CD33<sup>pos</sup>, have a phenotype that overlaps with basophils. For a proper quantification of e-MDSCs by flow cytometry, the gating strategy should specifically exclude basophils.

**Keywords:**

Myeloid-derived suppressor cells, basophils, myeloid cells, flow cytometry, phenotyping

**To the Editor:**

Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of myeloid cells characterized by their immune suppressive functions.<sup>1</sup> Circulating MDSCs are increased in inflammatory diseases, sepsis,<sup>2</sup> and numerous cancers, including lymphoid malignancies<sup>3</sup> and solid tumors. Their number increases with disease severity and predicts response to treatment and overall survival in various cancers.<sup>3,4</sup> In septic patients, polymorphonuclear (PMN)-MDSCs are specifically associated with the occurrence of nosocomial infections.<sup>2</sup> Consequently, these cells are becoming important biomarkers in several clinical situations and need as such to be accurately quantified. However, despite efforts in recent years to unify the nomenclature of MDSCs, it remains difficult to standardize their gating strategy by flow cytometry because of their lack of fully specific phenotypic markers.

In human peripheral blood mononuclear cells (PBMC), monocytic (M)-MDSCs are defined as CD14<sup>pos</sup> HLA-DR<sup>low</sup> CD15<sup>neg</sup> and PMN-MDSCs as CD11b<sup>pos</sup> CD14<sup>neg</sup> CD15<sup>pos</sup> (or CD66b<sup>pos</sup>) low-density granulocytes.<sup>1</sup> Recently, a third subset so-called early-stage subset of MDSCs (e-MDSC) comprising more immature progenitors has been identified as Lin<sup>neg</sup> (CD3/CD14/CD15/CD19/CD56) HLA-DR<sup>neg</sup> CD33<sup>pos</sup> CD11b<sup>pos</sup> cells lacking myeloid lineage markers of M-MDSCs and PMN-MDSCs.<sup>1,5</sup> The existence of e-MDSC has been

demonstrated in renal cell carcinoma, epithelial ovarian cancer, and head and neck cancer but their suppressive function remains unproven.<sup>4,6,7</sup>

Basophils represent 0.5-1% of peripheral leukocytes in normal conditions. They can be isolated within the PBMC fraction after a density gradient centrifugation, and are CD33<sup>pos</sup> CD11b<sup>pos</sup> HLA-DR<sup>neg</sup> CD3<sup>neg</sup> CD14<sup>neg</sup> CD19<sup>neg</sup> CD56<sup>neg</sup>, and for the most part CD15<sup>neg</sup> (i.e. Lin<sup>neg</sup>).<sup>8</sup> Thus, their phenotype overlaps with e-MDSCs, thereby leading to potential overestimation of respective cell type counts.

To address this issue, we first analyzed peripheral blood from healthy donors (HDs, n=8) by flow cytometry using CD203c and CRTH2 antibodies ordered from Miltenyi Biotec (Bergisch Gladbach, Germany), Lin-1 (CD3, CD14, CD16, CD19, CD20, CD56) from Becton Dickinson (BD Biosciences, San Jose, CA), and HLA-DR, CD33, and CD11b from Beckman Coulter (Brea, CA). We found that an average of 92.6% (range 78.5 to 98.2%) of Lin-1<sup>neg</sup> HLA-DR<sup>neg</sup> CD33<sup>pos</sup> CD11b<sup>pos</sup> cells expressed CD123 (interleukin 3 receptor  $\alpha$ -chain) and CRTH2 (CD294, receptor for prostaglandin D2), consistently with a basophil phenotype (Figure 1A).<sup>9</sup> Additionally, the expression of CD203c (ecto-nucleotide pyrophosphatase phosphodiesterase, specifically expressed by basophils and mast cells upon activation) was induced on CRTH2<sup>pos</sup> CD123<sup>pos</sup> cells from HDs (n=3) after 15 min of stimulation by an anti-IgE antibody (Allergenicity kit, Beckman Coulter) (Figure 1B). Moreover, these cells released histamine after activation by anti-IgE, as measured by ELISA (data not shown). To confirm these findings, we sorted Lin-1<sup>neg</sup> HLA-DR<sup>neg</sup> CD33<sup>pos</sup> CD11b<sup>pos</sup> cells from a HD sample (FacsAriaIII, BD). Microscopic examination of cytopsin slides stained with May-Grünwald-Giemsa and captured at 1000x magnification revealed a mixture of basophils (characterized by their large purple-black granules and double-lobed nucleus) and immature myeloid cells with agranular cytoplasm containing vacuoles (presumably e-MDSCs) (Figure 1C). Finally,



and in accordance with a previous paper,<sup>8</sup> we confirmed on 8 samples (n=6 HD and n=2 diffuse large B-cell lymphoma, DLBCL) that on average only 9.4% (range 4.8 to 14%) of basophils expressed CD15 (Figure 1D). Consequently, the Lin<sup>neg</sup> HLA-DR<sup>neg</sup> CD33<sup>pos</sup> phenotype encompasses a vast majority of basophils, which are not excluded by the CD15 marker.

Since circulating e-MDSC are increased in various types of cancers,<sup>4,6,7</sup> we further analyzed 20 DLBCL samples at diagnosis from a cohort already published by our group.<sup>3</sup> The research protocol was conducted under French legal guidelines and fulfilled the requirements of the local institutional ethics committee; all patients provided written informed consent (BMS-LyTRANS study; clinicaltrials.gov: NCT01287923). Clinical characteristics of DLBCL patients enrolled in this cohort are listed in Table 1. Within the Lin-1<sup>neg</sup> HLA-DR<sup>neg</sup> CD33<sup>pos</sup> CD11b<sup>pos</sup> population, we defined basophils and e-MDSCs as CD123<sup>pos</sup> and CD123<sup>neg</sup> fractions, respectively (Figure 1E). Basophils represented 68.3 % (range 4.1 to 97.6 %) of Lin-1<sup>neg</sup> HLA-DR<sup>neg</sup> CD33<sup>pos</sup> CD11b<sup>pos</sup> cells (Figure 1E).

Altogether, our results show that the Lin<sup>neg</sup> HLA-DR<sup>neg</sup> CD33<sup>pos</sup> CD11b<sup>pos</sup> phenotype includes a heterogeneous population of functionally active mature basophils and immature myeloid cells morphologically consistent with e-MDSCs. The presence of basophils, often in large proportions, might account for the absence of clear suppressive phenotype in previous functional assays targeting e-MDSCs.<sup>4,6,7</sup> Therefore, in addition to the recent recommendations,<sup>1</sup> we suggest to include basophil-specific exclusion markers such as CCR2/CD294 or CD123 in the reference panel, in order to refine the quantification of e-MDSCs and to better define the clinical relevance of these cells.

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Table 1 Patients characteristics of the lymphoma cohort

Characteristics		Patients (n=20)
Average age [range] (years)		54 [32-72]
Male (n)		12
Female (n)		8
IPI (n)	0-1	6
	2	3
	3	7
	4-5	4
Cell of origin (n)	GCB	5
	Non-GCB	9
	Unknown	6

IPI: International Prognostic Index; GCB: Germinal Center B-cells

**Figure legend****Figure 1: Lin<sup>neg</sup> HLA-DR<sup>neg</sup> CD33<sup>pos</sup> CD11b<sup>pos</sup> cells contain functional basophils**

A- On peripheral blood from healthy donors (HD), a subset of Lin<sup>neg</sup> HLA-DR<sup>neg</sup> CD33<sup>pos</sup> CD11b<sup>pos</sup> cells express basophil markers CD123 and CRTH2. B- Expression of CD203c on Lin<sup>neg</sup> HLA-DR<sup>neg</sup> CD33<sup>pos</sup> CD11b<sup>pos</sup> cells after anti-IgE stimulation. C- Lin<sup>neg</sup> HLA-DR<sup>neg</sup> CD33<sup>pos</sup> CD11b<sup>pos</sup> cells observed on cytospin. Basophils (▲) and mononuclear cell suggestive of e-MDSC (•). Scale bar represents 10 μm. D- Expression of CD15 on basophils (green) and neutrophils (blue). E- Lin<sup>neg</sup> HLA-DR<sup>neg</sup> CD33<sup>pos</sup> CD11b<sup>pos</sup> cells were marked as CD123<sup>neg</sup> e-MDSCs (red) or CD123<sup>pos</sup> basophils (green) (top panels). Significant plots from one patient are shown. Data were acquired on a Navios flow cytometer (Beckman Coulter) and analyzed with Kaluza software (Beckman Coulter).

