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The CLEC12A Receptor Marks Human Basophils; Potential Implications for Minimal Residual Disease Detection in Myeloid Malignancies

Marie Toft-Petersen, MD[1], Anne Stidsholt Roug, MD, PhD[1], Trine Plesner, MD, PhD[2,3], Lene Ebbesen, MD, PhD[1], Gordon D. Brown, PhD[4], and Line Nederby, MSc, PhD[1,5]

[1] Department of Hematology, Aarhus University Hospital, Aarhus, Denmark

[2] Department of Pathology, Aarhus University Hospital, Aarhus, Denmark

[3] Department of Pathology, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark

[4] Immunity, Infection and Inflammation Programme, Institute of Medical Sciences, University of Aberdeen, Aberdeen, UK

[5] Department of Clinical Immunology and Biochemistry, Lillebaelt Hospital, Vejle, Denmark

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Corresponding author:

Marie Toft-Petersen, MD

Department of Hematology, Aarhus University Hospital

Tage-Hansens Gade 2, entrance 4A, 2nd floor

DK-8000 Aarhus C, Denmark

Office: (+45) 78467395

Fax: (+45) 78467398

E-mail: marie.toft-petersen@clin.au.dk

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Abstract

Background: The transmembrane receptor C-type lectin domain family 12, member A (CLEC12A) is known to be highly expressed on monocytes and neutrophils and is a reliable leukemia associated marker in acute myeloid leukemia. Consequently, detailed knowledge of the various normal cell types expressing this receptor is essential. We have observed CLEC12A to be expressed on CD45lowSSClowCD14-CD123+ basophils in peripheral blood (PB) and in the present study, we aimed at verifying this observation and further delineate the CD45lowSSClowCD14-CD123+CLEC12A+ subpopulation.

Methods: We analyzed PB from 20 diagnostic chronic myeloid leukemia (CML) samples and 8 healthy donors in a 6 color multicolor flowcytometry (FCM) based assay. Furthermore, we performed fluorescence activated cell sorting on one CML sample to morphologically confirm the CD45lowSSClowCD14-CD123+CLEC12A+ subset to be highly enriched for basophils. Finally, to further delineate the CD45lowSSClowCD14-CD123+CLEC12A+ subpopulation in normal PB, we examined 3 healthy donors in a 10-color FCM assay enabling further separation of the cell subset into basophils and dendritic cells.

Results: The CLEC12A receptor is expressed on basophils.

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Conclusions: Identification and enumeration of basophils is of high relevance in diagnostic hematology and immunology. We here show that CLEC12A in a simple FCM assay consistently marks basophils. Importantly, since basophils are characterized by a CD45lowSSClow profile similar to the "blast-gate" employed for the evaluation of hematological disorders, awareness of minor normal CLEC12A+ subpopulations is crucial when using CLEC12A as a minimal residual disease marker in myeloid malignancies.

Introduction

The transmembrane receptor C-type lectin domain family 12, member A (CLEC12A) (also named hMICL, CD371 and CLL-1) was first described in 2004 to be abundantly expressed on monocytes and granulocytes, but absent on B- and T-lymphocytes as well as natural killer cells (1,2). In the field of hematology, we and others have described CLEC12A expression as a stable and reliable leukemia associated marker in acute myeloid leukemia (AML) (3,4). In addition, a 6-color multicolor flow cytometry (FCM) assay including CLEC12A proved highly valuable in AML minimal residual disease (MRD) detection (5). Using this assay in our routine diagnostics and follow-up of myeloid malignancies, we have observed CLEC12A to be useful in identifying basophils in PB. At present, immunophenotyping of basophils is mainly based on a characteristic CD45dim/SSClow profile

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– in some cases very similar to the leukemic “blast gate” - and a number of surface markers with the combination of CD123+HLA-DR- being widely used (6-8).

In the present study, we have investigated the expression of CLEC12A on basophils in detail using chronic myeloid leukemia (CML) samples, since a hallmark of CML is an elevated number of basophils in PB. Given the fact that dendritic cell (DC) subsets are known to express both CLEC12A and varying levels of CD123 (2,9-13), we also aimed to uncover the possible overlap of basophils and DC subsets in PB from normal donors.

Materials and methods

Patient samples and controls

Peripheral blood from 20 diagnostic stable phase CML samples was analyzed as part of the routine diagnostic procedure at Hemodiagnostic Laboratory, Department of Hematology, Aarhus University Hospital. The CML patients were diagnosed between May 2012 and June 2015. Manual differential counts of CML samples were performed with the use of a CellaVision DM9600 Digital Cell Morphology System (CellaVision AB, Lund, Sweden) counting 105 cells and subsequently confirmed by an experienced laboratory technician. Peripheral blood from 11 normal donors (NPB) was obtained from the Department of Clinical Biochemistry. Blood counts for normal donors were performed

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on a Sysmex XE-5000 (Sysmex, Kobe, Japan). Donor 1-8 presented with normal complete white blood counts (WBCs) and differentials, hemoglobin-levels and thrombocyte counts. Donor 9-11 presented with normal WBCs (differentials not performed), hemoglobin-levels and thrombocyte counts.

Flow cytometry

Fresh PB from CML patients and healthy donors was lysed using EasyLyse (DAKO, Glostrup, Denmark). As evident from Table 1, two tube designs were used; CML samples 1-20 and NPB samples 1-8 were analyzed in tube 1. Based on a standard nomenclature of DCs in humans (14), we defined three subsets of DCs in tube 2, namely plasmacytoid DCs (pDCs) (CD303+) (also named BDCA-2) and two subsets of myeloid DCs (mDCs) (CD1c+ (BDCA-1) and CD141+ (BDCA-3), respectively). The NPB samples 9-11 were analyzed in tube 2. The applied monoclonal antibodies, fluorochromes, clones and purveyors are provided in Table 1.

For tube 1, data acquisition was performed on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) with a 488 nm laser and a 635 nm laser. Compensation was performed using 7-Color Setup beads (BD Biosciences). The data was analyzed using FACSDiva Version 6.1.3 (BD Biosciences). For tube 2, data acquisition was performed on a Navios flow cytometer (Beckman-Coulter, Inc., Brea,

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CA, USA) equipped with a 488 nm laser, a 638 nm laser and a 405 nm laser. Compensation was set using UltraComp eBeads (eBioscience, San Diego, CA, USA) together with the relevant fluorochrome conjugated antibodies. The data from tube 2 was analyzed using FlowJo Data Analysis Software, version X (FlowJo, Ashland, OR, USA). Positive and negative gates were set with the use of internal controls.

The gating strategy used for the CML samples in tube 1 is depicted in Fig. 1A. Firstly, we defined the CD45lowSSClow gate and after selecting the CD14- cells within this gate, the CLEC12A+CD123+ cells were identified. The subset proved to be CD34- and CD117-. For NPB - in contrast to what was observed in the CML samples - the CD45lowSSClowCD14- subset showed two independent subpopulations, namely CD45lowSSClowCD14-CLEC12A++CD123+ and CD45lowSSClowCD14-CLEC12A+CD123++, when applying a similar gating strategy in tube 1. By back gating, the CD45lowSSClowCD14-CLEC12A++CD123+ cell subset extended into the CD45+ population. To study the CD45lowSSClowCD14-CLEC12A++CD123+ subpopulation further, the CD45lowSSClow gate was expanded to encompass a larger fraction of lymphocytes, henceforth named CD45+SSClow (Fig. 1B).

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For tube 2 the gating strategy is depicted in Figure 2. The CD45+SSClowCD14-CLEC12A++CD123+ and CD45+SSClowCD14-CLEC12A+CD123++ cell subsets were identified as for the NPB samples in tube 1 and were further analyzed for the expression of HLA-DR, CD1c, CD141 and CD303 to identify basophils, mDCs and pDCs, respectively.

Fluorescence activated cell sorting

Cryopreserved PB mononuclear cells from one selected CML case (CML patient 20) were thawed in 37 °C water bath and resuspended in RoboSep Buffer (StemCell Technologies, Vancouver, BC, Canada) with 15% heat inactivated fetal calf serum (FCS) (Biochrom, GmbH, Berlin, Germany) and stained with the monoclonal antibodies from tube 1 (Table 1). Fluorescence activated cell sorting was performed on a BD FACSAria™ III (BD Biosciences). Presumed basophils with the immunophenotype CD45lowSSClowCD14-CLEC12A+CD123+ were sorted according to the above-mentioned gating strategy. Cells were sorted into phosphate buffered saline containing 20% FCS. Sorted cells (aliquots of 10,000 and 20,000 cells, respectively) were cytospinned (500 rounds per minute for 1-3 minutes) onto poly-L-lysine coated slides (Thermo Fischer Scientific Inc., Waltham, MA, USA) and allowed to air-dry for subsequent Giemsa staining and morphological examination.

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Statistical analyses

All calculations were conducted in GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). Spearman's rank correlation coefficient was used to test if the percentage of basophils measured by FCM correlated with the percentage found by manual differential counts. A two-sided P-value less than 0.05 was considered significant.

Results

CLEC12A is expressed on basophils

In order to characterize the CD45lowSSClowCD14-CLEC12A+CD123+ cell subset observed in tube 1 and to verify the observation of these cells as basophils, we analyzed 20 stable phase CML samples by applying the gating strategy depicted in Figure 1A**.** Subsequently, we correlated these FCM based findings to the manual differential blood counts**.** For the CML samples, the manually counted absolute numbers of leukocytes and basophils together with the percentages of basophils determined by manual counting and FCM, respectively, are shown in Table 2**.** By FCM, the median percentage of basophils from CML patients was 2.3% of PB cells (range 0.5-10.1%). As evident in Figure 3, the percentage of basophils determined by FCM was highly correlated to the manual counts of basophils (r=0.69; p=0.0007).

To provide further evidence of the CD45lowSSClowCD14-CLEC12A+CD123+ subset to consist of basophils, we performed

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FACS of these cells in a representative CML case (CML patient 20, Table 2). The Giemsa stained cytospin preparations of the sorted subset morphologically confirmed the CD45lowSSClowCD14-CLEC12A+CD123+ cell subset to be highly enriched for basophils (Fig. 4).

In PB from healthy donors, the CLEC12A+CD123+ cell subset encompasses both basophils and dendritic cells

While the CD45lowSSClowCD14-CLEC12A+CD123+ cell subset was uniform when analyzing CML samples, it was divided into two clearly distinguishable subsets in NPB samples (Fig. 1B). The CD45+SSClowCD14-CLEC12A++CD123+ cells constituted a median of 0.7% of PB cells (range 0.5-1.3%) while the CD45+SSClowCD14-CLEC12A+CD123++ cells constituted a median 0.8% of PB cells (range 0.5-1.4%). For the NPB samples the percentage of neither the CD45+SSClowCD14-CLEC12A+CD123++ ($r=0.646$; $p=0.09$) nor the CD45+SSClowCD14-CLEC12A++CD123+ ($r=0.479$; $p=0.24$) correlated with the manual counts of basophils. This lack of correlation could be due to the very small fractions of cells, but since DCs also express CD123 we suspected these subsets to also contain mDCs and pDCs in addition to the basophils. To investigate this further, we examined PB from three additional normal donors in tube 2. As shown in Figure 2, the CD45+SSClowCD14-CLEC12A+CD123++ subset consisted of both HLA-DR- cells corresponding to basophils (median

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frequency of PB cells: 0.43%; range 0.35-0.67%) and HLA-DR+CD303+ pDCs (median frequency of PB cells: 0.21%; range 0.14-0.28%). While the basophils were clearly CLEC12A+ (median fluorescence intensity(MFI)/coefficient of variation (CV) for NPB samples 9-11: 7813/0.58, 6460/0.58 and 5735/0.62, respectively), the pDCs had a more varying expression of this antigen (MFI/CV for NPB 9-11: 4382/1.25, 3187/1.12 and 6296/1.25, respectively). The CD45+SSClowCD14-CLEC12A++CD123+ cell subset consisted of HLA-DR positive mDCs, primarily CD1c+ mDCs (median frequency of PB cells 0.14%; range 0.073-0.21%), but also a minute population of CD141+ mDCs (median frequency of PB cells: 0.0058%; range 0.0011-0.013%). The frequencies of the three DC populations were in accordance with the literature (15).

Discussion

In the literature, CLEC12A has been described to be abundantly expressed on monocytes and granulocytes, the latter distinguished by both CD45 and CD15 positivity together with high side scatter/autofluorescence properties (10). In contrast, basophils display a distinct CD45lowSSClow profile and are CD15 negative (8,16). Thus, to the best of our knowledge, no other studies have specifically addressed the CLEC12A expression on basophils.

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This knowledge is important in the hematological setting for several reasons. First of all, the basophilic CD45lowSSClow profile is often quite similar to the traditional CD45lowSSClow “blast-gate” employed in standard FCM based diagnostic and follow-up analyses in myeloid malignancies. In a study by Harrington et al, basophils were found to account for 25-35% of cells present within the CD45lowSSClow gate in bone marrow samples from patients with myeloproliferative disorders (17). Therefore, the amount of circulating blasts could be overestimated when using CD123 and/or CLEC12A as markers of leukemic blasts in cases, where basophils are present. Thus, even when applying a multicolor set-up, this remains important when leukemic blasts are negative for both of the immature markers CD34 and CD117 (18-20). Secondly, since the use of PB as a preferred and more accessible source of biological material in the follow-up of e.g. AML patients has proven applicable in the context of FCM based MRD-measurements (21,22), detailed knowledge of even minute fractions of non-malignant circulating cells is necessary. Lastly, the CD45lowSSClowCD14-CD123+CLEC12A+HLA-DR-immunophenotype could prove useful in the follow-up of other hematological malignancies where basophilia is present, since all of these markers are often used with advantage in the diagnostics and follow-up of myeloid disorders. Chronic myeloid leukemia is of course an obvious example, since an elevated percentage of basophils is a known prognostic factor (23). While acute basophilic leukemia is rare,

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other subtypes of acute myeloid leukemia (AML) with balanced translocations, e.g. t(6;9), inv(3), and AML associated with the *BCR-ABL1* fusion gene are also associated with elevated numbers of mature basophils in bone marrow and PB (24).

While the description of CLEC12A expression on basophils has clear implications for use in diagnostic hematology, it could also pave the way for further functional studies of this receptor in the context of immunology and allergology. In the recent years, basophils have been acknowledged as important players of the innate immune system, first and foremost in the protection against parasitic infections, but they also contribute to the development of allergic conditions (25). Although the complete function and role of CLEC12A in the immune system remains to be elucidated, recent studies indicate the receptor to have implications in a diverse spectrum of immunological mechanisms, including autoimmunity and infectious diseases (26-28).

In conclusion, we have shown CLEC12A to be unequivocally expressed on basophils and we have confirmed the expression of CLEC12A on CD1c+ and CD141+ mDCs and CD303+ pDCs. Since CLEC12A is a promising marker in the fields of both hematology and immunology, it is important to obtain detailed knowledge of the various normal cell types expressing this receptor. Importantly, since basophils and to some extent also DCs are immunophenotypically characterized

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by a distinct CD45lowSSClow profile similar to the traditional blast-gate, awareness of the different CLEC12A+ subpopulations is crucial when using CLEC12A and/or CD123 as an MRD marker, especially in CD34-/CD117- AML. Furthermore, this marker might also be of interest in basophil activation studies and in the characterization of basophils in non-hematological diseases in general.

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Author contributions

M.T.P., A.S.R. and L.N. designed the study, performed the experiments and analyzed the data. M.T.P. wrote the manuscript with input and critical reviews from L.N., and A.S.R.. T.L.P. performed morphological examination of the cytospin preparations. L.H.E. provided patients samples and monoclonal antibodies. G.D.B. provided the anti-CLEC12A hybridoma. All authors read and approved the final manuscript.

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Table 1. Flow Cytometry Panels

Tube 1				Tube 2			
MoAb	**Fluorochrome**	**Clone**	**Company**	**MoAb**	**Fluorochrome**	**Clone**	**Company**
α-CD123	FITC	AC145	Miltenyi	α-CD123	FITC	AC145	Miltenyi
α-CLEC12A	PE	HB3	Own lab	α-CLEC12A	PE	HB3	Own lab
α-CD14	APC-H7	MΦPP	BD	α-CD14	ECD	RMO52	BC
α-CD45	PerCP-Cy5.5	HI30	BioLegend	α-CD34	PerCP-Cy5.5	581	BioLegend
α-CD117	PE-Cy7	2B8	BD	α-CD141	PE-Cy7	M80	BioLegend
α-CD34	APC	Birma K3	DAKO	α-CD303	APC	201A	BioLegend
				α-CD19	APC-A700	J3-119	BC
				α-CD1c	APC-Cy7	L161	BioLegend
				α-HLA-DR	PB	L243	BioLegend
				α-CD45	Kr-O	J33	BC

FITC: fluorescein-isothiocyanate; PE: phycoerythrin, APC-H7: allophycocyanin-Hilite7; PerCP-Cy5.5: peridinin-chlorophyll-protein-cyanine5.5; PE-Cy7: phycoerythrin-cyanine7; APC: allophycocyanin; ECD: PE-Texas Red; APC-A700: allophycocyanin-Alexa700; APC-Cy7: allophycocyanin-cyanine7; PB: Pacific Blue; Kr-O: Krome-Orange. BD: BD Biosciences; BC: Beckman-Coulter.

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Table 2. CML patients. Counts of basophils.

Patient no.	Manual leukocyte count (x 10^9/L)	Manual basophil count (x 10^9/L)	Manual basophil count (%)	Flow cytometry basophil count [a] (%)
1	254.9	6.88	2.7	1.2
2	110.4	0.99	0.9	1.9
3	159.2	7.00	4.4	2.7
4	109.2	3.93	3.6	1.2
5	214.9	<0.02	0.0	1.8
6	139.5	2.65	1.9	1.0
7	253.7	2.03	0.8	1.5
8	171.5	1.54	0.9	2.1
9	96.8	5.42	5.6	6.1
10	265.8	11.70	4.4	3.9
11	122.6	3.19	2.6	0.5
12	304.5	<0.02	0.0	1.6
13	135.1	16.40	12.1	5.8
14	253.0	17.00	6.7	5.8
15	130.2	8.33	6.4	5.1
16	156.2	0.00	0.0	2.4
17	182.3	6.56	3.6	3.3
18	37.7	1.81	4.8	10.1
19	139.6	3.63	2.6	1.9
20	20.2	1.09	5.4	5.9

[a] Determined in tube 1.

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Figure Legends

Figure 1. Gating strategy in tube 1.

A. Gating strategy in a representative CML patient sample. In the first panel, the CD45low/SSClow population was defined. Next, CD14- cells were selected and the CLEC12A+CD123+ cells were identified. In the last panel, back gating of the CLEC12A+CD123+ subset displayed the characteristic CD45lowSSClow profile of basophils.

B. Gating strategy in PB from a healthy donor. In the first panel, a CD45+SSClow population was defined. Next, a selection of the CD14- cells was made. The CD14- cell population consisted of two independent subpopulations, namely CLEC12A++CD123+ and CLEC12A+CD123++, as depicted in the third panel. By back gating of these two cell subsets, the CLEC12A++CD123+ cells proved to be CD45+, as shown in the last panel.

Figure 2. Gating strategy in tube 2.

A. Representative plot of the CD45+SSClow/CD14- cells in a healthy donor, showing the CLEC12A++CD123+ and CLEC12A+CD123++ cell subsets.

B. The CLEC12A+CD123++ cell subset is clearly separated in the HLA-DR-CD303- basophils and the HLA-DR+CD303+ pDCs.

C. Back gating of the basophils (red) and the pDCs (blue) into the

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CD45+SSClow/CD14- cells showing the pDCs to have a more varying CLEC12A+ expression than basophils.

D and E. The CLEC12A++CD123+ cell subset consists of a minute fraction of HLA-DR+CD141+ mDCs (green) and a larger fraction of HLA-DR+CD1c+ mDCs (black).

G. Back gating of the two mDC subsets into the CD45+SSClow/CD14- cells, showing these to have similar high expression of CLEC12A.

Figure 3. Percentage of basophils in diagnostic CML samples determined by flow cytometry (in tube 1) versus manual blood differential counts.

Figure 4. Cytospin preparation of the CD123+CLEC12A+ subset in CML patient 20. The CD45lowSSClowCD14-CLEC12A+CD123+ cell subset is highly enriched for basophils (red arrows). Several cells are damaged, probably during the cytospin procedure, but the basophilic granules are visible (blue arrows).

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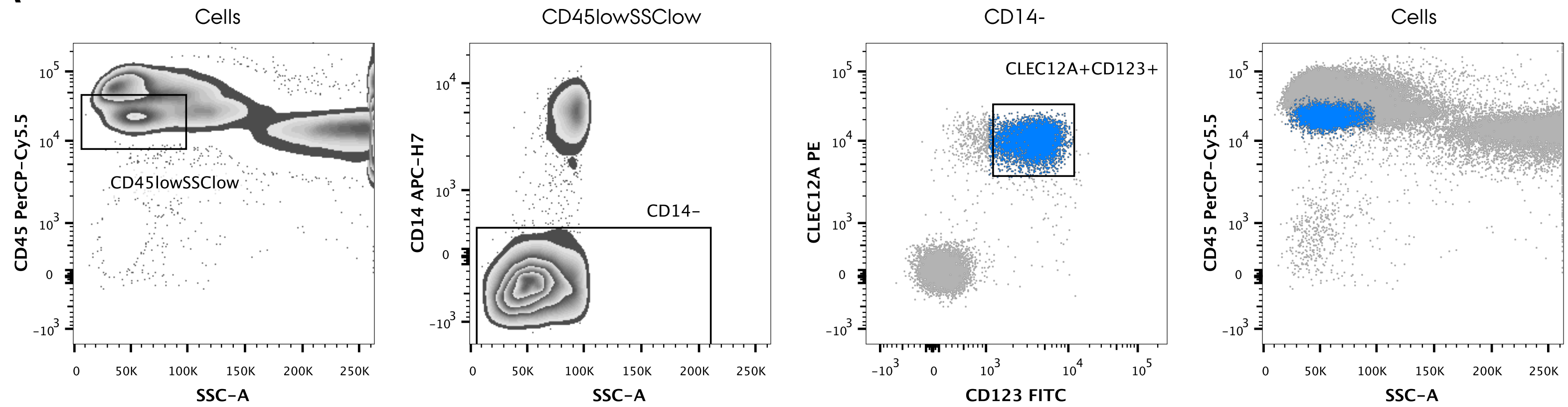

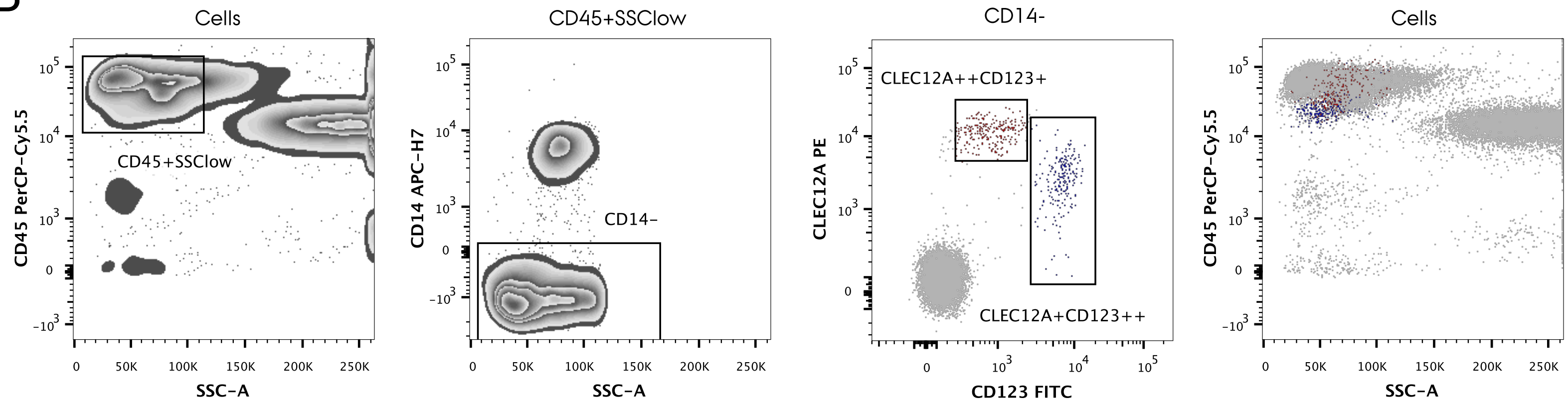

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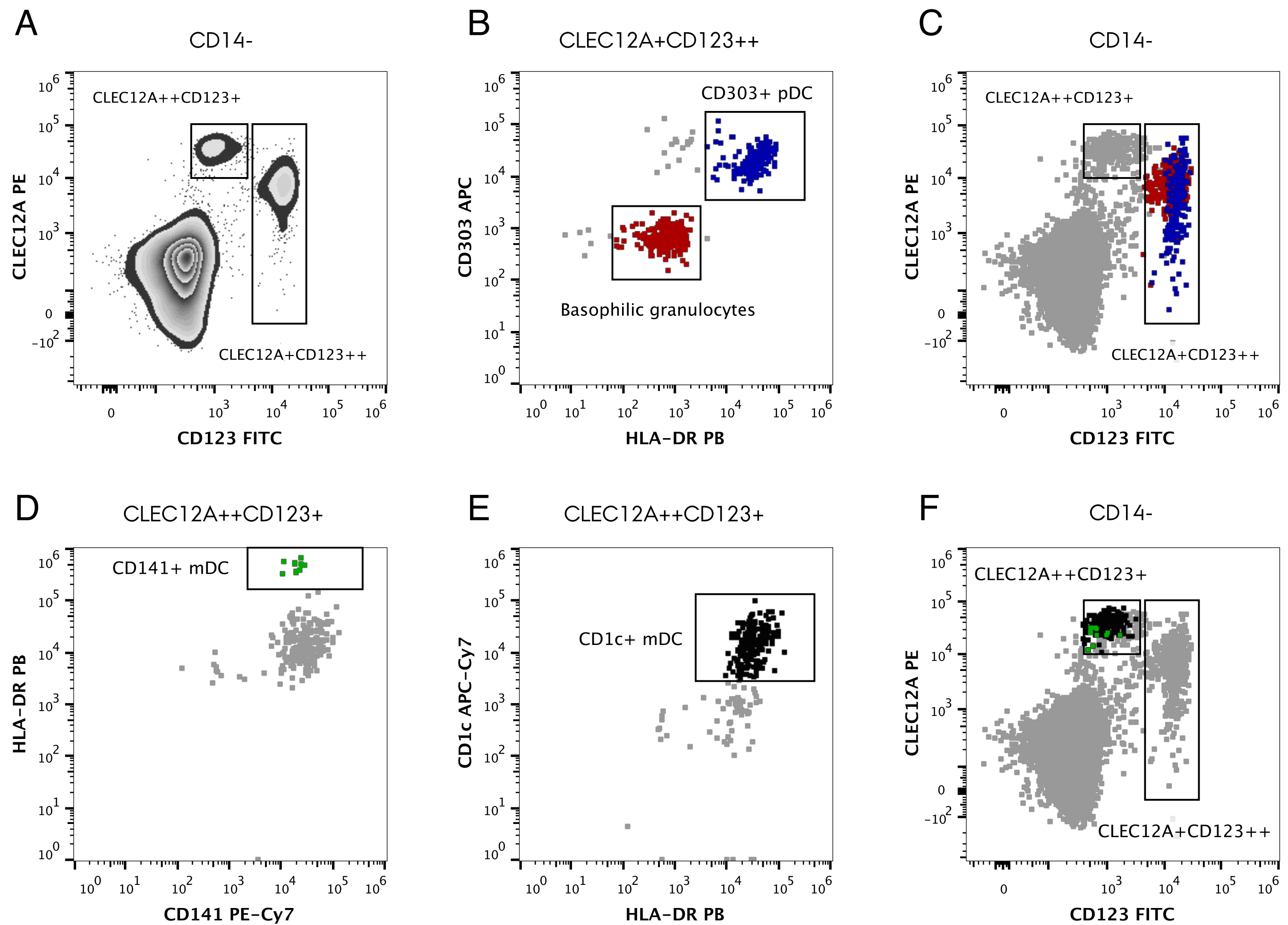

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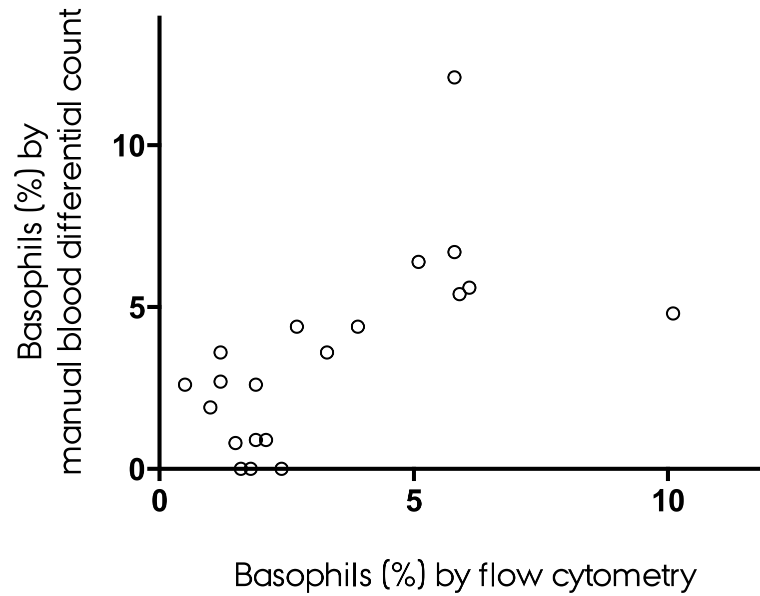

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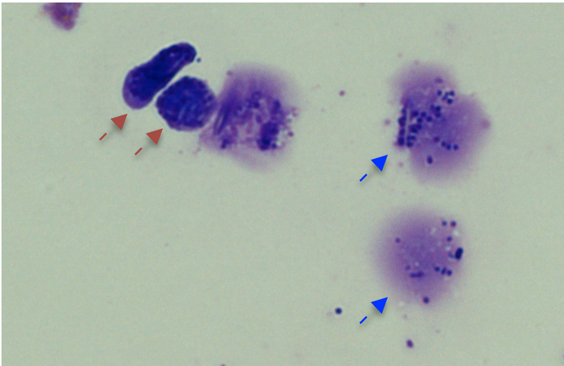

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