Characterization of the dendritic cell and monocyte-macrophage system through life: alterations in monoclonal gammopathies

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TESIS DOCTORAL

2023

CIC Centro de Investigación del Cáncer

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C E R T I F I C A:

Que el trabajo doctoral realizado bajo mi dirección por Doña. Daniela Pinto Damasceno titulado "CARACTERIZATION OF THE DENDRITIC CELL AND MONOCYTE-MACROPHAGE SYSTEM THROUGH LIFE: ALTERATIONS IN MONOCLONAL GAMMOPATHIES", reúne las condiciones de originalidad requeridas para optar al grado de Doctor en Medicina por la Universidad de Salamanca.

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Firmado por ALMEIDA PARRA JULIA MARIA - ***5355** el día 01/11/2023 con un certificado emitido por AC FNMT Usuarios

Fdo: Prof. Julia Almeida Parra

The present PhD thesis includes the following three original articles published in scientific journals indexed in the Science Citation Report:

1. **Expression profile of novel cell surface molecules on different subsets of human peripheral blood antigen-presenting cells.**
   Daniela Damasceno[1,2], Martín Pérez Andrés[1,2], Wouter BL van den Bossche[3], Juan Flores-Montero[1,2], Sandra de Bruin[4], Cristina Teodosio[4], Jacques JM van Dongen[4], Alberto Orfao[1,2] and Julia Almeida[1]
   Clinical & Translational Immunology (2016), 5, e100; doi:10.1038/cti.2016.54
   IF: 7.271 (Q1)

2. **Distribution of subsets of blood monocytic cells throughout life.**
   Daniela Damasceno, MSc[1], Cristina Teodosio, PhD[2], Wouter B.L. van den Bossche, MD[2,3], Martín Perez-Andres, PhD[1], Sonia Arriba-Méndez, MD, PhD[4], Luis Muñoz-Bellvis, MD, PhD[5], Alfonso Romero, MD, PhD[6], Juan F. Blanco, MD, PhD[7], Ana Remesal, MD, PhD[4], Noemi Puig, MD, PhD[8], Sergio Matarraz, PhD[1], José Luis Vicente-Villardón, PhD[9], Jacques J.M. van Dongen, MD, PhD[2*], Julia Almeida, MD, PhD[1*], Alberto Orfao, MD, PhD [1*] on behalf of the TiMaScan Study Group.
   Journal of Allergy and Clinical Immunology (2019), 144, number 1, Pages 320-323.e6; https://doi.org/10.1016/j.jaci.2019.02.030
   IF: 10.228 (Q1)

3. **Monocyte subsets and serum inflammatory and bone-associated markers in monoclonal gammopathy of undetermined significance and multiple myeloma.**
   Daniela Damasceno[1], Julia Almeida[1], Cristina Teodosio[2], Luzalba Sanoja-Flores[3], Andrea Mayado[1], Alba Pérez-Pons[1], Noemi Puig[4], Paula Arana[5], Bruno Paiva[6], Fernando Solano[7], Alfonso Romero[8], Sergio Matarraz[1], Wouter B.L. van den Bossche[2,9], Juan Flores-Montero[1], Brian Durie[10], Jacques J.M. van Dongen[2,†] and Alberto Orfao[1,†] on behalf of the TiMaScan Study Group
   Cancers (2021), 13(6): 1454, doi: 10.3390/cancers13061454
   IF: 6.126 (Q1)

Em memória da Avó Laura e da Avó Rita

Para o meu filho, José Guilherme

“The only limit to the height of your achievements is the reach of your dreams and your willingness to work hard for them”, Michelle Obama

Acknowledgements

Esta historia empieza en el Centro de Investigación del Cáncer, me habían concedido una beca en Portugal para irme a España, a Salamanca. Llegué acompañada de mi padre y apareció una chica de pelo negro que me dijo para pasar al despacho de Alberto. Empecé, muy nerviosa y formal (como somos los portugueses), hablando en portuñol. Tras unos minutos Alberto me contesta en portugués. Así empezó esta aventura que fue para mí mucho más que una tesis.

Gracias Profesor por tirar de mí, por hacerme resistente al trabajo. Gracias a eso, he crecido como estudiante y como persona. Quiero agradecerle también por haber apostado en mi formación, integrarme en un grupo de trabajo internacional, y confiar en mi para otros proyectos de los cuales tanto he aprendido. No solo como tutor, pero como humano, gracias por aconsejarme en lo que creía ser lo mejor para mi futuro.

No recuerdo fecha, pero recuerdo presentarme y tratarla de usted: "No me trates de usted que no soy una señora mayor!", me dijo. De ella he aprendido a luchar y a resistir, hasta que llegue la recompensa. Gracias Julia, no tengo palabras para agradecer toda tu implicación en este trabajo, hiciste de mí una alumna independiente, capaz de afrontar y solucionar problemas con una visión crítica. Gracias por contar siempre conmigo, eres para mí una "madre" científica.

To Prof. Jacques, I still remember when we met and he told me "this project can be a tunnel with no way out". I answered very confident that "there is always a light in the end of the tunnel". And the day has come, I've learned a lot from the TiMaScan meetings, they made me a better and well-prepared student.

Gracias al Dr. Luis Muñoz, al Profesor Juan Blanco, al Dr. Fernando Solano por vuestra disponibilidad para lograr las muestras y los datos clínicos de los pacientes. A Noemí Puig por ser siempre tan amable y predispuesta a ayudarme con toda la parte clínica del trabajo.

I would also like to thank Wouter for sharing this PhD experience from Rotterdam to Salamanca and for the amazing *stroopwafels*. They made the TiMaScan meetings much happier. Many thanks to Jane, Sandra, Rita, and Kyra, for the nice time in the meetings and the chocolate beads, and also to all the team involved in the TiMaScan Project.

Gracias a todos los pacientes por colaborar en la investigación y hacer posible esta tesis.

A mi familia de corazón Sara, Maribel y Manuel. A Sara por compartir conmigo los mejores momentos y también los peores, por tener siempre una solución. Son incontables e inmejorables los momentos que hemos pasado juntas, las aventuras que me recargaban las pilas para un año más, y otro, y otro. Y a Maribel y Manuel, que me abrieron las puertas de su casa, con quien compartí varias nocheviejas en la mejor compañía. Siempre dispuestos a ayudarme y aconsejarme. Gracias por todo, familia.

"I'll be there for you" (*Friends)*. A mi Luli, compañera de tantas guerras, por todo lo que me has enseñado, que la vida hay que llevarla con calma, que no por ir más deprisa llegamos antes. Gracias por estar siempre ahí y no dejarme desistir nunca, por alegrarte tanto o más por mis logros. Aunque ya tengas una tesis, esta es tuya también.

"¡Cambiaremos nuestros nombres!" (*Thelma&Louise). A*sí empieza la historia de una grande amistad que podría muy bien ser la nuestra. A mi amiga Andrea, la chica del pelo negro que antes os hablaba, llevare conmigo todas las conversaciones y momentos únicos. Gracias por la calma, por hacerme crecer como persona y por tu fiel amistad durante todo este tiempo. Esta tesis es tuya también amiga y de nuestros pequeños revolucionarios.

A Alvarito y Guille que sin darse cuenta han contribuido también para esta tesis doctoral. La energía y el amor que transmiten me hicieron sentir en casa y alegraron mis días. Os llevo en mi corazón pequeños. Gracias Su por confiar en mí y por ser una amiga tan dedicada y preocupada por mi bienestar, por insistir conmigo y darme fuerzas.

“Bueno pues ya te enseñé todo y más, te dejo sola en la campana, ahora vuelvo”. Cris es de las personas más inteligentes y trabajadoras que conozco y puedo decir que mucho de lo que he aprendido en estos años te lo debo a ti y a esas noches interminables de trabajo. Más allá de eso, gracias por tu amistad y por tu paciencia, por estar siempre disponible a escucharme y tranquilizarme.

“Mi sueño es la medicina, de toda la vida” “Pues hazlo, estas a tiempo”. Así empezó, con un “hazlo”. Gracias Sergi por dedicarme tu tiempo, tu amistad, por estar ahí en menos de un minuto siempre y por hacerme creer y luchar por mis sueños. No hay límites.

Compañero de café y de sitio de laboratorio, ha vivido mil vidas en la misma y sus historias podrían ser parte de una película. Gracias “tito” Guille por aguantarme (y yo a ti) y por cuidar a nuestro cactus que de tanta energía positiva que ha absorbido de su entorno, hoy es casi un árbol centenario.

A Jara, desde la historia de las rosquillas del convento de Zamora a las comidas diarias de risas que nos montábamos. Y si me preguntan “¿Conoces Zamora?” pues les tendré que contar la maravillosa historia de las rosquillas.

Muchas de las expresiones españolas las aprendí de María GG, muchas que ni existen porque se las inventa al momento, poco formales, pero muy útiles.

Pocos portugueses podrán decir que conocen Fresno de Alhándiga. Yo he estado en las fiestas de Fresno y he comido una paella muy rica. Gracias Sheila por ser siempre tan positiva y transmitirme esa energía todos los días.

A Luzalba, Elena, Nacho y Paula por empezar este viaje conmigo y arreglarlo todo con una cerveza, un té o una *bulk lysis*. A Laura por estar conmigo en momentos muy duros y por tirar de mí en esos momentos.

A Martin por enseñarme los principios básicos de la citometría y por el trabajo dedicado a esta tesis.

A Quentin, por su infinita paciencia para descodificar lo que necesitaba y por el tiempo dedicado a mi tesis para lograr todo lo que le pedía, siempre con humor, en portugués o en francés, daba igual.

Al arma de Andrea, Alba, por estar siempre predispuesta a ayudarme y por el empujón en la última etapa experimental cuando aún no veía la luz. Ánimo pequeña.

À Carolina, por poder falar contigo em português sobre música electrónica e por não entenderes por que é que os espanhóis acham que as portuguesas têm bigode.

A Nacho por ver todo con humor y hacerme reír incluso en días de muchas *bulk Lysis*. A Javi, mi aprendiz, por hacerme una mejor maestra. A Alejandro por haber heredado con dignidad mi sitio y ser un buen compañero.

A Carlos por contar conmigo para la docencia y por su amabilidad siempre.

A la nueva generación de citometristas: Julio, Noemi, Oihane, Alba, Blanca, Guille, Javi, Alicia, Pablo, que siguen caminando hacia el final. Deciros que muchas veces es difícil poner todo en perspectiva, pero cuando llegue el momento, veréis que al final merece la pena todo el esfuerzo.

Obrigada aos meus amigos brasileiros: à Elaine pela paciência nos meus primeiros passos no mundo da citometria; ao Vitor por me acalmar em momentos de crise e pela companhia e empatia que fomos construindo em todos os projetos que nos eram incumbidos; ao Carlos por conseguir decifrar o que lhe pedia; à Roberia pela amabilidade e por me ter recebido de braços abertos no Rio de Janeiro;

Gracias a Rosana, a las Miriam y a Virginia por hacer hueco en el citómetro bajo mis pedidos especiales.

A Juana por la creatividad, las historias y por la experiencia que ha compartido conmigo.

A todos mis compañeros del CIC y del IDI por amenizar los días de trabajo: Toño, Sara, Arancha, Basia, Andrés, Manuel, Rafa, Fernando.

A Mamen por tu serenidad a la hora de escucharme y por apoyarme. Gracias a todos los chicos y chicas del banco de tumores y del banco de ADN por participar en las cenas de navidad y en las locuras que se nos ocurrían.

A mi querida amiga Marga por todo el apoyo y preocupación a lo largo de toda esta aventura y también de las demás aventuras académicas que ha acompañado. Siempre dispuesta a ayudar.

A Marian por la energía, simpatía y felicidad que me transmite.

A Ricardo, Claros, Alfredo, Ángeles, y a todos los chicos y chicas de Immunostep por compartir los momentos de las comidas.

A todas las personas de Cytognos que se cruzaron conmigo y confiaron en mi para proyectos paralelos a la tesis doctoral.

À Raquel por estar lá nos últimos anos e pela cumplicidade, por entender as minhas rotinas intermináveis.

A mi querida amiga Isabel, gracias por los desahogos y por entenderme tan bien. Gracias por aconsejarme en el momento crucial que guardare siempre en mi corazón.

A Eva y a Carla, y a mi recién llegada tocaya Daniela por los mensajes de coraje y por estar ahí siempre.

A todo el equipo de baloncesto "Añover" por los momentos de ocio haciendo lo que más nos gusta: jugar y salir de fiesta. Gracias por hacerme sentir en casa.

"Longe da vista, mas nunca longe do meu coração"

À Teresa pela tua fiel amizade. Obrigada Maria Teresa, Maria Leonor e Manuel Maria por me deixarem entrar no vosso Mundo e por me fazerem sentir criança outra vez. Ao Zé por partilhar comigo momentos da vossa família tão bonita. Obrigada ao Manuel e à Tia Teresa por me acolherem como filha desde sempre.

À Majo, à Li, e à Seixas, por me apoiarem sempre e me recordarem a importância de ter os amigos sempre por perto e a todos os meus amigos que não mencionei mas contribuíram também para finalizar esta etapa.

À Tia Zeca e ao Herculano, aos meus padrinhos e a toda a minha família pela união e pelo apoio incondicional nas minhas aventuras.

Obrigada Pai pelos valores que sempre me transmitiste de constância, trabalho, perseverança, e por permitires que crescesse e continue a crescer com os meus erros. Obrigada pela exigência e por nunca desistires do meu sonho. Obrigada Telminha pela sua infinita paciência e calma, por ter sempre uma palavra amiga que me conforta. À avó Nanda por me receber sempre com um abraço cheio de amor e carinho.

Obrigada Mãe pelo coração enorme que tens e por acalmares as minhas lágrimas em tempos tão difíceis que tivemos que superar, juntas. Obrigada Paulo por amenizares os fins de semana para recarregar energias com os teus cozinhados de chef Michelin.

À minha irmã, a minha confidente. Obrigada por me ouvires a qualquer hora do dia, por me guiares sempre e por me fazeres sorrir, mesmo entre lágrimas. Obrigada Zé por seres um grande amigo e por me receberes sempre de braços abertos. Aos meus bebés Maria Laura e Eduardo, pela energia contagiante e o amor que me dão todos os dias. Ao meu irmão Francisco pela compreensão de estar longe de casa e pelas (poucas) conversas que me enchiam o coração só de o ver, e me tranquilizavam por saber que estava bem. Ao meu irmão mais novo, Zé Pedro, que acompanhou esta aventura desde pequenino. Obrigada pelas visitas, por vires festejar o meu aniversário e viveres esses dias como se fossem teus.

Aos amores da minha vida João e José Guilherme.

À minha querida avó Rita, obrigada por me teres feito mais forte, e ao mesmo tempo melhor pessoa, por deixares em mim tantos valores e tantas saudades que me fazem continuar apesar de todas as adversidades da vida. Esta tese é para ti, sei que estas comigo sempre no meu coração e em tudo o que faço.

Table of contents

---------- List of abbreviations ----------

BATF: basic leucine zipper ATF-like transcription factor

BTLA: B- and T-lymphocyte attenuator

BDCA: blood dendritic cell antigen

BM: bone marrow

BMSC: bone marrow stromal cell

CCL: C-C motif chemokine ligand

CCR: C-C motif chemokine receptor

CS: Carnegie stages

CB: cord blood

CD: cluster of differentiation

cDC: conventional dendritic cell

CDP: common dendritic cell progenitor

CLR/CLEC: c-type lectin-like receptor

cMo: classical monocyte

cMoP: common monocyte progenitor

CMP: common myeloid progenitor

CXCL: C-X-C motif chemokine ligand

CXCR: C-X-C motif chemokine receptor

CX3CR: C-X3-C motif chemokine receptor

DAMP: damage-associated molecular pattern

DC: dendritic cell

EMP: common erythroyd-myeloid progenitor

FcεRI: high-affinity IgE receptor type I

GEP: gene expression profile

GM-CSF: granulocyte-macrophage colony stimulating factor

GMDP: granulocyte-macrophage-dendritic cell progenitor

HIV: human immunodeficiency virus

HLA: human leucocyte antigen

HSC: hematopoietic stem cell

IFN: interferon

IFN-α: interferon alpha

IFN-γ: interferon gamma

IFN-λ: interferon lambda

Ig: immunoglobulin

IGH: immunoglobulin heavy chain

IGL: immunoglobulin light chain

IL: interleukin

iMo: intermediate monocyte

IMWG: International Myeloma Working Group

IRF: interferon regulatory factor

LC: Langerhans cell

LPS: lipopolysaccharide

LN: lymph node

MRC: mannose receptor c-type 1

MRI: magnetic resonance imaging

mDC: myeloid dendritic cell

MDCP: macrophage-dendritic cell progenitor

MDM: monocyte-derived macrophages

MGUS: monoclonal gammopathy of undetermined significance

MHC: major histocompatibility complex

Mo: monocyte

MPO: myeloperoxidase

MPS: mononuclear phagocytic system

MM: multiple myeloma

Mϕ: macrophage

ncMo: non-classical monocyte

NK: natural killer

NF-kB: nuclear factor-κB

OC: osteoclast

PAMP: pathogen-associated molecular pattern

pDC: plasmacytoid dendritic cell

PB: peripheral blood

PBMC: peripheral blood mononuclear cell

PC: plasma cell

PDL: programmed death ligand

PRR: pattern recognition receptor

PSGL-1: P-selectin glycoprotein ligand 1

RES: reticulo-endothelial system

RNA: ribonucleic acid

ROS: reactive oxygen species

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

SIGLEC: sialic acid-binding immunoglobulin-type lectin

Ss: single stranded

Slan: 6-sulfo LacNAc

SMM: smoldering multiple myeloma

TAMP: tumor-associated molecular pattern

Tc: cytotoxic T cell

TGFβ: transforming growth factor beta

Th: T-helper

TIM: T-cell immunoglobulin mucin receptor

TiMa: tissue macrophage

TNFα: tumor necrosis factor alpha

TNFRS21: tumor necrosis factor receptor superfamily member 21

TLR: toll-like receptor

Treg: T-regulatory cell

TRM: tissue resident macrophage

TSNE: t-distributed stochastic neighbor embedding

VEGFA: vascular-endothelial growth factor alpha

YSMPs: yolk sac-derived myeloid-biased progenitor

WBC: white blood cells

WHO: World Health Organization

Introduction

1. Monocytes, macrophages and dendritic cells

In 1892, Elie Metchnikoff and Paul Elrich identified phagocytes in marine invertebrates, a group of cells that included different cell types like the macrophages (Mϕ) and microcytes, later recognized as polymorphonuclear cells (1, 2). Already at that time, phagocytes were reported to play a role in the defense against foreign/external particles and microorganisms through a process analogous to inflammation in higher organisms (3). The relevance of these early findings was recognized in 1908, with a shared Nobel prize awarded to Elie Metchnikoff and Paul Ehrlich due to their pioneering work and findings about cellular and humoral immunology. In 1924, Ludwig Aschoff recognized the different phagocyte subtypes to be cells of a broader system, the reticuloendothelial system (RES) (4), known since 1969 as the mononuclear phagocytic system (MPS). The MPS consists of monocytes (Mo) and tissue-resident macrophages (TRM), which can be distinguished from polymorphonuclear leucocytes, because of their highly- specialized phagocytic capacity (2).

In turn, dendritic cells (DCs) were first identified in the mouse spleen in 1973, by Ralph M. Steinman and Zanvil A. Cohn, when both authors were investigating the induction of immune responses that require lymphocytes and "accessory cells" thought to be Mϕ, in a major lymphoid organ (5). These authors found that such accessory cells, had unusual shapes and migration patterns which had not been described before for Mϕ (5). Based on their unique morphologic appearance they named these cells as “dendritic cells” for the first time (5).

At present it is well-established that DCs, Mo and Mϕ are all key players in pathogen sensing, phagocytosis, and antigen presentation to T-cells. In addition, it is also well-known that they consist of multiple specialized cell populations which can be identified in blood and

in multiple human tissues, based on their unique phenotypic profiles and/or diverse effector and immunomodulatory functions, giving rise altogether to a complex network of cells capable of integrating (multiple) environmental signals leading to either immunity or tolerance (6). Accordingly, DCs are currently considered to act as immunomodulatory cells, involved in capturing and processing intracellular and extracellular particles (e.g., proteins) for adequate antigen presentation and subsequent priming of antigen-specific T-cells (7, 8). They play a pivotal role in connecting innate and adaptive immunity in multiple immune cell responses, ranging from inflammation to tolerance (8).

Despite tissue DC include a broad range of different cell populations, in blood there are two classical major subsets of human DC with clearly different functional and phenotypic properties: plasmacytoid DC (pDC) and conventional DC (cDC), also known as myeloid DC (mDC) (9). Similarly, three major subsets of Mo have been identified in human blood based on the expression of the cluster of differentiation (CD)14 lipopolysaccharide (LPS) receptor and the CD16 low-affinity immunoglobulin (Ig)G Fc receptor, defined as $CD14^{+}CD16^{-}$ classical monocytes (cMo), $CD14^{+}CD16^{+}$ intermediate monocytes (iMo) and $CD14^{-/lo}CD16^{+}$ non-classical monocytes (ncMo), with different phenotypic and functional properties (10-12). All above cell populations are immune effector cells, equipped with chemokine and pathogen recognition receptors (PRR), which allow them to recognize foreign particles and to migrate from blood to tissues, where they differentiate into tissue-mature DC or Mϕ, under different conditions (13-17). Once in a tissue, these cells are involved in the production of inflammatory cytokines and phagocytosis of foreign or toxic molecules which are presented on human leucocyte antigen (HLA) class II molecules on their surface membrane (16). DCs will subsequently migrate via blood to secondary lymphoid tissues where they present the processed antigens to T-cells (18, 19).

1.1. Origin and differentiation of dendritic cells and monocytes

The maturation pathway of both DC and Mo still remains to be fully understood. This is due to the current lack of knowledge regarding the specific precursor cells for the different populations of Mo and DC that have been identified so far. In contrast to other hematopoietic cell populations, tissue DC and Mϕ do not exclusively differentiate from bone marrow (BM) hematopoietic precursors and stem cells (HSC), some of the more mature populations of these cells (e.g., TRM, Langerhans cells -LC-) originating directly from the yolk sac during embryogenesis (20-22).

1.1.1. Maturation pathways of dendritic cells and monocytes in the bone marrow

Peripheral blood (PB) Mo and DCs originate from a $CD34^+$ HSC in the BM (23). However, at present controversial results exist about which are the exact precursors of Mo and DCs and the relationship that exists between them which has contributed to open the discussion about the rigid model of myeloid *vs* lymphoid segregation during the early stages of human hematopoiesis (24-29). Despite all the above, recent studies suggest that both circulating DCs and Mo originate from a BM HSC, which gives rise to a multipotent $CD34^+$ $CD45RA^-$ $CD90^-$ $CD49f^-$ progenitor (MPP); this MPP differentiates into a common $CD34^+$ $CD45RA^+$ $CD123^{lo}$ $CD38^+$ $CD10^-$ $CD135^+$ c-type lectin-like receptor –$CLEC\text{-}12A^{hi}$ (CD371) $CD64^{int}$ granulocyte/ Mϕ progenitor (GMP) and a common $CD34^+$ $CD45RA^+$ $CD123^{int}$ $CD115^+$ Mϕ/DC progenitor (MDP). This later MDP cell would give rise to a common $CD34^+$ $CD45RA^+$ $CD123^{hi}$ $CD38^+$ $CD10^-$ $CD135^+$ $CD117^+$ $CD11c^-$ DC progenitor (CDP) (and later to DCs), osteoclasts (OC) and Mϕ (28, 29). Likewise, Xiao *et al* (28) also described a human common Mo/Mϕ-OC-DC progenitor (MODP), with a $CD34^+$ $CD45RA^+$ $CD123^{hi}$ $CD38^+$ $CD10^-$ $CD135^+$ $CD117^+$ $CD115^-$ $HLADR^+$ $CD11c^-$ immunophenotypic profile among the common GMP cell fraction. The MODP would overlap with the previously described CDP and would give rise to DCs, Mo/Mϕ and OC (23) (Figure 1).

More recently, a minor population (0.02% of all blood leucocytes) of circulating cDC progenitors (pre-cDC) has also been identified in blood which displays a unique $CD100^{+}$ $CD34^{int}$ $CD45RA^{+}$ $HLADR^{+}$ $CD117^{+}$ $CD115^{-}$ $CD141^{-}$ immunophenotypic profile (24, 30, 31), whose precursor and precise ontogeny and relationship with the other different compartments of the more mature DC still remains to be elucidated.

Altogether, CDP, also referred to by Xiao *et al* (28) as MODP, are present in BM and cord blood (CB), but not in PB or secondary lymphoid tissues (23, 32). CDP progressively lose CD34 and give rise to Mϕ and OC, pDCs, and potentially also pre-cDCs. In turn, pre-cDCs might differentiate to $CD141^{+}$ cDC1 (24) and $CD1c^{+}$ cDC2 cells, (24, 30), supporting a model in which circulating pre-cDCs are capable of reconstituting the pool of organ resident cDCs (21, 24, 33, 34). In parallel, human GMP would also commit toward the monocytic lineage in BM, where they give rise to early $CD34^{+}$ $CD64^{+}$ monocytic precursors (monoblasts or clonogenic Mo progenitor; cMoP) with restricted Mo differentiation potential (Figure1) (29). These Mo progenitors will sequentially mature into promonocytes (with greater expression levels of CD11b and CD14) and finally, to mature $CD300e^{+}$ Mo (35) (Figure 1).

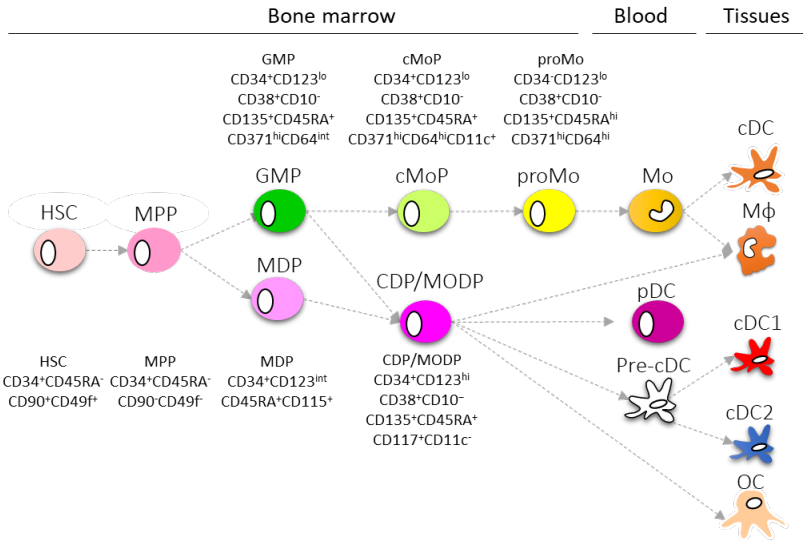

Figure 1. Schematic model of the ontogeny of dendritic cells (DC) and monocyte-macrophage (Mo-Mϕ) cells including osteoclasts (OC). HSC: hematopoietic stem cell; MPP: multi-potent progenitors; MDP: Mϕ-DC progenitor; GMP: granulocyte-Mϕ progenitor; CDP: common DC progenitor; MODP: Mϕ-OC-DC progenitor; cMoP: clonogenic monocyte progenitor; proMo: promonocytes; pDC: plasmacytoid DC; cDC1: conventional DC1; cDC2: conventional DC2; (modified from Xiao et al. 2017 and Kawamura et. al 2018)

1.1.2. Origin of tissue resident macrophages and Langerhans cells

Mϕ are known to be present in virtually all human tissues where they play multiple relevant roles and functions. These include a modulatory effect on the hematopoietic microenvironment and regulation of cell metabolism, in addition to tissue cleaning and repair (36). From the ontogenic point of view, at present it is well-established that human hematopoiesis starts during the early embryonic development in the yolk sac, outside the embryo, from where it subsequently extends to the liver before stabilizing, in the fetal period, around the seventh month of gestation, in the BM (37). Despite Tavian and Péault (38) showed that fetal HSC emerge in human embryos already at week +5 of gestation, they failed to report the subsequent maturation stages of human TRM (38). Later on, in 2015, Hoeffel *et al* (39) discovered that murine Mϕ can be raised from a c-Myb erythro-myeloid progenitor (EMP)-derived fetal Mo, recently identified also in (aborted) human embryos at 24–56 days of development by Bian *et al* (37), despite these later cells showed much less pronounced transcriptomics features related to the erythroid lineage than EMPs. In the reported experiments, these later authors isolated $CD34^+$ $CD44^+$ yolk sac-derived myeloid progenitors (YSMPs) from the pool of the first hematopoietic $CD45^+$ cells that emerge in the yolk sac (37). These cells showed two distinct fates based on gene expression profile (GEP) analysis of *ex vivo* YSMPs from the embryonic liver which point to the monocytic (*CD192* and *CD14*) and neutrophil (*S100A9* and *CD66b*) lineages (37). Furthermore, they reported a dual HSC-independent origin: i) a Mo-independent primitive (first) wave of $CD163^+$ and $MRC1^+$ (mannose receptor c-type 1) Mϕ in the early yolk sac, which contributed to the major fraction of TRM; and ii) a second fetal liver YSMPs-derived Mo wave (37) (Figure 2). Of note, the contribution of these HSC-independent waves of TRM should be considered for the characterization of the different subsets of Mϕ found in normal homeostatic and disease

conditions, based on their distinct transcriptomics and epigenetics identity *vs* their HSC-derived counterpart.

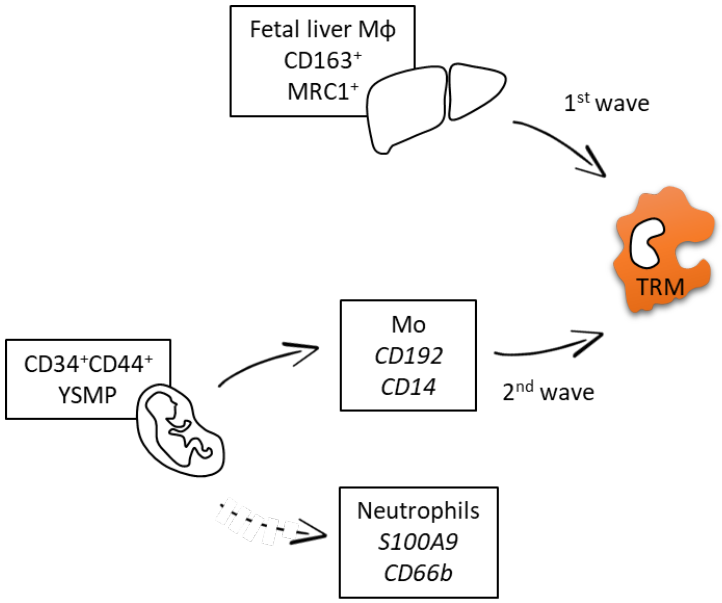

Figure 2. Two distinct waves of yolk sac and fetal liver-derived macrophages (Mφ) contribute to the establishment of tissue-resident Mφ (TRM) in human embryonic and fetal tissues. A first wave and a second wave derived from fetal liver Mφ contributes to the major fraction of TRM from yolk sac myeloid progenitors (YSMP) derived Mo. YSMP can also give rise to neutrophils. Mo: monocytes; TRM: tissue resident Mφ, modified from Bian et al. 2020 (39).

1.2. Human populations of blood circulating dendritic cells and monocytes

DCs are present and can be detected in human blood where they represent a minor population of all leucocytes (<1%). Typically, two main subsets of DC with clearly different functional and phenotypic properties have been described in human blood: pDC and cDC/mDC. Firstly, pDCs that comprise around 30% of the DC compartment (40) and are a uniquely distinct population of cells that have a high capacity to produce and secrete type I interferon (IFN-I), particularly in the context of viral responses. In turn, cDC consist of a heterogeneous population of cells that can be further subdivided based on the expression of CD141 -blood DC antigen (BDCA)3- and CD1c (BDCA1) into: i) $CD141^{+}CD1c^{-}$ cDC1 (6% of the total DCs), typically involved in antigen cross-presentation to $CD8^{+}$ T cells; and, ii) $CD141^{-}CD1c^{+}$ cDC2 (60% of the total DCs) involved in responses to e.g., bacterial challenges (8, 12, 40-42).

In PB, Mo are also a major immune cell population that acts as a key player during inflammation and pathogen challenge, via their ability to phagocytose, produce cytokines, and present antigens to T-cells (43). Overall, Mo represent around 4-5% of all white blood cells (WBC) in human PB and they are involved in the innate response to bacterial, fungal, parasitic, and viral infections (44, 45). The three major circulating subsets of Mo identified so far are distributed as follows: i) $CD14^{++}CD16^{-}$ cMo represent around 76%±6% of all blood Mo; ii) $CD14^{+}CD16^{+}$ iMo, 4%±2%; and iii) $CD14^{lo/-}CD16^{+}$ ncMo included around 9%±6% of blood Mo (10-12, 46).

Under physiological conditions, the balance between the production, proliferation, differentiation, tissue migration, survival, and death of DCs and Mo is critical to ensure a normal homeostasis. Despite this, and to the best of our knowledge, no study had reported at the moment of starting this doctoral thesis, on the kinetics of these subsets of human blood DC, and to a less extent also, some minor subsets of blood Mo. At the same time, the precise biological and functional roles and relationships, among all DC and Mo subsets remain only partially understood. In this regard, recent studies (47, 48) in which the kinetics of major populations of blood circulating Mo have been investigated in order to better understand the maturational relationship between the distinct subsets of Mo, have shown that cMo are retained approximately for two days in BM before they circulate for only ≈24h in blood, prior to their active migration to peripheral tissues (47, 48). In contrast, ncMo circulate for a longer period of approximately seven days, during which they act as sensors for monitoring endothelium damage (47, 48). While blood circulating cMo consist of recently produced BM-derived Mo that have been released to blood, the origin of ncMo remains controversial. Thus, while some authors claim that ncMo directly arise from cMo in blood (48, 49), others suggest that they correspond to cMo that had migrated from blood to inflamed tissues where they

have differentiated into tissue Mϕ (47, 50) capable of recirculating back to blood in order to maintain normal tissue homeostasis (50, 51).

Remarkably, several studies have reported on the counts of monocytic populations throughout life. Thus, higher counts of cMo have been found in human (neonatal) CB compared to healthy adults (52), while the numbers of blood circulating iMo and ncMo appear to be higher in older *vs* younger healthy donors (HD) (53), particularly in the elderly (53-55). However, contradictory data exits in this regard, since some authors also reported greater total $CD14^+$ $CD16^+$ Mo counts in elderly healthy controls *vs* young adults (52, 55).

1.2.1. Phenotypic characteristics of blood dendritic cells and monocytes

The phenotype of DCs and Mo has been extensively investigated in blood by both flow cytometry and RNA-sequencing studies, contributing to a better identification and understanding of the heterogeneity of the multiple distinct populations of blood circulating DC and Mo subsets (46, 56-58). Overall, human DC and Mo subsets characteristically express relatively high levels of HLA class II molecules (e.g., HLADR) at the same time they lack on markers specific of other hematopoietic lineages such as CD3 (a T cell marker), CD56-natural killer (NK) cell-associated marker-, CD19 and CD20 (B cell markers) (59, 60).

Although DCs, in general, are all capable of processing and presenting antigens to T cells and initiate/modulate the immune response, human blood pDCs can be distinguished from cDCs and other cells, based on their unique pattern of expression of cell surface membrane markers such as CD303 (BDCA-2), CD304 (BDCA-4), CD85k and CD85g together with the (more recently identified marker) CD358 -tumor necrosis factor receptor superfamily member 21 (TNFRSF21)-, the B- and T-lymphocyte attenuator (BTLA), and CD300A (8) markers, which are involved in the regulation of their major physiological function: the production of

IFN-I. The early GMDP-associated markers CD123 -interleukin (IL)3-Rα- and CD45RA are also characteristically expressed by pDCs, and they undergo down-regulation when DC progenitors differentiate to cDCs (8, 41, 61, 62) (Table 1). In addition, pDCs display an array of membrane receptors involved in proinflammatory and tolerogenic processes, which include the toll-like receptor (TLR)7 and TLR9 for the recognition of single stranded RNA (ssRNA) and ssDNA, respectively, and for promoting signal transduction after sensing viral and self-nucleic acids (8). Indeed, the lack of tolerance of pDC to self-nucleic acids has been frequently associated with autoimmune diseases such as systemic lupus erythematosus and psoriasis (8). Unlike cDCs, pDCs do not express the CD11c and CD11b adhesion molecules, neither the immunoregulatory receptor CD33, or the Axl, CD5, CD327 and CD13 antigens (Table 1). However, pDC shared with the cDC2 subset, expression of the C-C motif chemokine receptor (CCR)2 (i.e., CD192), the co-stimulatory molecule CD86, and the CLEC4A protein (8, 62).

Among blood circulating human cDCs, the cDC2 cells are the mostly represented subset. cDC2s and have been classically defined by the expression of the CD1c glycoprotein in the absence of CD141 (40). In addition, cDC2 cells generally expresses a broad variety of immune receptors for antigen uptake, transport, and presentation, including the TLR1 to TLR10 receptors, together with CD205, CD369 (CLEC7A), and CD370 (CLEC9A). Moreover, cDC2s express CD172α and CD32b in association with the CD11b, CD11c, CD13, and CD33 antigens. Unlike cDC1, cDC2 also express the CX3C-motif chemokine receptor (CX3CR)1, the high-affinity IgE receptor type I (FcεRI), and the scavenger receptors CD36 and CD163. The pattern of expression of CD5 and CD14 in cDC2 cells is heterogeneous and is currently used to the define new (potential) subsets of cDC2 cells (8, 27, 34, 57, 62).

The other major subset of human cDCs (i.e., cDC1 cells), were originally described based on their high levels of expression of CD141 in the absence of CD1c (41, 42). However,

this marker alone is not a robust marker for cDC1 cells in different environments outside blood, because Mo and cDC2 cells can also express CD141 both after tissue migration and following *in vitro* culture and stimulation (61, 63). Like cDC2 cells, cDC1 cells express CADM1, XCR1, CD13, CD33, CD86, CD192, CD205, CD272 and CD327, together with low CD11b and CD11c levels, but in the absence of both CD14 and CD172α (34, 60, 62, 64, 65) (Table 1). From the functional point of view human cDC2 cells are considered to play a critical role in antigen cross-presentation to T cells, with an enhanced ability to recognize apoptotic and necrotic cells (including cell debris) via the CD370 receptor (66).

As described above for DCs, extensive efforts have also been made aimed at improving our knowledge about the immunophenotypic profiles of the different subsets of blood Mo. Thus, apart from the classification of blood Mo into cMo, iMo and ncMo based on their pattern of expression of CD14 and CD16, detailed immunophenotypic studies have highlighted the great phenotypic heterogeneity of each of these three individual cell subsets, pointing out in parallel the possibility also of their potentially distinct functional roles (10, 11).

In common, blood Mo express chemokine receptors and PRR involved in tissue migration, and show an ability to produce inflammatory cytokines (44). Moreover, the three conventional subsets of blood Mo (in baseline, non-activated conditions) also share lack of reactivity for immature markers like CD34 and CD117, activation markers such as CD142 and CD169, cathepsin D, CD94, CD206, and CD273 (56). In contrast, all three subsets of blood Mo express lysosomal proteins (CD68 and NOX2), CD13, CD18, CD33, CD44, CD45, CD55, HLADR, CD300e (IREM2), CD172α, the CD11b and CD11c integrins, CD86 and TLR2 (56) (Table 1). However, some of the above markers are expressed at different levels in the three populations of conventional blood circulating Mo.

Accordingly, cMo are characterized by surface expression of several CLEC molecules (CLEC4A or CD367, CLEC4D or CD368, CLEC5A, CLEC7A or CD369, CLEC12A or CD371, and CLEC13A) and show a unique pattern of expression of chemokine receptors that include positivity for CD181 -C-X-C motif chemokine receptor 1 (CXCR1)-, CD182 (CXCR2), and CD192 (CCR2). In addition, cMo also express the IL13Rα1 and show low levels of expression of the CD86 co-stimulatory molecule (16, 46, 56, 67) (Table 1). These cells also express CD36, a potent scavenger receptor involved in the uptake of oxidated low-density lipoproteins (46). In general, the phenotypic profile of cMo is consistent with a high phagocytic capacity and a pro-inflammatory profile in line with the typical functional (*in vivo* and *in vitro*) behavior of these cells (45).

In turn, iMo show expression of CD74, the angiopoietin receptor Tie-2 (CD202B), the transforming growth factor beta (TGFβ) signaling molecule, endoglin (CD105) and several phagocytose-related molecules (FPR1, FPRL1/2), together with the T cell inhibitory molecule CD366 (16, 46, 56, 67) and the CD192 (CCR2), CD195 (CCR5), and CX3CR1 chemokine receptors (46). Of note, among blood Mo, iMo show the highest expression levels of HLADR, which support their major functional role as pro-angiogenic cells with high capacity for antigen processing and presentation to T cells (11) (Table 1).

Finally, ncMo have been found to show a characteristic CD192 (CCR2)-negative, $CX3CR1^{hi}$ phenotype, in association with an absent and augmented response to the respective chemokines (68). Other molecules that are typically expressed by ncMo include Siglec-10, CD43, and CD45RA (27, 56, 69) (Table 1). In addition, the heterogeneous levels and patterns of expression of CD61, CD9, CD36 and 6-sulfo LacNAc (Slan), an unsialylated O-linked carbohydrate modification of P-selectin glycoprotein ligand 1 (PSGL-1 or CD162) (70), critical for proper functioning of PSGL-1 (71), have been also reported in several studies. Altogether,

these findings highlight the heterogeneous phenotype and nature of this population of blood circulating ncMo (46, 57, 68, 69).

Table 1. Immunophenotypic profiles of the different populations of blood dendritic cells (DC) (8, 27, 34, 62) and monocytes (Mo) (16, 46, 56, 67-69).

Functional group of proteins	Surface antigen	CD code	DC subsets			Mo subsets		
			pDC	cDC1	cDC2	cMo	iMo	ncMo
CYTOKINE, CHEMOKINE AND GROWTH FACTOR RECEPTORS	XCR1	NA	-	+	+	ND	ND	ND
	CXCR1	CD181	-	-	-	+	-	-
	CXCR2	CD182	-	-	-	+	-	-
	CCR2	CD192	+	+	+	+	+	-
	CCR5	CD195	-	-	ND	-	+	-
	CX3CR1	NA	-	-	+	-	+	+
	IL-3Rα	CD123	++	-	-	-	-	ND
	IL13Rα1	CD213α1	ND	ND	ND	+	-	-
ADHESION MOLECULES	TSPAN-29	CD9	ND	ND	ND	-/+	+	-/+
	Integrin-alpha-M	CD11b	-	dim	+	+	+	lo
	Integrin alpha-X	CD11c	-	dim	+	+	+	+
	CADM1	NA	-	+	+	ND	ND	ND
	LEU1	CD5	-	-	-/+	-	-	-
	Vitronectin receptor	CD61	ND	ND	ND	-/+	dim	-/+
IMMUNOGLOBULIN RECEPTORS	FCGR2B	CD32b	-	-	+	ND	ND	ND
	FcγRIII	CD16	-	-	-	-	+	+
	FcεRI	NA	+	-	+	-/+	-	-
ACTIVATION-RELATED MARKERS	CD14 molecule	CD14	-	-	-/lo	+	+	lo/-
	Sialophorin	CD43	ND	ND	ND	ND	ND	+
	B7-2	CD86	+	+	+	lo	+	++
	TNFRSF21	CD358	+	-	-	ND	ND	ND
	BDCA-4	CD304	++	-	-	ND	ND	ND
	BTLA	CD272	+	+	+	ND	ND	ND
	TIM-3	CD366	ND	ND	ND	ND	+	ND
IMMUNOREGULATORY RECEPTORS	Siglec-3	CD33	-	+	++	++	++	+
	Siglec-6	CD327	-	+	+	ND	ND	ND
	Siglec-10	NA	ND	ND	ND	-	ND	+
	LILRB4	CD85k	+	-	-	ND	ND	ND
	LILRA4	CD85g	+	-	-	ND	ND	ND
ANTIGEN PRESENTATION	HLADR	NA	+	+	+	+	++	+
	HLADG	CD74	ND	ND	ND	-	+	-
	DEC-205	CD205	-	+	+	ND	ND	ND
	NA	CD1c	-	-	++	dim	dim	dim

Table 1. Immunophenotypic profiles of the different populations of blood dendritic cells (DC) (8, 27, 34, 62) and monocytes (Mo) (16, 46, 56, 67-69) (continuation).

Functional group of proteins	Surface antigen	CD code	DC subsets			Mo subsets		
			pDC	cDC1	cDC2	cMo	iMo	ncMo
SCAVENGER RECEPTORS	Glycoprotein IIIb	CD36	+	-	+	+	+	-/+
	M130	CD163	-	-	+	-	-	-
C-TYPE LECTINS	BDCA-3/Thrombomodulin	CD141	-	++	-	ND	ND	-
	BDCA-2/CLEC4C	CD303	++	-	-	-	-	-
	CLEC4A	CD367	+	-	+	+	ND	ND
	CLEC4D	CD368	ND	ND	ND	+	-	-
	CLEC5A	NA	ND	ND	ND	+	ND	-
	CLEC7A	CD369	-	-	+	+	-	-
	CLEC9A	CD370	-	+	+	ND	ND	ND
	CLEC12A	CD371	ND	ND	ND	+	ND	ND
	CLEC13A	NA	ND	ND	ND	+	ND	ND
PHAGOCYTIC-RELATED MOLECULES	$FPR1^+$	NA	-	-	-	ND	+	ND
	$FPRL1/2^+$	NA	-	-	-	ND	+	ND
OTHER MOLECULES	Aminopeptidase N	CD13	-	+	+	+	+	+
	SIRPα	CD172a	-	-	+	+	+	++
	LCA	CD45RA	+	-	-	ND	ND	+
	Axl	NA	-	-	-	ND	ND	ND
	CMRF35-H9	CD300A	+	-	-	ND	ND	ND
	Endoglin	CD105	ND	ND	-	-	+	-
	Tie-2/Angiopoietin receptor	CD202B	ND	ND	-	-	+	-
	Slan/6-Sulfo LacNAc	NA	-	-	-	-	-	-/+

pDC: plasmocytoid DC; cDCs: conventional DC; cMo: classical Mo; iMo: intermediate Mo; ncMo: non-classical Mo; ND: not determined

1.2.2. Functional characteristics of blood circulating dendritic cells and monocytes

DCs are critical players in the initiation and regulation of pathogen-specific adaptive immune responses at the same time they play a central role in the development of immunological cell memory and tolerance. In turn, Mo are major effectors and regulators of inflammation and the innate immune response, playing also an essential role in tissue homeostasis and repair.

From the functional point of view, pDCs are currently considered as the main producers of IFN-I (both IFN-α and INF-β) as well as IFN-III, particularly in response to viral pathogens via ssRNA and dsDNA-mediated TLR7 and TLR9 signaling, respectively (8, 62). Other soluble factors produced by pDCs following TLR-mediated stimulation include the C-C-motif ligand (CCL)3, CCL4, CCL5, IL8, the C-X-C-motif chemokine ligand (CXCL)10 and the CXCL1 chemokines (27). Additionally, pDCs have been shown to play a role in IL12 production and naïve allogenic T cell proliferation (24). However, following the recent identification of a new population of human DC (the Axl^+ DC) which expresses the typical pDC markers CD123, CD303 and CD304, but in association with functional features that overlap with those of cDC and with a greater T-cell stimulatory capacity, the later findings must be interpreted with caution, as in many studies Axl^+ DCs may have been considered as part of pDCs (24).

The DC population included in the heterogeneous compartment of cDCs, can all effectively activate and stimulate T-cytotoxic (Tc) and T-helper (Th) cells via efficient antigen presentation (59). Activation of the later Th cells results in their phenotypic polarization to proinflammatory Th1 and Th17, anti-inflammatory Th2 and/or immunoregulatory T (Treg) cells (72). While the cDC1 cell population mediates antiviral and antitumoral immunity through presentation of extracellular antigens to Tc cells via the major histocompatibility complex (MHC) class I molecules, cDC2 cells are specialized in presenting antigens to $CD4^+$ Th cells through HLA class II molecules (73, 74).

Since their first description, many different functions have been assigned to cDC1 cells, including an important role in cellular immunity against i) tumors and intracellular pathogens, ii) Tc and Th1 cell type immunity, iii) antigen cross-presentation and the production of IFN-I, IFN-III and IL12 (at lower levels) (62). In fact, one of the most important functional roles of cDC1 cells relies on their ability to cross-present antigens to T-cells, and thereby their capacity

to cross-prime Tc responses (62) and enable the generation of antigen-specific immune responses to tumor cells and viruses. Furthermore, the cDC1 subset ensures the induction of Th cells, as well as the secretion of IFN-λ after poly(I:C) stimulation (62). All above functions also emphasize the relevance of cDC1 cells in the immune defense against intracellular pathogens (62). Conversely, cDC2 cells have been suggested to play a more relevant role in the protection from extracellular pathogens due to their specialization in antigen presentation via MHC-II molecules, which is essential for the activation, proliferation and differentiation of naïve $CD4^+$ T cells into Th2 and Th17 cells (62).

Genome wide analyses of the three major subsets of human blood Mo have been reported in the literature and contributed to advance our knowledge about the common *vs* differential gene-expression background and function role of each subset (30, 45, 75, 76). Since Mo are key cells in the immune response and in inflammation, any gene which might affect signal transduction, chemotaxis, and apoptosis of these cells, will also have a direct impact on their contribution to the immune response and inflammation. Recently, Villani *et al* (30) investigated the heterogeneity of human blood Mo/DC through single-cell RNA sequencing. Overall, their findings revealed that, despite the different populations of Mo share a similar signature, their GEP are clearly different from those of DCs. Furthermore, in this study, cMo and ncMo also emerged as separated clusters, while iMo represented a heterogeneous population with GEP between the former two subsets of cMo and ncMo (30). Several studies (30, 75, 76) have demonstrated that cMo are heavily involved in the immune response including the inflammatory response, in addition to chemotaxis, tissue cleaning and repair. In turn, iMo typically expressed genes which might potentially affect the cell cycle, differentiation, and trafficking of Mo, together with genes associated with an increased production of reactive oxygen species (ROS), angiogenesis and the MHC class II-mediated

antigen processing and presentation activity. Finally, the GEP of ncMo was strongly related to changes (e.g., rearrangement) involving the cytoskeleton and cell movement, supporting the concept that these cells are constantly surveying the endothelium for signs of inflammation with an enhanced ability for fast (tissue) migration (30, 75, 76). In some studies ncMo have been functionally linked to pro-apoptotic signaling, based on the presence of prominent, large, end-stage granules with low amounts of myeloperoxidase (MPO) and lysozyme, suggesting that these cells are less capable of antigen digestion in comparison with cMo, and that they might correspond to end-stage monocytic cells (77, 78). Interestingly however, inflammatory cMo which are highly effective in phagocytosis, show a remarkable plasticity, being able to differentiate into both DC and Mϕ, in contrast to both iMo and ncMo, which have only been reported to show *in vitro* differentiation capacity to Mϕ (13-16, 67). In summary, while cMo and iMo possess pro-inflammatory properties, ncMo have been shown to display both pro-inflammatory (mainly attributed to the higher production of tumor necrosis factor –TNF-α) (45, 75, 79) and patrolling functions (45, 69, 80-82).

1.2.3. Immunophenotypic and functional characteristics of more recently described populations of blood DC and Mo

Recent studies have shown that the great heterogeneity of the different classical populations of blood circulating DC and Mo described above, might be due, at least in part, to the inclusion among a cell population of more than one cell subset with different immunophenotypic and functional properties.

In this regard, cDC2s have been reported to comprise more than one phenotypically and functionally distinct DC subsets. In fact, Villani *el al* (30) reported the existence of two different populations of cDC2: cDC2A and cDC2B cells (30, 58, 83-86). The transcriptional profile of cDC2A ($CD32b^+$ $CD1c^+$) cells is associated with higher levels of expression of HLA

molecules, while cDC2B ($CD163^+CD36^+$ $CD1c^+$) cells express CD14 in association with upregulation of genes involved in both acute and chronic inflammation (30) (Table 2). While both cDC2A and cDC2B cells express similar levels of HLADR and CD33, cDC2B cells were reported to express higher levels of CD11b, and the programmed cell death ligand (PDL)1 (CD274) molecules, through which they can suppress antigen-specific T cell responses (86, 87) (Table 2). Functionally, cDC2B cells have been also shown to produce higher amounts of TNFα and IL10 following LPS stimulation, and of IL1β (83, 84); in contrast, they display a lower ability to induce T cell proliferation (86) and to stimulate Th1 cell differentiation (87). In turn, cDC2A cells-but not cDC2B cells, except if stimulated with GM-CSF (granulocyte-macrophage colony-stimulating factor) or LPS-, mediate for an increased IFN-γ production by CD4 T cells, and efficiently produce TNFα, IL6, IL10, and IL23, with the ability to prime Th2, Th17 and Treg cells (85).

In parallel to the above studies, other authors have proposed alternative criteria for the subsetting of cDC2 cells, based on the differential expression of CD5 and CD14 (85). This includes a non-inflammatory $CD14^-$ cDC2 subset, with two distinct $CD5^-$ and $CD5^+$ subpopulations with different functional profiles related to cytokine production, migration profile, T cell activation and polarization capacity and a more pro-inflammatory $CD14^{-/lo}$ subset (8, 12, 57, 85). Interestingly, the $CD14^+$ subset of cDC2 cells has been shown to also express higher levels of other Mo-associated markers apart from CD14 (85), which has prompted Dutertre *et al* (58) to demonstrate that these cells did not have a monocytic origin. Interestingly, Dutertre *et al* (58) also described three different subsets of $CD5^-$ cDC2 which included: i) a $CD163^-$, in addition to ii) a $CD163^+$ $CD14^-$, and iii) a $CD163^+$ $CD14^+$ population with progressively higher pro-inflammatory abilities, potentially reflecting sequential progressively (more advanced) maturation stages (58).

In addition to the newly described subsets of cDC2 cells, other new populations of human DC have been recently described in blood. These include the Axl^+ DCs and the $CD100^+$ $CD34^+$ pre-cDCs (24, 30, 31). The former Axl^+ DC population represents a mature population of DC that express CD45RA, CD303, and CD12 (27), together with CD2, CD5, CD81, CD22, CD327 and CD169, associated with a significantly reduced ability to produce IFN-I (30, 58, 88). This subset of Axl^+ DC also shows intermediate levels of CD11c, CD304 and CD33 (Table 2). From the functional point of view, Axl^+ DCs show a unique GEP with intermediate features between those of pDC and cDC (30), associated with a high capacity to induce T cell proliferation (30). In contrast to pDC, they show a negligible ability to produce IFN-I upon stimulation via TLR9 (30) and secretion of higher IL8 levels together with a potent (*vs* undetectable) ability to induce T cell proliferation in response to LPS stimulation (30). Moreover, Villani *et al* also concluded that pDCs (depleted of Axl^+ DCs) do not upregulate CD86, and have a low ability to induce T cell proliferation, suggesting that contamination of Axl^+ DCs in the pDC (gated population) could potentially be responsible for the T cell stimulatory activity previously described in the literature for pDC (30), based on upregulation of the costimulatory CD86 molecule on pDC as a key co-stimulatory molecule among antigen presentation for T-cell stimulation (30).

In parallel, Villani *et al* (30) also described the existence of a potential cDC precursor (pre-cDCs) with an unique $HLADR^+$ $CD100^+$ $CD34^{+/int}$ immunophenotype, associated with a higher proliferation rate and more immature morphological, phenotypic, and GEP, and the ability to mature to cDC cells. These features were in contrast with those of Axl^+ DCs, which had a resting phenotype with low proliferative potential and high T cell stimulatory potential (24, 30). These $CD100^+$ $CD34^{+/int}$ pre-cDC were also shown to express CD117 and CD45RA, as

well as CD86^{lo} and CD197 (CCR7) in the absence of CD1c, CD115, CD116, CD141, CD14, CD11c, CD123 and CD135 (27) (Table 2).

Despite the great heterogeneity of DC in human blood and the relatively high number of studies in which they have been characterized, there is limited data on the potential relationship among the different (sub)populations identified.

Similarly to DC populations, the three major populations of blood circulating Mo that have long been identified, also typically comprise several functionally and phenotypically distinct (sub)populations. Thus, cMo can be further subdivided based on the pattern of expression of the L-selectin (CD62L) and FcεRI molecules into CD62L$^+$ and CD62L$^-$ cMo and into FcεRI$^+$ and FcεRI$^-$ cMo, respectively (57, 89) (Table 2). The leucocyte selectin CD62L has long been identified has an important cell adhesion molecule involved in cell migration and trafficking, enabling cMo to adhere to the vascular endothelium and to extravasate into tissues where they would act (mostly) as proinflammatory Mϕ (47, 48). Although FcεRI is long known to be constitutively expressed by human mast cells and basophils both in normal homeostatic conditions (90) and in the context of allergic diseases (91, 92), FcεRI expression on Mo has also been recently described. In these later cells FcεRI can be rapidly endocytosed and transported to the lysosomes following binding to IgE/Ag immunocomplex (89). Because of this, there is an increased interest on better understanding the exact role of FcεRI in the delivery of intracellular signals in Mo, which could have either i) a pro-inflammatory effect by upregulating cytokine production via activation of the nuclear factor-κB (NF-kB) and promoting the transcription of proinflammatory mediators such as IL6 and TNFα with the subsequent recruitment of inflammatory cells, or ii) an anti-inflammatory and immunosuppressive action through an increased production of IL10 as a negative regulator of

TNFα (89). Because of all the above, FcεRI^{+} cMo have been associated with a more prevalent immunomodulatory (than phagocytic) function (93), although their origin and precise functional role still remains to be fully elucidated. More recently, Hamers *et al* (46) have also reported the existence of up to four different subsets of cMo in human blood which might be identified based on their distinct pattern of expression of the FcεRI, CD61 (or CD9), CD93 and CD11a molecules on their cell surface membrane. These four subsets of cMo include: 1) cMo1, with an FcεRI^{-} CD61^{-} CD9^{+} CD93^{hi} CD11a^{+} phenotype; 2) cMo2, which display an FcεRI^{-} CD61^{-} CD9^{-} CD93^{lo} CD11a^{+} profile; 3) cMo3, characterized by an FcεRI^{-} CD61^{+} CD9^{hi} CD93^{+} CD11a^{+} immunophenotypic pattern; and 4) FcεRI^{hi} CD61^{-} CD9^{-} CD93^{+} CD11a^{+} cMo4. Altogether these results further highlight the great heterogeneity of cMo (Table 2).

Similarly to cMo, ncMo have also been found to be a highly heterogeneous cell population, which consists of more than one different cell subset based on the expression, among other markers, of e.g., CD61 and CD9, probably conferring different cell adhesion and platelet binding properties to ncMo. These distinct phenotypic profiles of ncMo are also associated with different patterns of expression of other molecules such as Slan, allowing the identification of up to three distinct subsets of ncMo: 1) CD61^{+} CD9^{+} Slan^{+} ncMo1; 2) ncMo2 with a CD61^{-} CD9^{-} Slan^{+} phenotype; and 3) CD61^{-} CD9^{-} Slan^{-} ncMo3 cells (46). Alternatively, combined expression of CD36 and Slan can also be used for the identification of (four) distinct (sub)populations of CD36^{+} Slan^{-}, CD36^{+} Slan^{+}, CD36^{-} Slan^{-} and CD36^{-} Slan^{+} ncMo (57) (Table 2).

Of note, some reports have investigated the functional behavior and role of Slan^{+} ncMo (94-101). Their results suggest that Slan^{+} ncMo act as cells that patrol blood vessels and selectively detect viral infected and damaged cells (94-101). Once activated via TLR stimulation, Slan^{+} ncMo would produce proinflammatory cytokines, such as IL1β, IL6, IL12, IL23, and TNFα (69) and they would ultimately enhance Th1 and Th17 T cell responses, which

play an important role in chronic inflammatory diseases and promote cytotoxic T and NK cell-mediated anti-tumor cell responses (94-101). Although the exact function and protein ligands of Slan still remain unknown, sulfated terminal glycotypes found in the Slan antigen were shown to generally serve as ligands for lectins (45, 69, 81, 100).

Table 2. Phenotype of more recently identified (sub)populations of dendritic cells (DC) (24, 27, 30, 31) and monocytes (Mo) in human blood (46, 57, 89).

Major population	Cell subset	Immunophenotype
DCs	cDC2A	$CD1c^{++}$ $CD5^{hi}$ $CD11b^{+}$ $CD11c^{++}$ $CD32b^{+}$ $CD33^{+}$ $CD172a^{+}$ $HLADR^{++}$
	cDC2B	$CD1c^{+}$ $CD5^{lo}$ $CD11b^{+}$ $CD11c^{+}$ $CD14^{+}$ $CD33^{+}$ $CD36^{+}$ $CD163^{+}$ $CD274^{+}$ $HLADR^{+}$
	Axl^{+} DC	$CD1c^{-}$ $CD2^{hi}$ $CD5^{+}$ $CD11c^{int}$ $CD12^{+}$ $CD22^{+}$ $CD33^{int}$ $CD45RA^{+}$ $CD81^{+}$ $CD123^{+}$ $CD169^{+}$ $CD303^{+}$ $CD304^{int}$ $CD327^{++}$ Axl^{++} $HLADR^{+}$
	Pre-cDC	$CD1c^{-}$ $CD11c^{-}$ $CD14^{-}$ $CD34^{+int}$ $CD45RA^{+}$ $CD86^{lo}$ $CD100^{+}$ $CD115^{-}$ $CD116^{-}$ $CD117^{+}$ $CD135^{-}$ $CD123^{-}$ $CD141^{-}$ $CD197^{lo}$ $HLADR^{+}$
cMo	cMo1	$CD9^{+}$ $CD11a^{+}$ $CD14^{+}$ $CD16^{-}$ $CD61^{-}$ $CD62L^{+}$ $CD93^{hi}$ $FcεRI^{-}$ $HLADR^{+}$
	cMo2	$CD9^{-}$ $CD11a^{+}$ $CD14^{+}$ $CD16^{-}$ $CD61^{-}$ $CD62L^{-}$ $CD93^{lo}$ $FcεRI^{-}$ $HLADR^{+}$
	cMo3	$CD9^{hi}$ $CD11a^{+}$ $CD14^{+}$ $CD16^{-}$ $CD61^{+}$ $CD62L^{-}$ $CD93^{+}$ $FcεRI^{+}$ $HLADR^{+}$
	cMo4	$CD9^{+}$ $CD11a^{+}$ $CD14^{+}$ $CD16^{-}$ $CD61^{-}$ $CD62L^{+}$ $CD93^{+}$ $FcεRI^{+}$ $HLADR^{+}$
ncMo	ncMo1	$CD9^{+}$ $CD14^{-}$ $CD16^{+}$ $CD36^{+}$ $CD61^{+}$ $HLADR^{+}$ $Slan^{+}$
	ncMo2	$CD9^{-}$ $CD14^{-}$ $CD16^{+}$ $CD36^{-}$ $CD61^{-}$ $HLADR^{+}$ $Slan^{+}$
	ncMo3	$CD9^{-}$ $CD14^{-}$ $CD16^{+}$ $CD36^{+}$ $CD61^{-}$ $HLADR^{+}$ $Slan^{-}$

DCs: dendritic cells; DCs: conventional DCs; cMo: classical Mo; iMo: intermediate Mo; ncMo: non-classical Mo; CD: cluster of differentiation; HLA: human leucocyte antigen; Ig: immunoglobulin;

1.3. Tissue localized dendritic cells and monocytes

As previously indicated, tissue DC and Mϕ consist of a pool of both yolk sac, fetal-derived tissue resident populations and BM-derived cells which had migrated to the tissues via the bloodstream (37, 47, 50, 51). Following their development and egress from the BM to the tissues, multiple DC and Mϕ populations can be detected in distinct locations such as CB, the spleen, thymus, lymph nodes (LN), tonsils, skin, the lung, and the intestine (40, 59-61, 102, 103).

Due to its key role in the front line to fight incoming pathogens and its accessibility, the skin has been one of the tissues which has been more extensively investigated and that at the same time shows one of the greatest diversities of DC and Mϕ populations, both localized in the dermis and, to a less extent also, the epidermal layer (27). Among skin DCs, dermal cDC1s are characterized by high expression amounts per cell of HLADR and CD141, moderate levels of CADM1, CD370, XCR1 and CD103 and low to absent levels of CD1c, CD11c, CD14, CD207, CD172α and CD135 expression (27) (Table 3). Functionally, dermal cDC1 cells display a high capacity of cross-presentation in skin draining LN (61), also expressing the CXCL10 chemokine which allows crosstalk with Th1 cells and activated $CD183^+$ (CXCR3) NK cells (27, 61, 64, 104). In contrast with cDC1 cells, dermal cDC2 cells express CD1a, CD1c, CD103, together with CD172α in the absence of CD209 (DC-SIGN) (27). The absence of Birbeck granules, as well as the higher CD11c and CD11b levels found on dermal cDC2 (*vs* cDC1) cells, which further allow their clearcut separation from a third population of skin DCs, which can be found in the suprabasal epidermis within the interstices of keratinocytes: the LCs (27) (Table 3). Globally, LCs are phenotypically similar to dermal cDC2s ($HLADR^+$, $CD1a^+$, $CD1c^+$, $CD209^-$, $CD172\alpha^+$), but they characteristically express CD207 in addition to a broad range of molecules such as CD324, CD326, CD83 and CD32 (27, 40) not present in cDC2 cells (Table 3). Interestingly, both pDCs and Axl^+ DCs are absent in the skin (40).

Immune cells that express CD14 in the skin have limited stimulatory ability on naive T cells, as compared to tissue cDC2s; conversely, they can strongly stimulate memory $CD4^+$ T cells and induce comparable responses to those observed after stimulation by tissue cDC2s and Mϕ (135). Up till now, three different populations of $CD14^+$ Mϕ have been described in the skin. These consist of a $CD1c^{-/lo}$ $CD209^-$ Mϕ1 subset defined previously by McGovern *et al* (105), and a $CD1c^+$ $CD209^+$ Mϕ2 subset, both of which express HLADR and CD11c, in the

absence of CD16 and CD11b and minimal autofluorescence levels; in addition, a third Mϕ3 autofluorescent subset, characterized by co-expression of CD64, together with CD14, HLADR and CD209 (believed to be an exclusive DC marker), in the absence of the fibrin stabilizing factor FXIII,$^{-}$ has also been identified among CD14^{+} antigen-presenting cells in the skin (27, 105) (Table 3).

DCs play a critical role in normal homeostatic conditions (via a balance between tolerogenic and inflammatory immune responses) also in tissues other than the skin challenged by a myriad of different antigens and microorganisms such as the lung or the intestine. Thus, in the lung, cDC1 and cDC2 cells can also be identified, like in blood and the skin, by their unique CD141^{+} CD1c^{-} and CD141^{-} CD1c^{+} expression profile, respectively (27, 61, 102, 106). Although lung cDCs can be identified by their specific key markers, CD141^{+} cDC1s from the lung are also characterized by the lack on CD207 and CD103 expression, whereas lung cDC2 cells are CD207^{+} CD103^{-} (19, 102) (Table 3). Interestingly, Granot *et al* (19) have demonstrated the presence of migratory CD197^{+} (CCR7) DCs, particularly cDC2 cells, in the lung, intestine and several associated draining LN including lung and mesenteric LN, which supports the capacity of tissue DCs of migrating from barrier tissues to lympho-hematopoietic organs, where they can efficiently start T cell-mediated adaptative immune responses. Of note, human intestinal DCs typically display a tolerogenic and (immature) phenotype once they are present in resting conditions (107). This phenotype includes low expression of PPRs and lower expression of both HLA-II molecules and cell surface co-stimulatory molecules, compared to DCs from other tissues (107). Based on the expression of CD103 and CD172α, intestinal DCs can be further subclassified into CD103^{-} CD172α^{+} DC1, CD103^{+} CD172α^{+} DC2 and CD103^{-} CD172α^{-} DC3 cells, whose functional roles still remain to be elucidated. Interestingly,

the αVβ8 integrin required to activate latent TGFβ, is expressed by the DC2 subset in the intestine, which also expresses $CD1c^+$ (107-109) (Table 3).

Together with DCs, tissue (including mucosal) Mϕ are also essential cellular components for the maintenance of immune homeostasis and tissue integrity, through the removal of apoptotic/damaged cells and foreign components, such as microorganisms and their pathogenic factors. In general, TRM are present in virtually every tissue and exhibit a great functional plasticity, depending on the tissue microenvironment. Depending on the specific tissue where they are located, different TRM have been identified so far. Among others, these include $CD206^+$, $CD169^+$ alveolar Mϕ, and $CD206^+$ $CD169^-$ interstitial Mϕ in the lung (Table 3) or intestinal Mϕ (110). cMo ($CD14^+CD16^-$) and iMo ($CD14^+CD16^+$), as well as ncMo ($CD14^-CD16^+$), have also been detected in the lung of healthy volunteers, where all subsets express CD71 in the absence of CD206 and the co-stimulatory CD80 and CD86 molecules (27, 111) (Table 3). In turn, intestinal Mϕ consist of four distinct populations which can be separated among them based on their differential expression of CD11c, HLADR and CD11b (17). Intestinal Mϕ1 and Mϕ2 cells are both $CD11c^+$, the former showing lower expression of HLADR compared to Mϕ2 cells; conversely, Mϕ3 and Mϕ4 cells are either $CD11c^-$ $CD11b^-$ and $CD11c^-$ $CD11b^+$, respectively (Table3) (17). Kinetic studies performed by Bujko *et al* (17) suggest that these four types of intestinal Mϕ are in fact derived from PB Mo, following sequential differentiation to Mϕ1 and Mϕ2 cells and finally, either Mϕ3 or Mϕ4 cells.

In case of massive inflammatory conditions, the plasticity of TRM allows them to adopt different phenotypes, in a process termed Mϕ repolarization (112). Thus, intestinal TRM can differentiate into classical (activated) M1-type or alternatively, M2-type Mϕ, depending on the triggering pathogen and the surrounding tissue microenvironment (112, 113). Generally,

M1 Mϕ efficiently contribute to the recognition and elimination of bacteria and cancer cells through phagocytosis and cytotoxicity-mediated effector mechanisms, following the release of proinflammatory Th1 cytokines such as IFN-γ, TNFα or LPS (114). In turn, M2 Mϕ are induced by Th2 cytokines (IL4, IL13, and IL10, CCL12, CCL17, CCL22) and promote tissue repair and tumor growth (10, 114, 115). Based on this (simplified) model, the increased frequency of M1 Mϕ has been long associated with anti-tumor immune surveillance, while a M2 Mϕ predominance has been related with an increased tumor growth and a decreased patient survival (10, 115, 116).

All above findings confirm that DC and Mo subsets show an extremely high phenotypic and functional plasticity, which depends on the surrounding microenvironment that should be considered in future studies, devoted to the correct identification of individual DC and Mo/Mϕ cell populations.

Table 3. Phenotypic profile of dendritic cell (DC) and monocyte (Mo) populations in the skin (27, 40) , lung (110, 111), and intestine (17, 27, 107, 117).

Tissue	Cell subset	Immunophenotype
Skin (27,40)	cDC1	$CD1c^{-/lo}$ $CD11c^{lo}$ $CD14^{-}$ $CD103^{+}$ $CD135^{lo}$ $CD141^{++}$ $CD172a^{-}$ $CD207^{-}$ $CD370^{+}$ $CADM1^{+}$ $HLADR^{+}$ $XCR1^{+}$
	cDC2	$CD1a^{+}$ $CD1c^{+}$ $CD11b^{+}$ $CD11c^{+}$ $CD103^{+}$ $CD141^{-}$ $CD172a^{+}$ $CD207^{-/+}$ $CD209^{-}$ $HLADR^{+}$
	Mϕ1	$CD1c^{-/lo}$ $CD11b^{-}$ $CD14^{+}$ $CD209^{-}$ $Autofluorescence^{-}$ $HLADR^{+}$
	Mϕ2	$CD1c^{+}$ $CD11b^{-}$ $CD11c^{+}$ $CD14^{+}$ $CD16^{-}$ $CD209^{+}$ $Autofluorescence^{-}$ $HLADR^{+}$
	Mϕ3	$CD11c^{+}$ $CD14^{+}$ $CD16^{-}$ $CD64^{+}$ $CD209^{+}$ $Autofluorescence^{+}$ $HLADR^{+}$ $FXIIIA^{-}$
	LCs	$CD1a^{++}$ $CD1c^{+}$ $CD11c^{lo}$ $CD32^{+}$ $CD83^{+}$ $CD172a^{+}$ $CD207^{+}$ $CD209^{-}$ $CD324^{+}$ $CD326^{+}$ $Birbeck\ granules^{+}$ $HLA\text{-}DR^{+}$
Lung (110, 113)	cDC1	$CD103^{-}$ $CD141^{+}$ $CD207^{-}$
	cDC2	$CD1a^{+}$ $CD1c^{+}$ $CD103^{-}$ $CD141^{+}$ $CD197^{+}$ $CD207^{+}$
	Alveolar Mϕ	$CD14^{-}$ $CD45^{+}$ $CD71^{++}$ $CD80^{+}$ $CD86^{+}$ $CD169^{+}$ $CD206^{+}$
	Intersticial Mϕ	$CD14^{+}$ $CD45^{+}$ $CD80^{+}$ $CD86^{+}$ $CD169^{-}$ $CD206^{+}$
	cMo	$CD14^{+}$ $CD16^{-}$ $CD71^{+}$ $CD80^{-}$ $CD86^{-}$ $CD206^{-}$
	iMo	$CD14^{+}$ $CD16^{+}$ $CD71^{+}$ $CD80^{-}$ $CD86^{-}$ $CD206^{-}$
	ncMo	$CD14^{-}$ $CD16^{+}$ $CD71^{+}$ $CD80^{-}$ $CD86^{-}$ $CD206^{-}$

cDCs: conventional DCs; Mφ: macrophage; LCs: Langerhans cells; cMo: classical Mo; iMo: intermediate Mo; ncMo: non-classical Mo; CD: cluster of differentiation; HLA: human leucocyte antigen.

Table 3. Phenotypic profile of dendritic cell (DC) and monocyte (Mo) populations in the skin (27, 40) , lung (110, 111), and intestine (17, 27, 107, 117) (continuation).

Tissue	Cell subset	Immunophenotype
Intestine (17, 27, 107, 117)	DC1	$CD103^{-}$ $CD172a^{+}$ $CD192^{+}$
	DC2	$CD1c^{+}$ $CD103^{+}$ $CD172a^{+}$ $\alpha V\beta 8^{+}$
	DC3	$CD103^{+}$ $CD172a^{-}$
	Mϕ1	$CD1c^{+/lo}$ $CD11b^{+}$ $CD11c^{+}$ $CD14^{+}CD206^{-}$ $HLADR^{+}$
	Mϕ2	$CD1c^{+}$ $CD11b^{+}$ $CD11c^{+}$ $CD14^{+}CD206^{+}$ $HLADR^{++}$
	Mϕ3	$CD1c^{-}$ $CD11b^{-}$ $CD11c^{-}$ $CD14^{+}CD206^{+}$ $HLADR^{+}$
	Mϕ4	$CD1c^{-}$ $CD11b^{+}$ $CD11c^{-}$ $CD14^{+}CD206^{+}$ $HLADR^{+}$

cDCs: conventional DCs; Mφ: macrophage; LCs: Langerhans cells; cMo: classical Mo; iMo: intermediate Mo; ncMo: non-classical Mo; CD: cluster of differentiation; HLA: human leucocyte antigen.

1.4. Altered numbers and/or phenotypes of blood dendritic cells and monocytes in different disease conditions

Under pathological conditions, the fine balance among the different populations of DCs and Mo circulating in blood might be disrupted, leading to numerical and/or phenotypic alterations involving one or more cell populations, as previously reported for e.g., several infectious diseases and inflammatory conditions (118) including sepsis, human immunodeficiency virus (HIV) infection, tuberculosis, hepatitis B and C and dengue, where a common tendency for the expansion of the iMo and/or ncMo cellular compartments in blood has been recently reported (56, 82, 119-121). More recently, significantly increased numbers of iMo, associated with lower counts of ncMo and normal cMo numbers have been found also in patients with COVID-19 (122), in association with decreased DC counts during the acute phase of infection (123). Other disease conditions in which altered counts of different subsets of Mo have been reported include liver cirrhosis (increased iMo counts in association with recruitment of cMo from BM to blood to maintain the pool of circulating cMo at the expense of lower ncMo counts in acute decompensated cirrhotic patients) (124), cardiovascular diseases such as myocardial infarction (125, 126) or vascular inflammation and atherosclerosis

(127, 128), where increased numbers of iMo are found in association with a greater risk of cardiovascular events and higher mortality rates (129). In patients suffering from hematological neoplasms (e.g., acute leukemia), significantly increased numbers of ncMo have been associated with a higher rate of disease progression (130), while in chronic myelomonocytic leukemia a clear predominance of cMo (≥94% of all Mo) exists at the expense of iMo and ncMo and contributes to the differential diagnosis between chronic myelomonocytic leukemia and other (e.g., reactive and neoplastic) monocytosis (131, 132). The expansion of the $Slan^+$ compartment of ncMo has also been recently reported in many disease conditions such as psoriasis (94), atopic dermatitis (133), lupus erythematosus (95), multiple sclerosis (134), Crohn's disease (135), HIV infection (136, 137), or diffuse large B cell lymphoma (138), among others. In contrast, limited data exists about the relative distribution and absolute counts of other minor subsets of Mo (e.g. $CD62L^+$, $CD62L^-$, $FcεRI^+$, and $FcεRI^-$ cMo), and particularly about DCs, both in normal homeostatic conditions and in specific diseases. Likewise, in patients with indolent systemic mastocytosis, reduced total circulating Mo counts, due to decreased iMo and ncMo, has been recently described, in parallel to decreased numbers of circulating pDC and cDC both in indolent and aggressive forms of the disease (139). In contrast, Axl^+ DCs were expanded in patients with BM mastocytosis (139). In turn, evaluation of the kinetics of the distinct subsets of the PB leucocytes, including Mo, following hip replacement surgery has revealed rapid Mo responses immediately after (2h) surgery with significantly decreased Mo counts in blood, following by a rebound with maximum levels of twice those found at baseline, detected 24-hour post-surgery. From then, total Mo and cMo levels remained elevated for over 24 hours before they gradually returned to pre-operative baseline levels after 48 hours; in contrast, several days were required until iMo and ncMo recovered and reach normal baseline levels (140).

2. Plasma cell neoplasms

Plasma cells (PCs) are terminally differentiated cells originated from B lymphocytes during primary and secondary immune responses, whose main function relies on the production of antibodies of different isotypes (i.e., IgG, IgM, IgD, IgE, and IgA) in response to an antigenic stimulus. Following a primary immune response, long-lived PCs are generated in the germinal centers of secondary lymphoid tissues, from where they migrate as plasmablasts, through the bloodstream, to the BM and other peripheral tissues such as the mucosal layers, where they differentiate into mature long-lived PCs. In the BM, PCs survive in specific niches provided by BM stromal cells (BMSCs), representing between 0.1% and 1% of the whole BM cellularity of adult healthy individuals (141-143).

PC neoplasms (and other diseases with paraproteins) (144) consist of a heterogeneous group of tumors in which abnormal clonal PC capable of producing a monoclonal Ig which becomes detectable in the serum are present (144, 145). This results in the production and further accumulation of a monoclonal protein (i.e., M-component) that can be detected either as a whole Ig protein in the serum or plasma, or as a free light chain in the serum or the urine, whose tissue accumulation might contribute to organ damage, in the more advanced malignant stages of the disease (141, 144).

In PC neoplasms the first oncogenic event leading to the neoplastic transformation of a PC might take place in the germinal center, when an antigen-activated B-cell undergoes the immunoglobulin heavy (*IGH*) and light (*IGL*) chain gene somatic hypermutation and *IGH* isotype class-switching events, typically leading to either DNA hyperdiploidy or chromosomal translocations involving the *IGH* gene rearrangements (143, 146-148). These cytogenetic alterations would increase PC survival and facilitate the acquisition of secondary cytogenetic

abnormalities including other numerical chromosome gains and losses –e.g., $1q^{+}$, del(1p), del(17p), del(13)-, RAS mutations, and even additional chromosomal translocations and gene rearrangements (143, 146-148). Of note, several of these genetic alterations, including the presence of del(17p), gains of chromosome $1q^{+}$, t(4;14), t(14;16), and t(14;20) are considered to confere a higher risk of malignancy (143, 146-148).

According to the current classification of the World Health Organization (WHO 2022) of PC neoplasms, three PC neoplasms emerge as the most prevalent: monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM) and clinical/symptomatic MM (144).

Patients with MGUS are often diagnosed accidentally, during a routine analysis of serum proteins because of another underlying clinical condition, which reveals a serum M-component <3g/dL, associated with a low (<10%) PC infiltration of the BM in the absence of clinical manifestations of a PC malignancy -i.e., lack on CRAB criteria: hypercalcemia (>1mg/dL), renal insufficiency (creatinine clearance <40mL/min or serum creatinine >2mg/dL), anemia (hemoglobin<100g/L) and osteolytic lesions (detected via x-ray or computed tomography)- (149) (Table 4). Despite the disease in most MGUS patients might remain stable for several decades, a small fraction of MGUS patients display a higher risk of progression to malignant disease such as SMM or MM, with a rate of malignant transformation of ≈1% per year (150).

Like MGUS, SMM also represents a biological and clinical heterogeneous entity with intermediate features (and prognosis) between MGUS and MM (144). Thus, SMM patients typically display a serum M-component of ≥3g/dL and/or a BM infiltration by (tumor) PC of between 10% and 59%, still in the absence of myeloma-defining (i.e., CRAB) events (149) (Table 4). From the prognostic point of view SMM displays a risk of progression to MM that

varies between 1% and 3% per year for low and intermediate risk cases, to >10% per year for high risk patients (150).

In contrast to MGUS and SMM, at diagnosis, MM patients show a clear malignant behaviour, with an urgent need for cytotoxic therapy, currently representing the second most prevalent hematological malignancy (151). At diagnosis, the vast majority of MM patients show high levels of M-component in serum, associated with BM infiltration by >10% PC, and one or more of the four CRAB criteria mentioned above (149). In a smaller fraction of MM patients however, the CRAB criteria is not present. According to the International Myeloma Working Group (IMWG), in such cases, additional criteria are required for the diagnosis of MM which include the presence of at least one of the following findings: BM infiltration by PC >60%, involved: uninvolved serum free light chain ratio ≥100 and/or >1 focal bone lesion on magnetic resonance imaging (MRI) studies (142).

Table 4. Diagnostic criteria of the International Myeloma Working Group (IMWG) for the most prevalent PC neoplasms (MGUS, SMM and MM).

	MGUS	SMM	MM
Monoclonal component	<3g/dL serum	≥3g/dL serum	present (serum/urine)
	and	and/or	and
BM PC infiltration (%)	<10	10-59	>10
	and	and	and
Myeloma-defining events (CRAB)	absent	absent	present*

*in the absence of CRAB findings, additional criteria are needed for the diagnosis of MM: BM infiltration by PC >60%, involved: uninvolved serum free light chain ratio ≥100 and/or >1 focal bone lesion on magnetic resonance imaging (MRI) studies.

MGUS: monoclonal gammopathy of undetermined significance; SMM: smouldering multiple myeloma; MM: multiple myeloma; BM: bone marrow; PC: plasma cell; CRAB: calcium elevated, renal failure, anemia, bone lesions.

The MM defining events (i.e., CRAB) result from: 1) tissue accumulation of PC, including an increased infiltration of the BM by tumor cells leading to decreased production of normal hematopoietic cells (i.e., anemia); 2) an enhanced interaction between the malignant PC and both the BMSCs and/or bone cells (osteoblasts and osteoclasts) leading to an

imbalanced/altered bone formation and resorption because of an increased osteoclast activity that leads to the development of lytic bone lesions, hypercalcemia and ultimately also to renal failure by nephrotoxicity, also triggered by the overload of monoclonal free light chains in the kidney (152). In parallel to the increased production of the M-component in MM, a significant decreased production of normal Igs, associated with immunodeficiency also exists, which leads to an increased patient susceptibility to infections (142) (Figure 3). Of note, such immunodeficiency might be seen not only in MM, but already in its precursor states (e.g., SMM and even MGUS) (142). These findings indicate that in patients with PC neoplasms, progressive accumulation of neoplastic PC has an impact on the surrounding tumor cell microenvironment, which significantly affects immune cells, such as normal B-cell and PC, and innate immune cells involved in bone metabolism.

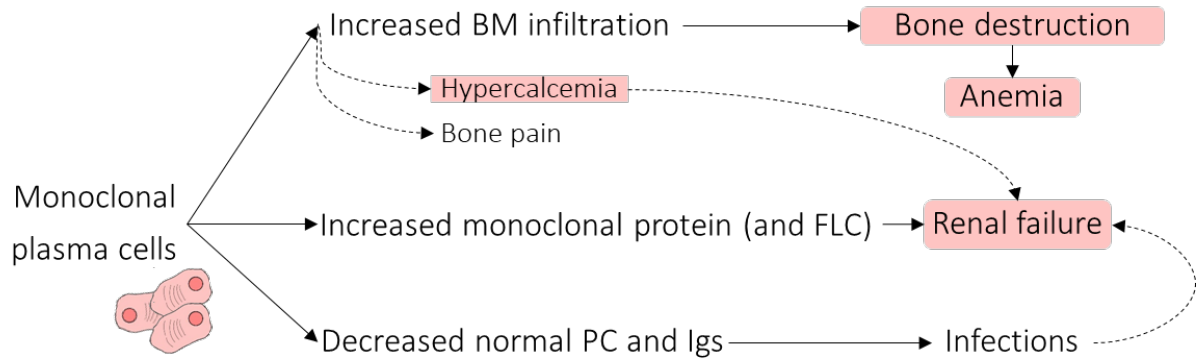

Figure 3. Illustrative summary graphical representation of the impact of tumor progression in the development of myeloma defining events in patients with PC neoplasms. Igs: immunoglobulins; BM: bone marrow; FLC: free Ig light chains; PC: plasma cell. Adapted from Rajkumar et. al 2014

2.1. The bone marrow and immune cell microenvironment in patients with PC neoplasms

The BM microenvironment consists of a broad variety of different cell types and their (secreted) products, together with the extracellular matrix. In general, the cellular composition of the tumor PC microenvironment in the BM consists of BMSCs, osteoblasts,

myeloid and lymphoid cells, fibroblasts, pericytes, adipocytes, endothelial cells and blood vessels (153). In turn, the non-cellular compartment includes the extracellular matrix, soluble factors such as cytokines, chemokines and growth factors, exosomes, microvesicles and other environmental factors, nutrients and tissue debris (153, 154). Altogether, hematopoietic and non-hematopoietic cells, as well as the non-cellular components of the BM, ensure that the HSC pool and the lineage-committed hematopoietic progenitor cells (HPCs) function normally, as reflected by the production and release of hematopoietic cells and their products to the bloodstream in normal daily levels.

The interaction between the tumor microenvironment and neoplastic cells in patients with PC neoplasms is long known to play a critical role in the control and growth of tumor PC (153, 155). In this regard, at present it is well-established that survival of malignant PC requires an altered microenvironment, including an altered homeostasis of the immune and bone microenvironment that would facilitate tumor growth and survival and contribute to drug resistance, local angiogenesis and bone resorption (153, 155). Thus, increased levels of the RANKL (receptor activator of NFκB ligand) pro-osteoclastogenic factor, in association with decreased osteoprotegerin (OPG) levels, have been previously described in the BM of MM (156-160), and also MGUS patients (161). Likewise, an enhanced turnover of BM osteoblasts, together with increased bone alkaline phosphatase (BALP) and RANKL serum levels, have also been found in both MGUS and MM patients (162, 163). These findings might be due to an increased differentiation of Mo into osteoclasts (164), or because of a higher secretion of IL6 (165), CXCL12 (166, 167) and RANKL by both BMSC (168) and locally activated T cells (169). As a consequence, an increased recruitment of osteoclasts to the BM endosteal niche would occur, followed by an inhibition of its decoy receptor OPG, and locally increased bone resorption, as (typically) observed in MM (153, 155).

In patients diagnosed with PC neoplasms that infiltrate the BM, both normal and malignant PC use BM sinusoids to enter the BM, based on the interaction between the chemokine receptor CXCR4 expressed on the PC membrane and its ligand CXCL12 highly expressed in the BM microenvironment (153, 170, 171). Other mediators that appear to be involved in PC homing to the BM include (the interaction between) the (upregulated) α4β1 integrin and its ligand (in the BM microvasculature) VCAM-1, the fibronectin receptor α4β7 and CD44 (153, 172). These later molecular interactions are critical for anchoring and retaining malignant PC in their BM niches (153, 172). At the earliest stages of interaction between neoplastic PC and BM endothelial cells, the P-selectin (CD62P) also interacts with PSGL-1 allowing malignant PC to move in the microvasculature (153, 170, 173). Once inside the BM milieu, malignant PC further interact with the tumor microenvironment leading to an immunosuppressive tumor microenvironment that protects the neoplastic PC clones from immunesurveillance mechanisms, which ultimately favors tumor survival and progression of the disease (170). This is associated with an increased occupancy of the BM PC niches which leads to a decreased normal B-cell and PC production, associated with immunoparesis, and even, colonization of different organs by the continuous trafficking and spreading of the increasingly high number of malignant PC that accumulate in the bloodstream, leading to a greater rate of dissemination of the disease throughout the axial skeleton. This later process is no longer dependent on the growth signals provided by the BM milieu and might be associated with a decreased expression of CXCR4 on the surface of MM cells or its binding to the Mϕ migration inhibitory factor (MIF) that might prevent adhesion of malignant PC to the BM stroma (153, 174, 175). Alternatively, it has also been suggested that a proliferation arrest in caused by hypoxic BM niches (due to massive infiltration by tumor PC), which together with the pro-inflammatory microenvironment, would force malignant PC to egress from BM into

the bloodstream in order to survive, with a decreased ability to return (home) into the BM (175).

2.2. Dendritic cell and monocyte subsets in PC neoplasms

Numerous immune cells have been implicated in the pathogenesis of PC neoplasms. Among them DCs (176-178), Mo and Mϕ (179-182), myeloid-derived suppressor cells (MDSC) (183-185), Tregs (183, 186-189), NK cells (190-193), and T cells (194, 195) are included, due to their important role in the interaction between tumor PC and their immune/BM microenvironment.

In this regard, previous studies suggest that the major subsets of PB DCs (i.e., cDCs and pDCs) from MM patients are functionally defective in antigen-presentation and T cell stimulation, and progressively accumulate in BM from the earlier stages of the disease (e.g., MGUS) to active MM (196, 197). Thus, data from the literature indicate that a direct interaction between cDCs and malignant PC induces a downregulated expression of proteasome subunits in tumor PC, turning them resistant to T-cell-mediated-cytotoxic immune responses (196). As for pDCs, their frequency in the BM of MM patients has been found to be increased with respect to normal BM, particularly in association with progression of the disease (197).

The intrinsic plasticity of TRM allows Mϕ repolarization, depending on the stimuli and the tissue microenvironment. Thus, an M2 immunosuppressive Mϕ phenotype appears to be significantly expanded in the BM of MM patients compared to both normal healthy volunteers, and also MGUS and SMM patients (179). BM infiltration by M2 Mϕ in MM has further been associated with a poorer patient outcome, in comparison with cases with a predominant M1 Mϕ pattern (153, 180). In this regard, it has been shown that the CXCL12,

CCL2, CCL3 and CCL14 chemokines produced by malignant PCs and BMSCs, might stimulate *in vitro* migration of M2-polarized Mϕ to the tumor niche, which could contribute to explain at least in part, the M2 Mϕ polarization observed in a significant fraction of MM patients (153).

In addition to the anti-tumoral *vs* pro-tumoral effects of innate cells in the BM microenvironment of patients with PC neoplasms, the altered interaction between DC, monocytic cells and other cells of the BM immune microenvironment of MGUS, SMM and MM patients, such as the osteoblasts and osteoclasts, also plays a relevant role in the pathogenesis of PC neoplasms (176-182). In recent years, several studies have investigated whether these alterations might be reflected in altered numbers and phenotypes of the major Mo subsets in patients with PC neoplasms, not only in BM, but even in blood. Thus, altered counts of both cMo (*vs* healthy donors) and Slan^{+} ncMo (*vs* healthy donors and MGUS) have been reported in blood of MM, in parallel to a higher BM tumor PC burden (181, 198). In addition, increased serum levels of pro-inflammatory cytokines such as IL1β, IL10, TNFα and IL6 (199), together with an enhanced spontaneous *ex vivo* secretion of inflammatory cytokines (*vs* HD) by blood Mo (182) have been reported in MM. Despite all the above findings, previous studies reported in the literature have restricted their analyses to the major populations of blood Mo and/or DC, and they did not take into account the increasing high phenotypic heterogeneity and diverse cellular composition of these compartments of Mo and DC.

Hypothesis and objectives

Mo and Mϕ, together with DCs, belong to the MPS composed by functionally related immune cells ("mononuclear phagocytes") that play a critical role as antigen-presenting cells in connecting the innate and adaptative immune responses. In recent years, accumulating evidences demonstrate that both Mo/Mϕ and DCs consist of several heterogeneous, functionally specialized, subsets of cells with diverse effector and immunomodulatory functions giving rise to a complex cellular network capable of integrating multiple environmental signals that lead to either immunity or tolerance. Thus, two major subsets of circulating human DCs have been identified in blood: the pDCs and the cDCs, also known as mDCs. Similarly, diverse subsets of Mo with different phenotypic profiles and functional roles have also been described in human blood which include cMo, iMo and ncMo.

Despite our knowledge about this complex DC and Mo/Mϕ cell system has greatly increased in recent years, the precise nature and the maturational links among the different populations of blood DC and Mo, their differential and functional roles, and the relationship with their tissue-counterparts are not yet fully understood. In addition, little is known about the distribution of the different Mo and DC subsets (and their precise phenotype) in blood throughout human life from newborns to the elderly. The establishment of age-associated normal/reference ranges for blood Mo and DC is essential to better identify when they are altered and define the nature of such alterations in specific immune, neoplastic or other disease conditions. Currently, there is still limited information available on the distribution of the different populations of Mo and DC in PB and BM of healthy individuals, but also in different disease conditions including those where these immune cells have been claimed to play a critical role, such as in PC neoplasms.

PC neoplasms include a group of several neoplastic disorders with variable levels of infiltration of BM, blood and/or other tissues by neoplastic PC, with a variable clinical behavior

and a heterogeneous outcome, ranging from MGUS to SMM and active MM. Of note, innate immune cells, such as Mo/Mϕ and DC, have been claimed to play an important role in clonal PC growth, plasticity and escape from the normal immunesurveillance mechanisms in patients with PC neoplasms. Precise knowledge about the specific alterations of the different populations of Mo in PB and the BM of patients diagnosed with MGUS, SMM and MM, and the potential relationship between such alterations and other immune-associated and bone-associated serum markers related to the tumor environment, might contribute to a better understanding of the clinical and biological differences (and heterogeneity) observed between (and among) patients with different diagnostic subtypes of PC neoplasms.

Based on all the above, the present doctoral thesis aimed at investigating more in depth the phenotype of blood DC and Mo and their kinetics in human blood *vs* BM, LN and spleen throughout life, in order to better identify their alterations and clinical correlates in patients with different diagnostic subtypes of PC neoplasms (i.e., MGUS, SMM and MM). In order to accomplish this general goal, we addressed three specific objectives:

1. To analyse the reactivity of different subpopulations of circulating human DCs and Mo in blood for a large panel of new monoclonal antibodies tested as part of the Tenth Human Leucocyte Differentiation Antigen Workshop, in the DC section (HLDA10-DC task);
2. To investigate the distribution of different populations of circulating Mo in CB and PB throughout human life, from newborns to elderly subjects, in order to define normal age- and sex-matched reference ranges and to understand the relationship between the cellular compartments of blood circulating Mo *vs* BM, LN and spleen Mo/ Mϕ.
3. To identify potential alterations in the distribution and phenotype of the different populations of Mo present in the BM and PB of patients with MGUS, SMM and MM

and their contribution, together with a broad panel of serum immune-related and bone-associated biomarkers, to the definition of altered cellular and soluble biomarkers profiles of immune-bone interactions, present in patients with different diagnostic subtypes of PC neoplasms.

Materials, methods and results

In this section, the healthy volunteers and patients included in this study, as well as the samples collected from them, together with the materials and methods used, and the results obtained, are described for each of the three specific objectives of this doctoral thesis and compiled in three chapters that correspond to the three manuscripts published as a result of the work performed:

The **first objective of the study**, focused on investigating in more depth the immunophenotypic profile of the major populations of blood circulating human DCs and Mo and the results derived from the work undertaken are presented in chapter 1:

Chapter 1: Expression profile of novel cell surface molecules on different subsets of human peripheral blood antigen-presenting cells.

Daniela Damasceno, Martín Pérez Andrés, Wouter BL van den Bossche, Juan Flores-Montero, Sandra de Bruin, Cristina Teodosio, Jacques JM van Dongen, Alberto Orfao and Julia Almeida.
Clinical & Translational Immunology (2016), 5, e100; doi:10.1038/cti.2016.54

The **second goal of this study** addressed the establishment of normal reference ranges for the major and minor subpopulations of circulating Mo in CB and PB throughout human life, and the comparison of the normal Mo phenotypes in blood *vs* BM, LN and spleen:

Chapter 2: Distribution of subsets of blood monocytic cells throughout life.

Daniela Damasceno, Cristina Teodosio, Wouter B.L. van den Bossche, Martín Perez-Andres, Sonia Arriba-Méndez, Luis Muñoz-Bellvis, Alfonso Romero, Juan F. Blanco, Ana Remesal, Noemi Puig, Sergio Matarraz, José Luis Vicente-Villardón, Jacques J.M. van Dongen, Julia Almeida, Alberto Orfao.
Journal of Allergy and Clinical Immunology (2019), 144, number 1, Pages 320-323.e6; https://doi.org/10.1016/j.jaci.2019.02.030

The **third objective of this doctoral thesis** aimed at investigating the distribution of the different populations of Mo in the BM and PB of patients with different diagnostic types of PC neoplasms, together with a broad panel of serum biomarkers, in order to identify altered profiles characteristic of MGUS, SMM and/or MM patients:

Chapter 3: Monocyte subsets and serum inflammatory and bone-associated markers in monoclonal gammopathy of undetermined significance and multiple myeloma.

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Cancers (2021), 13 (6); doi:10.3390/cancers13061454

Clinical & Translational Immunology (2016) 5, e100; doi:10.1038/cti.2016.54
Official journal of the Australasian Society for Immunology Inc.
www.nature.com/cti

ORIGINAL ARTICLE

Expression profile of novel cell surface molecules on different subsets of human peripheral blood antigen-presenting cells

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Although major steps have been recently made in understanding the role of the distinct subsets of dendritic cells (DC)/antigen-presenting cells (APC), further studies are required to unravel their precise role, including in-depth immunophenotypic characterisation of these cells. Here, we used eight-colour flow cytometry to investigate the reactivity of a panel of 72 monoclonal antibodies (including those clustered in seven new Cluster of Differentiation, CD) on different subsets of APC in peripheral blood (PB) samples from five healthy adults. These experiments were performed in the context of the Tenth International Workshop on Human Leukocyte Differentiation Antigens (HLDA10). Plasmacytoid DC was the only cell population that expressed CD85g and CD195, whereas they lacked all of the other molecules investigated. In contrast, myeloid DC mostly expressed inhibitory C-type lectin receptors (CLRs) and other inhibitory-associated molecules, whereas monocytes expressed both inhibitory and activating CLRs, together with other phagocytosis-associated receptors. Within monocytes, progressively lower levels of expression were generally observed from classical monocytes (cMo) to SLAN$^-$ and SLAN$^+$ non-classical monocytes (ncMo) for most of the molecules expressed, except for the CD368 endocytic receptor. This molecule was found to be positive only in cMo, and the CD369 and CD371 modulating/signalling receptors. In addition, the CD101 inhibitory molecule was found to be expressed at higher levels in SLAN$^+$ vs SLAN$^-$ ncMo. In summary, the pattern of expression of the different signalling molecules and receptors analysed in this work varies among the distinct subsets of PB APCs, with similar profiles for molecules within each functional group. These findings suggest unique pattern-recognition and signalling capabilities for distinct subpopulations of APCs, and therefore, diverse functional roles.
Clinical & Translational Immunology (2016) **5,** e100; doi:10.1038/cti.2016.54; published online 16 September 2016

'Mononuclear phagocytes' (that is, dendritic cells (DCs), monocytes and macrophages) are functionally related immune cells that have an essential role as antigen-presenting cells (APCs).[1] Among them, DCs are widely recognised as the most potent APCs capable of efficiently activating naive T cells. In turn, the main role of macrophages is to remove apoptotic/damaged cells and foreign components, such as microorganisms and their pathogenic factors, to ensure tissue integrity.[1] Despite these general roles, it is well known that both DCs and monocytes/macrophages are heterogeneous cell populations.[2] In fact, both consist of different functionally specialised subsets of cells with diverse effector and immunomodulatory functions that give rise to a complex cellular network capable of integrating multiple environmental signals leading to either immunity or tolerance. Regarding this, two clearly different subsets of human circulating DCs have been identified: plasmacytoid DCs (pDCs), a unique subset of DC that secrete large amounts of type I interferon in response to viruses,[3] and myeloid DCs (mDCs). mDCs can be further subdivided into a major subset of CD1c$^+$(BDCA1$^+$) mDCs, which are highly effective at the uptake of antigens, migration and antigen presentation to CD4$^+$ T cells, and a minor subset of CD141$^+$ (BDCA3$^+$) mDCs that are specialised in antigen cross-presentation.[1] Similarly, circulating peripheral blood (PB) human monocytes are also heterogeneous, consisting of three distinct populations based on their differential expression of the CD14 lipopolysaccharide co-receptor and the CD16 Fcγ receptor. These populations include: (a) CD14^{++}/CD16$^-$ or classical monocytes (cMo) that represent 90–95% of all PB monocytes in healthy subjects; (b) CD14$^{+/lo}$/CD16^{lo} or intermediate monocytes (iMo); and (c) CD14$^{lo/-}$/CD16$^+$non-classical monocytes (ncMo). The latter two subsets represent ~5–10% of all circulating monocytes in healthy adults.[4,5]

All PB subsets of DCs and monocytes are functionally related cells, which share antigen-presentation functions, among other roles.

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Received 28 January 2016; revised 1 August 2016; accepted 2 August 2016

Despite all of the above, the precise role of each subset and the exact relationship among them still remains to be fully understood. For example, the number of ncMo has consistently been found to be increased in response to microbial and/or inflammatory stimuli, and under these conditions they display phenotypic and functional features intermediate between those of cMo and DCs.[6] Thus, further studies are still needed, including a more detailed analysis of the phenotypic profiles of PB APCs, to better understand their precise roles and functional interactions.

Since the first Human Leukocyte Differentiation Antigen (HLDA) Workshop and Conference that took place in 1982, efforts have been made to identify and characterise a large number of molecules present in hematopoietic cells. Indeed, the increase of knowledge regarding the phenotypic profiles of different subsets of Leukocytes has permitted a more rational understanding of their functions. Here, we analyse the reactivity of different subpopulations of human circulating PB DCs and monocytes for a large panel of new monoclonal antibodies. This work forms part of the Tenth Human Leukocyte Differentiation

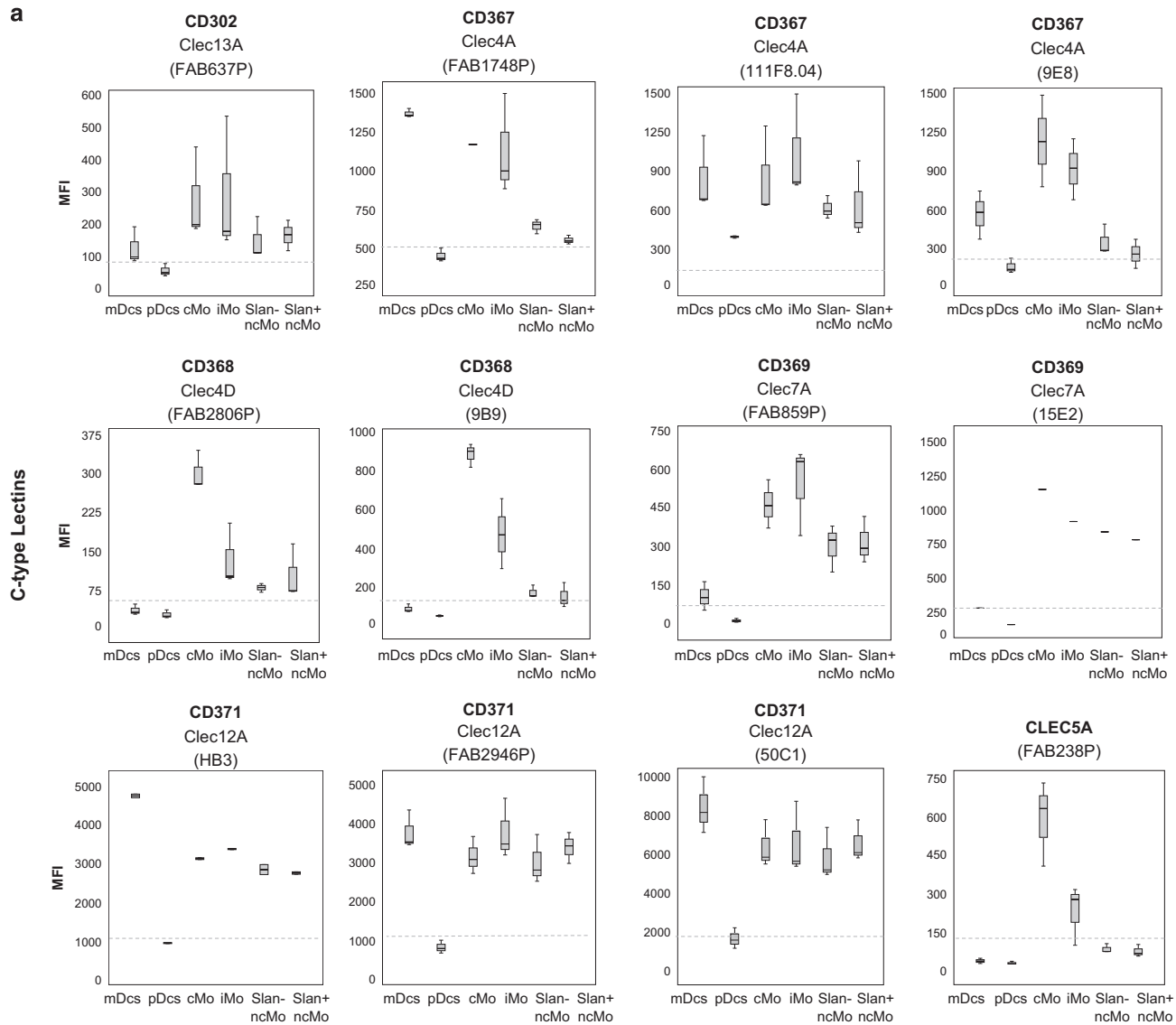

Figure 1 Immunophenotypical characterisation of the different subsets of circulating DCs and monocytes in normal PB. Panel **a** shows the expression of C-type lectins, and Panel **b** shows the expression of T-cell inhibitory/stimulatory molecules, DC-related markers, phagocytic-related molecules, other myeloid markers, chemokine/cytokine receptors and other cell surface molecules, in the different APC subpopulations of mDCs, pDCs, cMo, iMo, SLAN$^-$ ncMo and SLAN$^+$ ncMo present in normal adult PB in basal conditions. Within each group of molecules, only those markers found to be positive in at least one cell subset in most samples stained are represented. They are classified according to the 'CD' code and/or their alternative name, as well as the different monoclonal antibodies used (in brackets). Results are expressed as MFI (mean fluorescence intensity) for each whole cell population (arbitrary relative mean units, scaled from 0 to 262 144). Notched boxes represent 25th and 75th percentile values; the line in the middle and vertical lines correspond to the median value and the minimum-maximum values (without the extreme values and outliers), respectively. The dotted lines correspond to the cutoff value for positivity in each plot. The graph for CD369 (clone 15E2) includes data from one single case. Data represented in graphs for CD371 (clone HB3), CD366 (clone F38–2E2) and TREM2 correspond to two cases, not to five cases, owing to an insufficient amount of the corresponding antibody available. Abbreviations: mDCs: myeloid dendritic cells; pDCs: plasmacytoid dendritic cells; cMo: classical monocytes; iMo: intermediate monocytes; ncMo: non-classical monocytes; MFI: mean fluorescence intensity (arbitrary mean units scaled from 0 to 262 144).

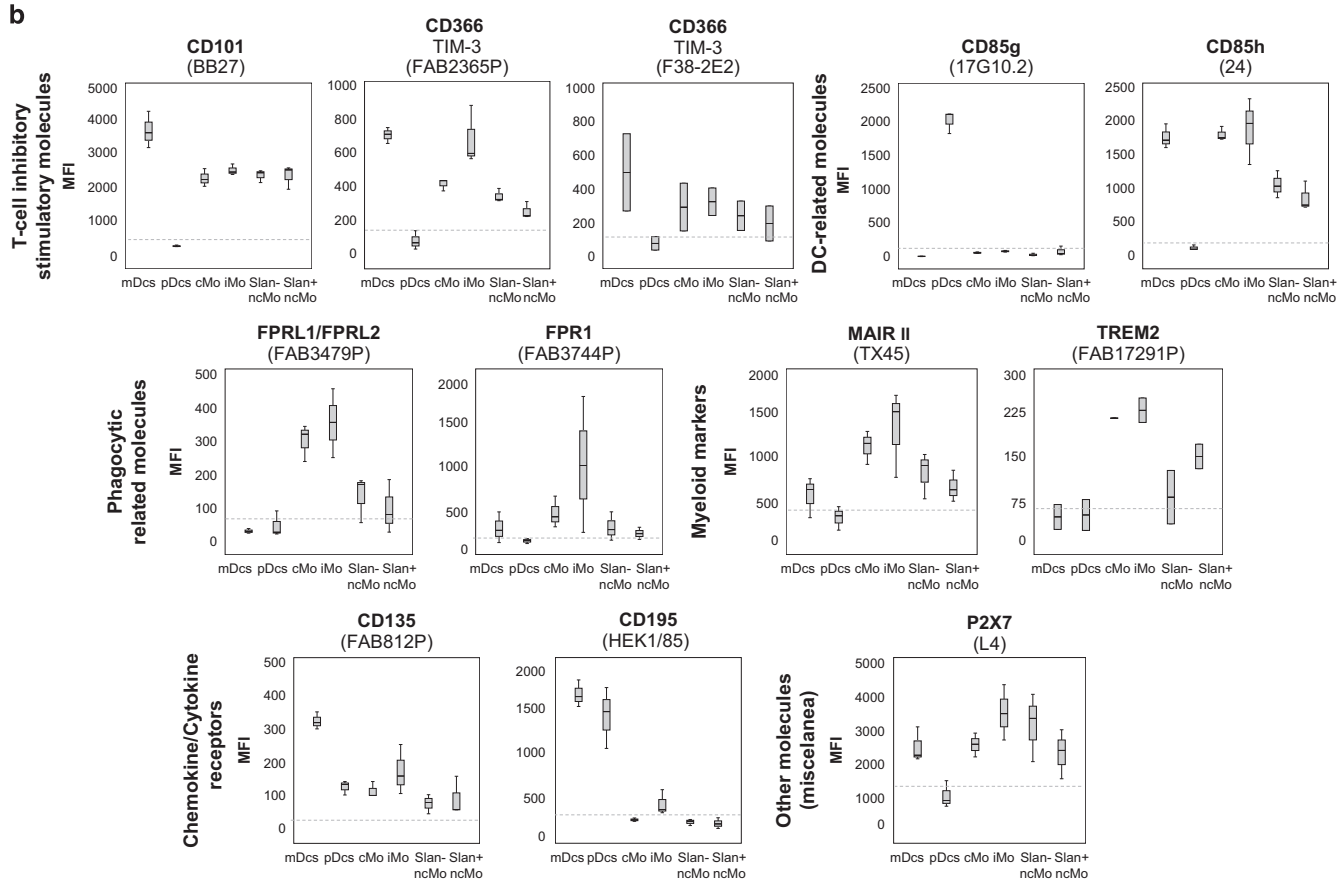

Figure 1 Continued.

Antigen Workshop—DC *section (HLDA10-DC* task), which aims to contribute to a better understanding on their precise functional roles.

RESULTS

Expression of C-type lectin receptors (CLRs) on different subsets of normal human PB APCs

Overall, CLRs showed variable patterns of expression on the different subsets of PB APC investigated. However, this was not the case for pDCs, in which none of the CLRs analysed were found to be clearly positive on the cell surface membrane. Actually, for pDCs, only low levels of expression of CD367 (CLEC4A/DCIR) and CD371 (CLEC12A/MICL/CCL-1) were observed on the PB by using the 111F8.04 and 50C1 monoclonal antibodies (mAb), respectively (Figure 1a). Interestingly, all mDCs from the five samples analysed were found to be CD367^{+} and CD371^{++}, the two molecules known to have inhibitory roles in the immune response.[7] In addition, mDCs partially expressed the CD302 (CLEC13A/DCL-1) endocytic receptor (65% of CD302^{+} mDCs from one out of the three samples analysed) at low levels, corresponding mostly to activating CLRs (Figure 1a and Table 1), where the rest of the CLRs studied lacked reactivity (Figure 1a and Table 1). Conversely, monocytes expressed the majority of the CLRs analysed, including CD302, and both inhibitory (for example, CD367 and CD371) and activating (for example, CD368, CD369 and CLEC5A) CLRs, although they were negative for CD370, CLEC2D, CLEC8A and CLEC14A (Figure 1a and Table 1). Interestingly, the different subsets of PB monocytes showed distinct patterns of expression for the CLRs: (a) some molecules (CD302, CD367 and CD369) were expressed on all subsets of monocytes, but with progressively lower levels from cMo to SLAN^{-} and SLAN^{+} ncMo; (b) other CLRs (for example, CD371) were expressed at high and similar levels in all subsets of monocytes; (c) CD368 and CLEC5A expression was restricted to some monocyte subsets, being clearly positive on cMo, while weakly expressed on iMo and negative in both SLAN^{+} and SLAN^{-} ncMo.

Expression of other phagocytosis-associated proteins, myeloid molecules and DC-related markers on different subsets of normal human PB APCs

The expression of the phagocytosis-associated proteins and myeloid molecules studied, other than the CLRs, was also restricted to monocytes and, to a lesser extent, to mDCs, whereas pDCs did not express any of these. Accordingly, mDCs were found to dimly express FPR1 and MAIR II and monocytes expressed FPRL1/FPRL2 and TREM2 (in addition to FPR1 and MAIR II), with progressively lower levels of expression from cMo/iMo to SLAN^{-} and SLAN^{+} ncMo for all the above molecules, except TREM2 (Figure 1b and Table 1).

The CD85h immunoglobulin-like transcript-activating factor was expressed at high levels on mDCs, cMo and iMo, whereas the intensity of expression was markedly lower on ncMo, particularly in the SLAN^{+} subset, and negative on pDCs (Figure 1b). In contrast, CD85g, a molecule known to mediate signals that negatively modulate IFNα production, was selectively expressed by pDCs (Figure 1b).

The other molecules from these groups of markers were systematically absent on all of the APC subsets analysed (Table 1).

Table 1 Biological and technical features of the *HLDA10-DC* monoclonal antibody reagents tested on normal human PB DC and monocyte subsets

Specificity	*Target molecule*	*Clone*	*Antibody (workshop codes)*	*Reactivity*
C-type lectins	CD302	FAB637P	10–54	mDC^{d+}, cMo^{+}, $iMo^{-/+}$, $ncMo^{d+}$
	CD367	FAB1748P	10–13	mDC^{+++}, cMo^{++}, iMo^{+}, $ncMo^{d+}$
	CD367	111F8.04	10–71	mDC^{++}, pDC^{d+}, cMo^{++}, iMo^{++}, $ncMo^{+}$
	CD367	9E8	10–72	mDC^{+}, cMo^{++}, iMo^{++}, $ncMo^{d+}$
	CD368	FAB2806P	10–21	cMo^{++}
	CD368	9B9	10–78	cMo^{++}, iMo^{+}
	CD369	GE2	10–01	–
	CD369	FAB1859P	10–35	cMo^{+}, iMo^{+}, $ncMo^{d+}$
	CD369	15 E 2	10–79	cMo^{++}, iMo^{++}, $ncMo^{+}$
	CD370	8F9	10–02	–
	CD370	9A11	10–09	–
	CD370	FAB6049P	10–45	–
	CD370	8F9	10–65	iMo^{+}
	CD371	HB3	10–17	mDC^{+++}, cMo^{++}, iMo^{++}, $ncMo^{+}$
	CD371	FAB2946P	10–51	mDC^{++}, cMo^{+}, iMo^{++}, $ncMo^{+}$
	CD371	50C1	10–73	mDC^{+++}, pDC^{d+}, cMo^{++}, iMo^{++}, $ncMo^{++}$
	Clec2D	FAB3480P	10–06	–
	Clec5A	FAB238P	10–28	cMo^{+}
	Clec5C	FAB1900P	10–31	–
	Clec8A	FAB1798P	10–40	–
	Clec14A	FAB7436P	10–57	–
T-cell co stimulatory inhibitory molecules	CD101	BB27	10–34	mDC^{+++}, cMo^{++}, iMo^{++}, $ncMo^{++}$
	CD245	DY12	10–43	–
	CD245	DY35	10–48	–
	CD273	ANC8D12	10–61	–
	CD365	FAB1750P	10–14	–
	CD365	1D12	10–67	–
	CD366	FAB2365P	10–24	mDC^{++}, cMo^{+}, iMo^{++}, $ncMo^{d+}$
	CD366	F38–2E2	10–75	$mDC^{-/+}$, cMo^{d+}, iMo^{d+}, $ncMo^{d+}$
	ULBP-3	FAB1517P	10–52	mDC^{+}, iMo^{+}
	B7-H4	MIH43	10–64	–
	TSLP-R	1B4	10–68	–
	TIM4	9F4	10–81	–
DC-related markers	CD1a	010e	10–03	–
	CD1a	0619	10–10	–
	CD1b	0249	10–18	–
	CD1c	L161	10–26	–
	CD85g	17G10.2	10–66	pDC^{+++}
	CD85h	24	10–74	mDC^{++}, cMo^{++}, iMo^{++}, $ncMo^{+}$
	CD209	118A8.05	10–83	–
Phagocytic-related molecules	FPRL1/FPRL2	FAB3479P	10–36	cMo^{+}, iMo^{++}
	FPR1	FAB3744P	10–47	mDC^{d+}, cMo^{d+}, $iMo^{-/+}$, $SLAN^{-}ncMo^{d+}$
	Calreticulin	FMU-CRT-2	10–23	–
	Calreticulin	FMU-CRT-8	10–29	–
	Calreticulin	FMU-CRT-17	10–42	–
Myeloid markers	MAIR II	TX45	10–80	mDC^{d+}, cMo^{++}, iMo^{++}, $ncMo^{d+}$
	TREM2	FAB17291P	10–07	cMo^{++}, iMo^{++}, $SLAN^{+}ncMo^{d+}$
	FDF03	36H2	10–84	-
Chemokine and cytokine receptors	CD135	FAB812P	10–15	mDC^{++}
	CD195	HEK/1/85	10–76	mDC^{+++}, pDC^{++}, iMo^{d+}
	CD213a2/ IL13Ra2	FMU-IL-13RA2–7	10–30	–
	CD213a2/ IL13Ra2	FMU-IL-13RA2–8	10–37	–
	CD213a2/ IL13Ra2	FMU-IL-13RA2–14	10–41	–
Other molecules (miscelanea)	Tie-2	FAB3131P	10–56	–
	P2X7	L4	10–70	mDC^{+}, cMo^{+}, iMo^{++}, $SLAN^{-}$ $ncMo^{++}$, $SLAN^{+}$ $ncMo^{+}$
	LPAP	CL3	10–04	–
	LPAP	CL4	10–11	–
	LPAP	CL7	10–19	–
	FAT1 cadherin	FMU-FAT-6	10–08	–

Table 1 (Continued)

Specificity	*Target molecule*	*Clone*	*Antibody (workshop codes)*	*Reactivity*
Other molecules (miscelanea)	FAT1 cadherin	FMU-FAT1–7	10–16	–
	unknown	BGA69	10–38	–
	Axl	FAB154P	10–50	–
	IL-1RAcP	AY19	10–53	–
	Vimentin	SC5	10–55	–
	unknown	MDR64	10–59	–
	unknown	CMRF-44	10–82	–
	GARP	ANC8C9	10–62	–
	GARP	ANC10G10	10–63	–
	unknown	CMRF-56	10–69	–
	DORA	104A10.01	10–77	–
	Tetanus toxoid	CMRF-81	10–85	–

Abbreviations: cMo, classical monocytes; iMo, intermediate monocytes; mDCs, myeloid dendritic cells; ncMo, non-classical monocytes; pDCs, plasmacytoid dendritic cells; .
Reactivity for each cell population is displayed as superscripts; symbols used for the assessment of the expression are based on median values of fluorescence intensity:–(negative); –/+ (variable reactivity, from negative to positive); d+ (dim positive);+(positive); ++ (strong positive); +++ (very strong positive). Peripheral blood cell subsets other than APCs were used as internal controls, to establish the cutoff for positivity per marker/monoclonal antibody reactivity.

Expression of T-cell inhibitory/stimulatory molecules on different subsets of normal human PB APCs

Only a minor number of the T-cell-related molecules investigated were expressed by both mDCs and monocytes. These molecules were absent on pDCs: CD101, CD366 (TIM-3/Hepatitis A virus cellular receptor 2) and, to a lesser extent, ULBP-3 (Table 1). The two former molecules (CD101 and CD366) are known to exhibit modulatory/co-regulatory functions related to tolerance induction, and were found to be expressed at higher levels on mDCs vs monocytes. Within the monocyte population, expression of CD101 was similar among cMo, iMo and ncMo, whereas CD366 expression progressively decreased from cMo/iMo to SLAN^{-} and SLAN^{+} ncMo (Figure 1b and Table 1).

Expression of chemokine and cytokine receptors on PB subsets of normal human APCs

CD135 (the receptor for Flt3L) and CD195 (CCR5) were both expressed by mDC, and the latter marker was also present on pDCs. In contrast, the different populations of monocytes were negative or weakly positive for CD135 and CD195 (Figure 1b). Likewise, none of the APC subsets analysed expressed the interleukin 13 receptor alpha2 (CD213a2) (Table 1).

Other molecules

None of the molecules included within the group 'miscellanea' were expressed by the APC subsets studied (Table 1), with the exception of P2X7 (Figure 1b). Accordingly, this latter molecule was expressed at high levels in mDCs and at lower levels in the different monocyte subsets, but was absent in pDCs.

DISCUSSION

Here we report on the result of an immunophenotypic analysis of a large panel of mAb directed against DC-related molecules, submitted to the *HLDA10-DC* workshop. Notably, the pattern of reactivity of the antibodies received by our lab was blindly tested on normal human PB subsets of DCs and monocytes, using non-cultured whole blood specimens from five healthy adults. Overall, our results show strikingly different patterns of expression of the different molecules analysed on the different subsets of PB APC. Common expression profiles were detected, although this depended on the functional group of the molecules studied and the specific subset of APC analysed.

CLRs comprise > 1000 receptors, which are typically found on phagocytes. These receptors are essential for antigen capturing,[8,9] and the subsequent activation of intracellular signalling cascades, triggering numerous cellular and immunological responses for maintaining the homoeostasis of the immune system and controlling immune responses during infection. In addition, CLRs also have an important role in diseases and conditions such as autoimmunity, allergy and cancer.[10] CLRs are associated with carbohydrate binding (that is, mannose, *N*-acetylglucosamine, L-fucose, glucose, galactose and *N*-acetyl-galactosamine, among other pathogen-related carbohydrates) and act as highly effective pattern-recognition receptors. This leads to the activation and/or modulation of immune functions upon encountering ligands from 'non-self' (pathogen-associated molecular patterns), 'damaged self' (damage-associated molecular patterns) or 'altered self' (tumour-associated molecular patterns).[11] CLRs are usually classified into two groups, based on their dominant signalling potential (activating receptors and inhibitory receptors), depending on whether they mediate cell activation or suppression of cellular activation, respectively.[11] pDC did not express any of the CLRs studied, except for partial and low expression of the CD367 and CD371 inhibitory molecules. In contrast, mDCs expressed CD367 and high levels of CD371, together with partial and low expression of CD302. Interestingly, CD367 (CLEC4A/DCIR) and CD371 (CLC12A/MICL/CCL-1) contain ITIMs (immunoreceptor tyrosine-based inhibitory motifs) in their cytoplasmic tails and both act as inhibitory receptors, havig an important role in the interaction between innate and adaptive immunity during tolerogenic immune responses.[11,12] In addition, CD367 also drives antigen cross-presentation in DCs[13,14] and CD371 has been recently shown to sense dead cells, and therefore, regulates inflammation in response to cell death.[12] These two inhibitory markers were also expressed by the different subsets of normal PB monocytes, but with a distinct immunophenotypic profile: mDCs and both cMo and iMo showed relatively high reactivity for CD367 expression, whereas ncMo expressed this marker at markedly lower levels (particularly on the subset of SLAN^{+} ncMo). Interestingly, SLAN^{+} ncMo have been found to induce potent proinflammatory Th1 and probably Th17 immune responses,[15] which could be consistent with the low expression of the CD367 tolerogenic marker. Alternatively, the lower expression of CLEC4A/DCIR observed on ncMo may reflect a more mature phenotype for this cell subset as CD367 expression has been shown to decrease on DCs by signals that

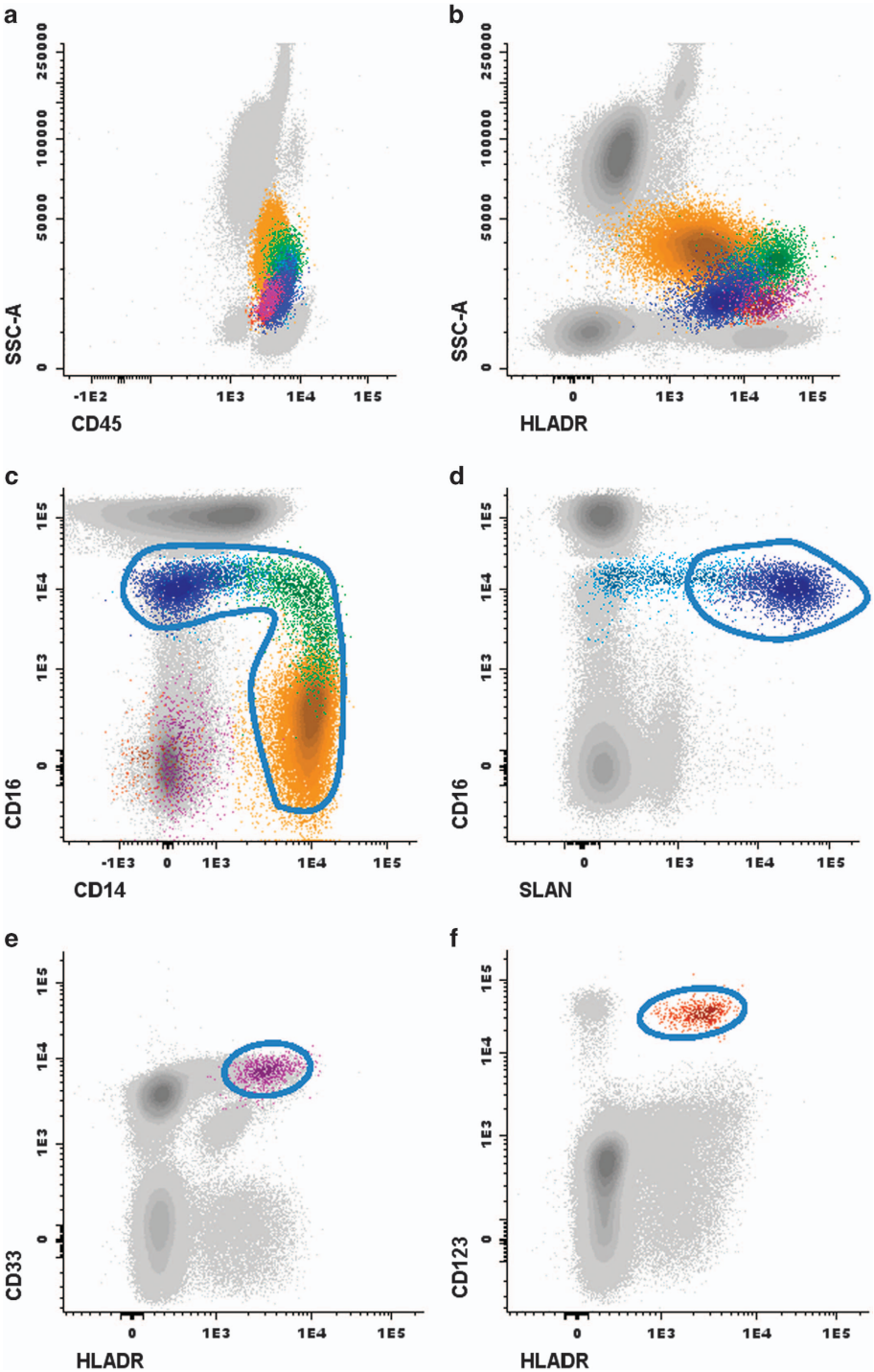

Figure 2 Gating strategy for the identification of the different subsets of circulating monocytes and dendritic cells (DCs) in a representative peripheral blood (PB) sample from a healthy donor. Coloured events in Panels **a** and **b** correspond to all the antigen-presenting cell (APC) subsets under study (that is, total PB monocytes and DCs), firstly gated from leukocytes (grey dots) according to their typical expression pattern of sideward scatter (SSC^{int}) together with CD45 (Panel **a**) and HLADR (Panel **b**). Next, monocytes were identified within total APCs, based on their expression of $CD33^{+/++}$ (not shown), together with their expression profile for CD14 and CD16 (coloured dots included in the gate in Panel **c**). Their differential expression of CD14 and CD16 further allowed the identification of three major subpopulations of monocytes (Panel **c**): $CD14^{++}/CD16^{-}$ classical monocytes (cMo), $CD14^{+/lo}/CD16^{lo}$ intermediate monocytes (iMo) and $CD14^{lo/-}/CD16^{+}$non-classical monocytes (ncMo). Within this latter monocytic subset, both $SLAN^{-}$ and $SLAN^{+}$ ncMo were identified, as shown in Panel **d**: the region including $SLAN^{+}$ ncMo (blue dots included in the gate) was set based on internal negative controls for SLAN (grey dots in Panel **d**). Myeloid DCs (mDCs, Panel **e**) were identified based on their high expression of CD33 and HLADR in the absence of CD14 and CD16, to exclude monocytes, while plasmacytoid DCs (pDCs, Panel **f**) were identified by the strong CD123 expression and positivity for HLADR, also in the absence of CD14 and CD16. Colour codes: yellow dots correspond to cMo, iMo are painted in green, whereas blue dots correspond to ncMo; dark purple and orange dots represent mDC and pDC, respectively.

have been induced to mature (for example, CD40 ligand, lipopolysaccharide and tumor necrosis factor α). In turn, CD371 was highly expressed by mDCs and, to a lesser extent, by all monocyte subsets, potentially reflecting a shared role for both cMo and ncMo as sensors of necrotic/dead cells and/or as regulators of inflammatory responses.

In contrast to the inhibitory CLRs, expression of activating CLRs such as CD368, CD369, involved in cellular activation through the Syk kinase,[16] and CLEC5A (a receptor associated with proinflammatory effects[17]) was restricted to monocytes, although higher levels of expression for the three molecules were observed on cMo and iMo vs ncMo. This suggests that these activating receptors might be associated with a specific monocyte-dependent immunological response. It is now known that CD368 (CLEC4D/Dectin-3) acts as a receptor for mycobacteria,[18] CD369 (CLEC7A/Dectin-1) functions as a receptor specific for beta glucans from fungal cell walls[19] and CLEC5A is a Dengue virus receptor,[20] although little is known about the ligands for this receptor. However, independently of their pathogen partner, these three receptors are Syk-coupled CLRs and, when they are stimulated, all lead to hem-ITAM-mediated signalling cascades inside the cell, triggering phagocytosis, production of reactive oxygen species, and proinflammatory chemokines/cytokines.[19,21–23] The fact that ncMo had lower expression levels of activating Syk-coupled CLRs versus both cMo and iMo, could suggest that the former monocyte subset has less-phagocytic activity, together with a lower capacity for producing both reactive oxygen species and proinflammatory chemokines/cytokine. The results obtained regarding the pattern of expression of the CLEC13A/CD302 endocytic receptor, involved in phagocytosis, would further support this hypothesis.[24]

Expression of other phagocytic-related molecules (FPR1 and FPRL1/FPRL2), myeloid molecules (MAIR II and TREM2) and the CD85h DC-related marker here analysed, were restricted to monocytes and, to a lesser extent, also to mDCs. Although absent on pDCs, ncMo systematically had lower levels of expression for these proteins as compared to both cMo and iMo. Therefore, ncMo seem to represent a final stage of maturation when antigen presentation or phagocytosis events no longer take place, at least via CLRs, Ig-like V-type receptors (that is, TREM2) or G protein-coupled receptors (that is, FPR1). Overall these results could indicate that these cells may be more prone to undergo apoptosis and die. As expected, CD85g was selectively expressed by pDCs, as it mediates signals that negatively modulate IFNα production, CD85g expression potentially representing a homoeostatic regulatory mechanism on immature circulating PB pDC.[25]

The T-cell immunoglobulin mucin receptors (TIM) investigated in this study (TIM-1, initially cloned as hepatitis A virus cellular receptor 1, and TIM-3 or hepatitis A cellular receptor 2) were allocated as belonging to the CD365 and CD366 new CD codes. Both molecules are expressed on activated/effector T cells.[26,27] However, although the former molecule is able to associate with the TCR complex and mediate T-cell activation signals,[28] the latter has been shown to inhibit Th1-mediated autoimmune and alloimmune responses and to promote immunological tolerance.[26] In addition to their classical roles, TIM receptors have been recently suggested to mediate phagocytosis of apoptotic cells by APCs.[29] In addition, none of the populations of APC analysed were found to express CD365, whereas both mDCs and monocytes (but not pDCs) expressed CD366, together with two other T-cell inhibitory molecules tested (CD101 and ULBP-3). The expression on mDCs was significantly higher than on the monocytes. Therefore, as for the above referred molecules, mDCs seemed to display a more pronounced inhibitory receptor molecule-associated profile than the monocytes.

As expected, the CD135 chemokine/cytokine receptor was highly expressed on mDCs, where it has a central role in cell proliferation and differentiation.[30] In turn, CD195 was expressed both on mDCs and pDCs. Monocytes showed a lower expression for both markers, suggesting they both might play a preferential role on mDCs and pDCs immune-dependent responses. Finally, P2X7, a purinergic receptor for extracellular ATP that triggers downstream events such as activation of membrane metalloproteases and intracellular caspase activation leading to apoptosis,[31] was highly expressed on iMo and SLAN- ncMo, further supporting the notion that ncMo could be an end-stage maturation subset of monocytes that could easily be triggered to undergo apoptosis. Interestingly, we observed slight differences within ncMo, according to the expression of SLAN (O-linked sugar modification of P-selectin glycoprotein ligand-1). Previous studies showed that antigens properly processed and presented by $SLAN^+$ APCs were successfully taken up by $CD4^+$ T cells.[32] Moreover, $SLAN^-$ and $SLAN^+$ ncMo have recently been studied as separate cell subpopulations based on their different gene expression profiles and immunophenotypes.[33] $SLAN^+$ cells were found to show a higher level of expression of the ubiquitin C transcript, which is associated with cell signalling, transcription, apoptosis, among other cellular functions. In addition, the immunophenotype of $SLAN^+$ ncMo was characterised by a higher expression of tumor necrosis factor-α, CX3CR1, and lower CCR2, contrasting with the $SLAN^-$ ncMo expression profile for these molecules.[33] These differences would suggest that $SLAN^+$ and $SLAN^-$ ncMo would have different functional roles, also consistent with the phenotypic differences observed in our study.

In summary, we show that the patterns of expression of the different signalling molecules and receptors evaluated (and recognised by the APC-related mAb submitted to the *HLDA10-DC* workshop) vary substantially among the different subsets of DC and monocytes analysed. Such differences potentially reflect distinct pattern-recognition and signalling as well as different antigen uptake and phagocytic activities for the different subsets of APCs evaluated. Further investigations regarding the functional correlation of the immunophenotype reported here are required to better understand the precise and unique roles of the different subsets of PB.

METHODS

Subjects and samples

A total of five healthy adult volunteers (three males and two females, with a median age of 42 years, ranging from 25 to 78 years) were included in this study. PB samples from the donors were collected into tubes containing K3-EDTA. The study was approved by the Ethics Committee of the University Hospital of Salamanca and performed following the Declaration of Helsinki. Each participant gave his/her informed consent prior to entering the study.

Flow cytometry immunophenotypic studies

A total of 72 new mAb were submitted to our laboratory (Cancer Research Center –CIC/IBMCC– and Service of Cytometry, Nucleus Platform for Research Support, University of Salamanca, Salamanca, Spain), being one of the contributors to the HLDA10-DC. These included mAb clustered in seven new CD that recognise cell surface molecules expressed by PB APC subsets. The remaining mAb tested, either recognised already established CDs or they required further validation. Details on the specificity, target molecules, clones, formats and workshop codes of the HLDA10-DC mAb tested in our laboratory are shown in Table 1.

Immunophenotypic studies were performed on fresh, whole PB samples, using eight-colour flow cytometry; antigen expression was analysed at the cell

surface membrane level by staining $\sim 5 \times 10^5$ cells in 100 µl per test. Seven common markers (backbone) were constantly present in all sample aliquots for identifying the different subsets of PB APC for which reactivity; for each mAb being studied, was tested. These included: anti-HLADR-Pacific Blue (PacB)-(Clone L243); CD45-Pacific Orange (PacO)-(Clone HI30); CD33-peridinin chlorophyll protein-cyanin 5.5 (PerCPCy5.5)-(Clone P67.6); CD123-allophycocyanin (APCy)-(Clone AC145); CD16-phycoerythrin-cyanine 7 (PECy7)-(Clone 3G8); CD14-APC-Hilite 7 (APCyH7)-(Clone MφP9) and anti-SLAN-(Clone DD1), conjugated with either phycoerythrin (PE) or fluorescein isothiocyanate, depending on the conjugate format of the mAb reagent available for testing. Accordingly, anti-SLAN-fluorescein isothiocyanate was used for testing PE-conjugated primary mAb, whereas anti-SLAN-PE was used with primary mAb conjugated with Alexa Fluor 488, as well as with unconjugated antibodies (biotinylated, purified or unpurified reagents). The secondary antibody used against the primary mAb was fluorescein isothiocyanate-conjugated (see below). Backbone reagents were all purchased from Becton Dickinson Biosciences (BD; San Jose, CA, USA), except for the anti-HLADR-PacB (Biolegend, San Diego, CA, USA), CD45-PacO (Invitrogen, Carlsbad, CA, USA) and anti-SLAN reagents (Milteny Biotech, Cologne, Germany).

In all samples, a direct immunofluorescence stain-lyse-and-then-wash procedure was used for the 8-colour combination tubes including PE or Alexa Fluor 488-conjugated primary mAb, following well-established techniques, which have been described elsewhere.[34] In the case of the eight-colour mAb combinations with unconjugated primary mAb, the following technique was strictly followed in order to avoid unspecific staining: first, indirect labelling was performed (using a fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig $F(ab)_2$ antibody fragments; Dako, Glostrup, Denmark), followed by two washing steps in phosphate buffered saline (PBS; pH = 7.6) to remove the residual soluble secondary antibody; then, labelling with the directly conjugated mAb was carried out.[35] In two additional normal PB samples, a cocktail of PECy7-conjugated anti-CD3 (Clone SK7; BD), *plus* anti-CD19 (Clone J3-119; Beckman Coulter, Brea, CA, USA), *plus* anti-CD56 (Clone N901 (NKH-1); Beckman Coulte) monoclonal antibodies, was added together with the backbone markers, to confirm that the strategy of identification of the different APC subsets in the remaining tubes was robust enough to unequivocally identify DC subsets without the presence of T-, B- and NK-cell-related markers (Supplementary Figure 1).

Immediately after the completion of the sample preparation, the samples were acquired in a FACSCanto II flow cytometer (BD), using the FACSDiva software programme (BD). For data analysis, the INFINICYT software programme (Cytognos SL, Salamanca, Spain) was used. The different DC and monocyte cell subsets, for which reactivity against the *HLDA10-DC* mAb was assessed, were identified following the gating strategy illustrated in Figure 2. The minimum number of clustered events considered to constitute an APC cell population to be accurately characterised was 50.[36] In addition, T cells were also identified and used as internal controls (data not shown).

Statistical methods

For each parameter under study, median, range and the 25th and 75th percentiles were calculated using the SPSS.21 software programme (IBM SPSS Statistics, IBM, Armonk, NY, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work has been partially supported by the following grants: RTICC RD12/0036/0048-FEDER and PI13/01412-FEDERfrom the *Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Ministerio de Economía y Competitividad*, Madrid, Spain.

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The Supplementary Information that accompanies this paper is available on the Clinical and Translational Immunology website (http://www.nature.com/cti)

increased proliferative capacity of cells expressing the exon 2 mutation. Our finding of bimodal FOXP3 expression in the carrier differs from the only other published report of a carrier with an exon 2 mutation (c.227delT) who expressed only one population of $CD4^+CD25^+CD127^{lo}$ cells, all expressing WT FOXP3.[7] Our results are the first to confirm the natural ability of isoforms lacking exon 2 to promote their own transcription, resulting in expression of a functional isoform of FOXP3 that can support Treg cell development.

In conclusion, we report a family with autoimmunity across 3 generations, including 2 affected male subjects. We have shown that a milder IPEX phenotype was due to selective deletion of FOXP3 exon 2 expression, resulting in loss of FOXP3fl but retained expression of FOXP3Δ2. This report confirms that FOXP3Δ2 can support Treg cell development *in vivo* and mitigate at least some of the clinical features of complete FOXP3 deficiency, as observed in patients with classic IPEX syndrome. These findings provide powerful patient-derived evidence for the functional capabilities of the FOXP3Δ2 isoform. The variable penetrance is important because it suggests that future patients might be identified in populations with milder autoimmunity not reaching the criteria for IPEX syndrome.

We thank the Clinical Trials & Biorepository Team, St Vincent's Centre for Applied Medical Research, for storage and handling of PBMCs for *ex vivo* analysis; the Clinical Immunogenetics Research Consortium Australia (CIRCA); the Department of Clinical Genetics, Vejle Hospital, Lillebaelt Hospital, Denmark; and the Immunology Laboratory, Children's Hospital at Westmead, Australia.

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Supported by the Swedish Cancer Society and Swedish Childhood Cancer Fund and the Jeffrey Modell Foundation through Sydney Children's Hospital. C. S. Ma is supported by an Early-Mid Career Research Fellowship from the Department of Health of the New South Wales Government. S. G. Tangye is supported by grants (1113904) and a Principal Research Fellowship (1042925) awarded by the National Health and Medical Research Council of Australia.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

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Available online March 21, 2019.
https://doi.org/10.1016/j.jaci.2019.03.003

Distribution of subsets of blood monocytic cells throughout life

To the Editor:

Currently, it is well established that monocytes are a heterogeneous type of cell consisting of phenotypically and functionally distinct subpopulations found to be numerically altered in blood in patients with a wide variety of disease conditions, such as infection, autoimmunity, respiratory and cardiovascular diseases, and inflammatory disorders.[1,2] Thus, 3 subpopulations of circulating monocytic cells have been identified based on expression of the CD14 LPS receptor and the CD16 low-affinity Fc IgG receptor: (1) $CD14^{hi}CD16^-$ classical monocytes (cMos), (2) $CD14^{hi}CD16^+$ intermediate monocytes (iMos), and (3) $CD14^{-/lo}CD16^+$ nonclassical monocytes (ncMos).[3] Monocytes circulate in blood for up to 3 days until recruited to virtually any human tissue, where they differentiate into either tissue macrophages or myeloid dendritic cells.[4] Then, tissue macrophages can migrate from their tissue location through the lymph system[5] before they potentially die outside the circulation.[6]

Despite our knowledge of the biology of monocytes increasing in recent years, normal reference ranges for the distinct monocyte subsets in blood throughout life (eg, from cord blood [CB] and newborns to elderly subjects) have never been systematically defined. Moreover, the precise maturational and functional relationship between the distinct populations of blood monocytes and their tissue distribution profiles remains unknown.

To provide a frame of reference for future identification of disease-associated altered profiles, we investigated the distribution of distinct monocytic cell subsets in normal blood versus secondary lymphoid tissues, such as bone marrow (BM), lymph node, and spleen. Our aim was to shed light on changes in the distribution of these monocytic cell subsets in blood and other lymphoid tissues. For this purpose, a total of 188 EDTA-anticoagulated blood samples were studied: 11 CB specimens from full-term neonates and 177 blood samples from 164 healthy subjects and 13 solid organ donors (102 male and 75 female

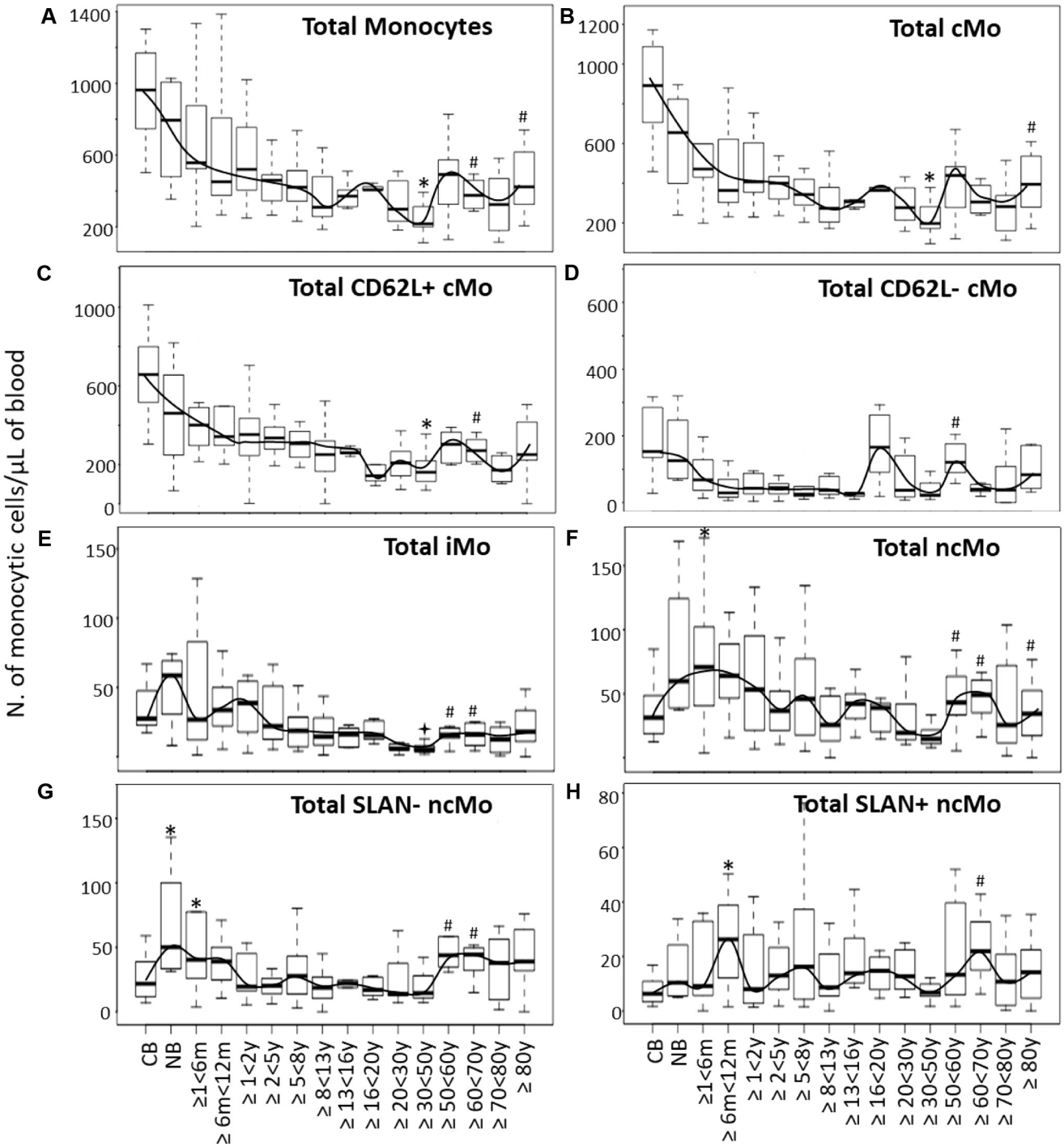

FIG 1. Distribution of different subsets of circulating monocytes in CB and peripheral blood samples from healthy subjects through life. *$P < .05$ versus CB. #$P < .05$ versus subjects aged 30 or more to less than 50 years. +$P < .05$ versus newborns. *NB,* Newborns.

subjects) with a median age of 26 years (range, 4 days to 92 years) distributed by age group, as shown in Table E1 in this article's Online Repository at www.jacionline.org. In addition, 9 BM samples (6 from male and 3 from female subjects; median age, 49 years [age range, 21-83 years]) from 9 of the above referred healthy donors, together with 13 lymph node and 13 spleen paired samples (9 from male and 4 from female subjects; median age, 69 years [age range, 49-81 years]) collected in parallel with blood specimens from solid organ donors, were studied. All organ donors were in brain death at the moment of tissue collection (performed within the first hour after heart failure), with the organs maintained as viable throughout the procedure.

Identification of different monocytic cell subsets was performed by using highly sensitive 10-color flow cytometry. The immunophenotypic criteria used for identification of monocytic subpopulations, as well as the precise flow cytometry

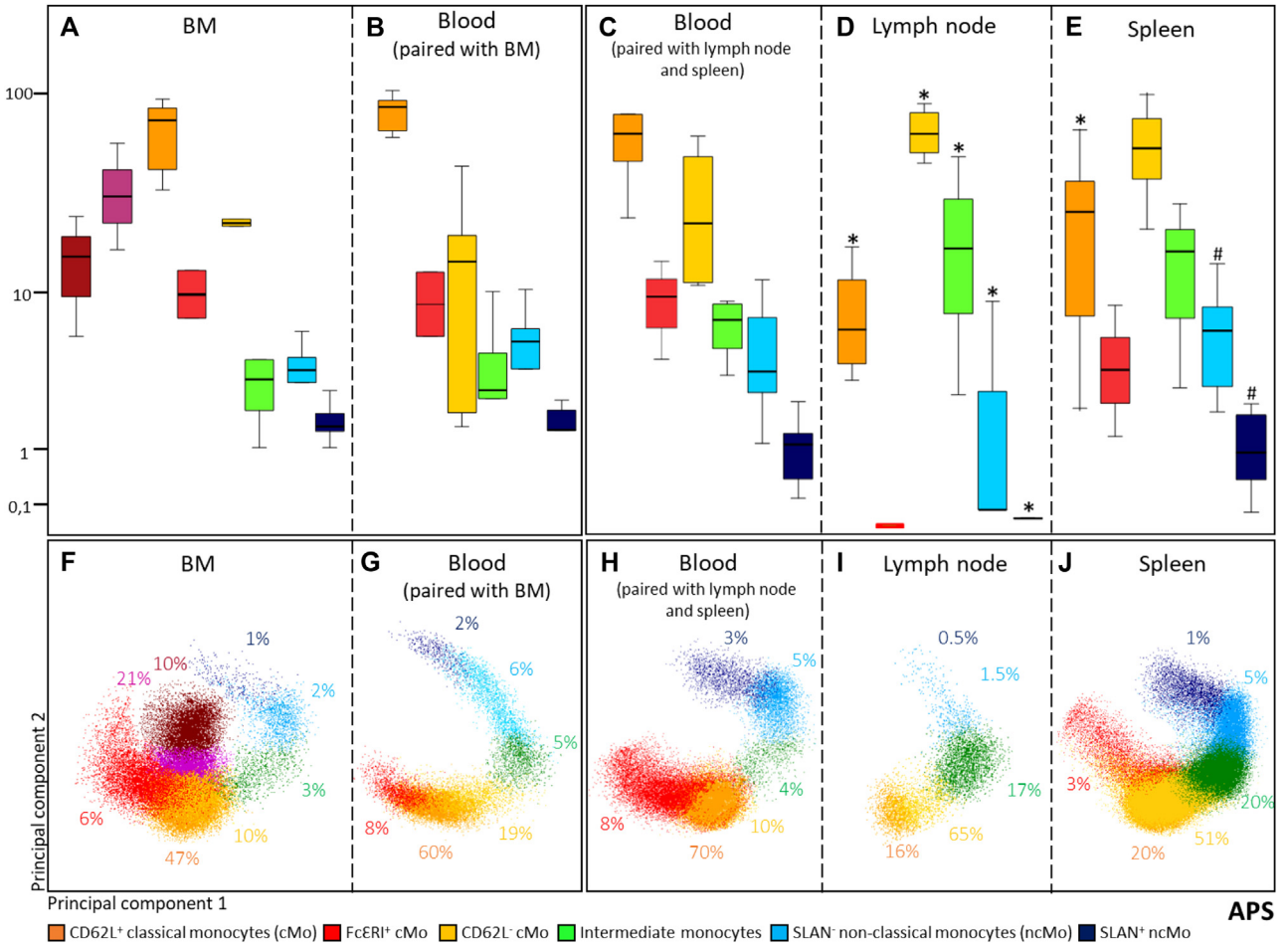

FIG 2. Relative distribution of different subsets of monocytes in adult blood versus paired BM and paired lymph node and spleen samples. **A-E,** $*P < .05$ versus blood. $\#P < .05$ versus lymph nodes. **F-J,** Multidimensional representations of distinct monocyte cell subsets in different tissues. $P > .05$ for comparison of the 2 blood sample groups for all monocyte subsets.

protocols, panels, and reagents used, are detailed in the Methods section in this article's Online Repository at www.jacionline.org (Tables E2 and E3). Five distinct subsets of monocytes were systematically identified in every sample analyzed: (1) $CD62L^+$ and (2) $CD62L^-$ cMos; (3) iMos; and (4) $SLAN^-$ and (5) $SLAN^+$ ncMos. An additional population of $Fc\varepsilon RI^+CD14^{hi}CD16^-$ monocytes was also identified in a subset of samples stained with anti-FcεRI (see Fig E1 in this article's Online Repository at www.jacionline.org).

Overall, our results show that the distribution of monocyte subsets in blood varies substantially during life, particularly during the first 6 months of life, when all monocyte subsets peaked (Fig 1); however, although cMos reached their highest numbers in CB samples, iMo and ncMo numbers peaked in newborns. These homeostatic changes most likely reflect the high production rate and release of recently generated $CD62L^+$ classical BM monocytes into blood[7] and thereby the early-life requirement for sufficient numbers of monocytes to fill the distinct tissues. Afterward, the 3 major monocyte subsets decreased until the age of 8 to 13 years, subsequently increased again at adolescence (particularly $CD62L^+$ cMos), and remained high in younger adults, decreasing thereafter until the age of 30 to 50 years. From 50 years onward, all of the above monocyte subsets increased in blood, which might reflect an increased tissue turnover, apoptosis, and/or "immunosenescence."[8] Based on the overall pattern of distribution of monocytic subsets in blood, with sequential peaks for cMos, iMos, and ncMos, particularly during the earliest periods of life and after the age of 50 years, our results would support the notion that iMos and ncMos might correspond to monocytic cell subsets at more advanced stages of maturation than cMos, which is also in accordance with previous reports.[9] Whether such maturation occurs in blood or outside the bloodstream is still a subject of debate, although some reports support the notion that the differentiation steps of cMos into iMos and ncMos more likely occur outside the blood compartment rather than in the blood.[6,9]

To gain insight into the potential tissue relationship between cMos and both iMos and ncMos, we further investigated the distribution of the distinct subsets of monocytes in normal BM, lymph node, and spleen samples. Our results showed that their relative distribution varies substantially in BM, lymph node, and spleen compared with that in blood (Fig 2). Thus, although $CD62L^+$ cMos were by far the most abundant subset of cMos in blood and BM, they were outnumbered by $CD62L^-$ cMos in lymph node and spleen. Moreover, higher percentages of iMos were found in lymph node and spleen versus both blood and BM, in which this cell subset represented a minor monocytic

population. In contrast, ncMos were particularly represented in blood and spleen samples, although they were found at very low percentages in BM and lymph node; interestingly, no (or very small numbers of) $SLAN^+$ ncMos were found in lymph node samples. The predominance of $CD62L^-$ cMos in lymph node and spleen would most likely reflect the more mature nature of cMos in these lymphoid tissues (vs BM and blood). Altogether, these findings suggest that, consistent with *in vivo* 6,6-$^2H_2$ glucose monocyte tracking studies,[6,9] cMos might lose CD62L and differentiate into iMos in lymphoid tissues. iMos might then recirculate through the lymph system[2] and sequentially give rise to $SLAN^-$ and $SLAN^+$ ncMos. Despite this, there were no statistically significant differences between the phenotypic profile of the different monocytic subsets in blood versus lymph node and spleen (see Fig E2 in this article's Online Repository at www.jacionline.org).

In summary, our results show that the number of circulating blood monocytes and their subsets varies significantly throughout life, which provides a frame of reference for further studies in distinct disease conditions. The differential distribution of the distinct monocyte subsets evaluated in different human tissues might reflect distinct functional features and kinetics of monocytic cell subsets throughout the body. Further studies are required to confirm this hypothesis.

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This work has been partially supported by the following grants: RTICC RD12/0036/0048-FEDER, Biomedical Research Networking Center Consortium–CIBER-CIBERONC (CB16/12/00400-FEDER), PI13/01412-FEDER, PI16/00787-FEDER from the Instituto de Salud Carlos III (ISCIII), Ministerio de Economía y Competitividad, Madrid, Spain, and the European Research Council (ERC) under the European Union's Horizon 2020 Research and Innovation Programme (grant agreement ERC-2015-AdG 695655 [TiMaScan]).

Disclosure of potential conflict of interest: J. J. M. van Dongen and A. Orfao report being the inventors on patent PCT/NL2012/050132 ("Methods and means for monitoring disruption of tissue homeostasis in the total body"); report being chairmen of the EuroFlow scientific foundation, which receives royalties from licensed patents that are collectively owned by the participants of the EuroFlow Foundation; and report an Educational Services Agreement between BD Biosciences and their universities. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online March 16, 2019.
https://doi.org/10.1016/j.jaci.2019.02.030

Autonomous regulation of IgE-mediated mast cell degranulation and immediate hypersensitivity reaction by an inhibitory receptor CD300a

To the Editor:

Although phosphatidylserine (PS) confined to the inner leaflet of plasma membrane is exposed on the cell surface when cells undergo apoptosis, viable cells, including mast cells (MCs), also externalize PS in certain cellular states.[1,2,E1-E3] However, the pathophysiological significance of PS exposure on viable cells remains elusive. To address the role of PS externalization on the cell surface of viable MCs, we monitored PS surface exposure on bone marrow–derived cultured MCs (BMMCs) by confocal microscopy after stimulation with trinitrophenyl (TNP)-specific IgE and TNP-ovalbumin (OVA) in the presence of PSVue 643, a fluorescent dye with rapid binding capacity for PS. The dye began to accumulate on the cell surface of live BMMCs within 600 seconds after FcεRI stimulation, whereas the nonstimulated BMMCs remained negative for the staining (Fig 1, *A*; see Video E1 in this article's Online Repository at www.jacionline.org), indicating that PS is externalized within 10 minutes after activation.

MCs abundantly express CD300a, an inhibitory immunoreceptor for PS.[3-5] By imaging flow cytometry analyses, we found that both PS and CD300a showed a polarization and

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Article

Monocyte Subsets and Serum Inflammatory and Bone-Associated Markers in Monoclonal Gammopathy of Undetermined Significance and Multiple Myeloma

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Citation: Damasceno, D.; Almeida, J.; Teodosio, C.; Sanoja-Flores, L.; Mayado, A.; Pérez-Pons, A.; Puig, N.; Arana, P.; Paiva, B.; Solano, F.; et al. Monocyte Subsets and Serum Inflammatory and Bone-Associated Markers in Monoclonal Gammopathy of Undetermined Significance and Multiple Myeloma. *Cancers* **2021**, *13*, 1454. https://doi.org/10.3390/cancers13061454

Academic Editor: Sylvie Hermouet

Received: 16 February 2021
Accepted: 18 March 2021
Published: 22 March 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Simple Summary: We investigated the distribution of different subsets of monocytes (Mo) in blood and bone marrow (BM) of newly-diagnosed untreated monoclonal gammopathy of undetermined significance (MGUS), smoldering (SMM) and active multiple myeloma (MM), and its relationship with immune/bone serum-marker profiles. Our results showed decreased production of BM Mo with decreased counts of classical Mo (cMo) in BM and blood of SMM and MM, but not MGUS. Conversely, intermediate and non-classical Mo were significantly increased in MGUS, SMM and MM BM. In parallel, increased levels of interleukin (IL)1β were observed in a fraction of MGUS and SMM, while increased serum IL8 was characteristic of SMM and MM, and higher serum IL6, RANKL and bone alkaline phosphatase concentrations, together with decreased counts of FcεRI$^+$cMo, were restricted to MM presenting with bone lesions. These results provide new insights in the pathogenesis of plasma cell neoplasms and the potential role of FcεRI$^+$cMo in normal bone homeostasis.

Abstract: Background. Monocyte/macrophages have been shown to be altered in monoclonal gammopathy of undetermined significance (MGUS), smoldering (SMM) and active multiple myeloma (MM), with an impact on the disruption of the homeostasis of the normal bone marrow (BM) microenvironment. Methods: We investigated the distribution of different subsets of monocytes (Mo) in blood and BM of newly-diagnosed untreated MGUS (n = 23), SMM (n = 14) and MM (n = 99)

patients vs. healthy donors (HD; $n = 107$), in parallel to a large panel of cytokines and bone-associated serum biomarkers. Results: Our results showed normal production of monocyte precursors and classical Mo (cMo) in MGUS, while decreased in SMM and MM ($p \leq 0.02$), in association with lower blood counts of recently-produced CD62L$^+$ cMo in SMM ($p = 0.004$) and of all subsets of (CD62L$^+$, CD62L$^-$ and FcεRI$^+$) cMo in MM ($p \leq 0.02$). In contrast, intermediate and end-stage non-classical Mo were increased in BM of MGUS ($p \leq 0.03$), SMM ($p \leq 0.03$) and MM ($p \leq 0.002$), while normal (MGUS and SMM) or decreased (MM; $p = 0.01$) in blood. In parallel, increased serum levels of interleukin (IL)1β were observed in MGUS ($p = 0.007$) and SMM ($p = 0.01$), higher concentrations of serum IL8 were found in SMM ($p = 0.01$) and MM ($p = 0.002$), and higher serum IL6 ($p = 0.002$), RANKL ($p = 0.01$) and bone alkaline phosphatase (BALP) levels ($p = 0.01$) with decreased counts of FcεRI$^+$ cMo, were restricted to MM presenting with osteolytic lesions. This translated into three distinct immune/bone profiles: (1) normal (typical of HD and most MGUS cases); (2) senescent-like (increased IL1β and/or IL8, found in a minority of MGUS, most SMM and few MM cases with no bone lesions); and (3) pro-inflammatory-high serum IL6, RANKL and BALP with significantly ($p = 0.01$) decreased blood counts of immunomodulatory FcεRI$^+$ cMo-, typical of MM presenting with bone lesions. Conclusions: These results provide new insight into the pathogenesis of plasma cell neoplasms and the potential role of FcεRI$^+$ cMo in normal bone homeostasis.

Keywords: plasma cell neoplasms; MGUS; multiple myeloma; monocytes; FcεRI monocytes; tumor microenvironment; inflammatory cytokines; immunosenescence; bone markers

1. Introduction

Plasma cell (PC) neoplasms consist of a wide spectrum of end-stage antibody-producing B-cell tumors [1,2] that range from pre-malignant conditions such as monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM), to symptomatic multiple myeloma (MM) and PC leukemia [3].

Tumor PC control and growth kinetics in both MGUS and MM depend both on the intrinsic characteristics of neoplastic PC and their close interaction with the tumor microenvironment [4,5]. Thus, malignant BM PC in MM, and to a less extent also in MGUS and SMM, have the ability to modify their surrounding immune and bone microenvironment, interfere with immune surveillance and ultimately lead to bone resorption and lysis via direct local cell-to-cell and cytokine-mediated interactions between monocyte/macrophages, stromal cells, osteoclasts and osteoblasts [6,7]. Such interactions contribute to increased survival of malignant PC [8], promote drug resistance and induce local angiogenesis, all of which favor tumor growth, in parallel with activation of osteoclasts and osteolysis [6,7]. Thus, increased numbers in BM of M2-polarized macrophages have been reported in MM compared to healthy donors (HD), MGUS and SMM patients [9], in association with a poorer patient outcome [10]. In turn, upregulation of the RANKL-receptor activator of nuclear factor κ B ligand-pro-osteoclastogenic factor, in parallel to decreased osteoprotegerin (OPG) levels, have been described in BM of MM [11–15] and also MGUS patients [16], in association with an accelerated turnover of BM osteoblasts and increased bone alkaline phosphatase (BALP) and RANKL levels in serum of these patients [17,18]. These alterations might result from an increased differentiation of monocytes (Mo) into osteoclasts [19] associated with higher secretion of interleukin (IL)6 [20], CXCL12 [21,22] and RANKL by both BM stromal cells [23] and activated T cells [24]. Altogether, this leads to increased recruitment of osteoclasts to the BM endosteal niche, followed by an inhibition of its decoy receptor OPG, and locally increased bone resorption which is typically observed in MM.

The functionally altered interactions between Mo (and other immune cells) and both osteoblasts and osteoclasts in BM of MGUS, SMM and MM might become detectable in blood at already early phases of the disease, via redistribution of specific subsets of blood Mo associated with the increase of specific serum biomarkers. Thus, altered counts of both classical (c)Mo (vs HD) and Slan$^+$ non-classical (nc)Mo (vs HD and MGUS) have been reported in the blood of MM [25] in association with a higher BM tumor load [26]. In addition, patients with MM have been reported to show strongly increased levels of pro-inflammatory cytokines in serum such as IL1β, IL10, TNFα [27] and IL6, together with an enhanced spontaneous ex vivo secretion of inflammatory cytokines (vs HD) by blood Mo [28]. Apart from BM Mo and macrophages, other immune cells have also been implicated in the pathogenesis of MM [4,29]. Among other cells, these include myeloid-derived suppressor cells (MDSC) [30–32], regulatory T cells (Tregs) [30,33–36], natural killer (NK) cells [37–40], T cells [41,42] and dendritic cells (DC) [43–45], which further supports a relevant role for the interaction between tumor PC and their immune/bone microenvironment in the pathogenesis of PC neoplasms.

Despite all the above, at present, there are still limited data about the distribution of distinct subsets of Mo in blood and BM of MGUS, SMM and MM, and the potential relationship between these alterations and other immune and bone-associated serum markers, which might contribute to better understand the clinical and biological differences between distinct diagnostic categories of these PC neoplasms and the underlying pathogenic mechanisms.

Here, we investigated in detail the distribution of different subsets of cMo, intermediate Mo (iMo) and ncMo in blood and BM of newly-diagnosed untreated MGUS, SMM and MM patients, in parallel to a large panel of immune and bone-associated serum markers. Our ultimate goal was to identify altered cellular and soluble immune/bone interaction profiles that are associated with distinct diagnostic categories of the disease (MGUS vs. SMM vs. MM) and that contribute to determining their different clinical behavior.

2. Materials and Methods

2.1. Patients, Controls, and Samples

A total of 128 aspirated BM and 220 peripheral blood (PB) EDTA-anticoagulated samples were studied. These included: (i) 15 normal BM (median age: 59 y; range: 31–83 y) from HD that underwent orthopedic surgery, and 97 normal PB (median age: 62 y; range: 32–92 y) samples recruited from the general population for a total of 107 HD (including 10 paired samples); (ii) 19 BM (median age: 70 y; range: 44–85 y) and 22 PB (median age: 67 y; range: 31–85 y) specimens from 23 MGUS patients (36 paired samples); (iii) 13 BM (median age: 71 y; range: 61–82 y) and 13 PB (median age: 73 y; range: 61–82 y) samples of 14 newly-diagnosed untreated SMM cases (26 paired samples); and, iv) 81 BM (median age: 71 y; range: 45–85 y) and 88 PB (median age: 72 y; range: 45–85 y) specimens from 99 newly-diagnosed untreated MM patients (140 paired samples) (Table S1). Whole-body computerized tomography was used to evaluate the presence of osteolytic lesions at diagnosis. All samples were collected at diagnosis at each participating center—University Hospital of Salamanca (HUS; Salamanca, Spain); University Clinic of Navarra (Pamplona, Spain); and Hospital Nuestra Señora del Prado (Talavera de la Reina, Spain)- and processed locally within 24 h after collection (Table S1). None of the patients included in the study had been previously diagnosed with a monoclonal gammopathy. All participants gave their informed consent to participate in the study in accordance with the guidelines of the local Ethics Committees and the Declaration of Helsinki.

2.2. Flow Cytometric Analysis of BM and PB Subsets of Monocytes

Immunophenotypic identification, enumeration and characterization of the distinct subsets of Mo present in BM and PB samples were performed using an 11-color antibody combination based on the TiMaScan^{TM} tube (Cytognos, SL, Salamanca, Spain) as backbone, and that consisted of the following reagents: CD45-pacific orange (PacO; clone HI30), CD62L-brilliant violet 650 (BV650; clone DREG-56), CD16-BV786 (clone 3G8), CD36-fluorescein isothiocyanate (FITC; clone CLB-IVC7), CD14- plus CD34-peridinin chlorophyll protein-cyanin 5.5 (PerCPCy5.5; clones MφP9 and 8G12, respectively), anti-Slan-phycoerythrin (PE; clone DD-1), CD117-PE-CF594 (clone YB5.B8), anti-HLADR-PECy7 (clone G46–6), CD64-allophycocyanin (APC; clone 10.1), and CD300e (IREM2)-APCC750, (clone UP-H2). In a subset of 43 BM samples and 65 PB specimens, an additional PE-conjugated anti-FcεRI antibody reagent (clone: AER-37) was also added. All antibody conjugated reagents were purchased from Becton/Dickinson Biosciences (BD; San Jose, CA), except for the CD45-PacO (Invitrogen; Carlsbad, CA, USA), CD36-FITC (Cytognos; Salamanca, Spain), anti-Slan-PE (Milteny Biotech; Cologne, Germany), CD300e and anti-FcεRI (Immunostep; Salamanca, Spain) antibodies. For sample preparation, the EuroFlow bulk lyse standard operating protocol (SOP) was used as previously described in detail [46,47]. Briefly, bulk erythrocyte lysis with ammonium chloride was performed. Subsequently, nucleated cells were washed and placed in phosphate-buffered saline solution (PBS) at a concentration of 10^7 cells/200 μL. Concentrated 10^7 BM and PB nucleated cells were then stained, washed and fixed with FACS lysing solution (BD) as per the EuroFlow SOP for staining of cell surface-only markers [46,47], available at www.EuroFlow.org (accessed on 4 May 2018). Stained samples were immediately measured in an LSR Fortessa X-20 flow cytometer (BD) using the FACSDiva^{TM} software (BD). Prior to acquisition, instrument setup, calibration and monitoring were performed using the EuroFlow instrument setup and compensation SOP for 12-color measurements [46] available at www.EuroFlow.org. For data analysis, the Infinicyt^{TM} software (Cytognos S.L) was employed. For every sample, the following subsets of Mo were systematically identified: (1) CD62L$^+$ and, (2) CD62L$^-$ cMo (CD14^{hi} CD16$^-$); (3) iMo (CD14^{hi}CD16$^+$); and (4) CD36$^+$Slan$^-$, (5) CD36$^-$Slan$^-$, (6) CD36$^+$Slan$^+$, and (7) CD36$^-$Slan$^+$ ncMo (CD14$^{-/lo}$CD16$^+$). An additional population of FcεRI$^+$ cMo (CD14^{hi}CD16$^-$) was also identified in the subset of samples stained with anti-FcεRI (n = 108). For the identification and enumeration of Mo and monocyte subsets, a previously defined gating strategy was used, as illustrated in Figure 1 [48].

2.3. Quantification of Soluble Cytokine plasma Levels Using the Cytometric Bead Array Platform

Soluble IL1β, IL6, IL8, IL10, IL12p70 and TNF-α levels were measured in a subset of 40 (IL1β, IL8 and IL12p70) to 50 (IL6 and TNF-α) freshly-frozen plasma samples from an identical number of subjects from the same patient cohort. The Cytometric Bead Array immunoassay (CBA) and the human inflammatory cytokine kit (BD) were used to quantify soluble IL1β, IL6, IL8, IL10, IL12p70 and TNF-α plasma levels, strictly following the recommendations of the manufacturer. Briefly, 50 μL of thawed plasma samples were incubated for 1 h at room temperature (RT) with 50 μL of a mixture of each of the anti-cytokine antibody-coated beads. Afterward, 50 μL of the PE-conjugated detection antibody reagent was added to each sample, followed by incubation for 2 h at RT. Once this incubation was completed, the unbound antibody was washed out (1×) and the washed beads were (immediately) measured in a FACSCanto II flow cytometer (BD). Data on 3000 events per bead population per sample were measured and stored for a total of 30,000 beads. For data analysis, the FCAP Array v3 (BD) and the CBA (BD) software programs were used, as described elsewhere [49]. The limit of quantitation (LOQ) for the distinct cytokines evaluated was as follows: IL1β, 7.2 pg/mL; IL6, 2.5 pg/mL; IL8, 3.6 pg/mL; IL10, 3.3 pg/mL; IL12p70, 1.9 pg/mL; TFN-α, 3.7 pg/mL.

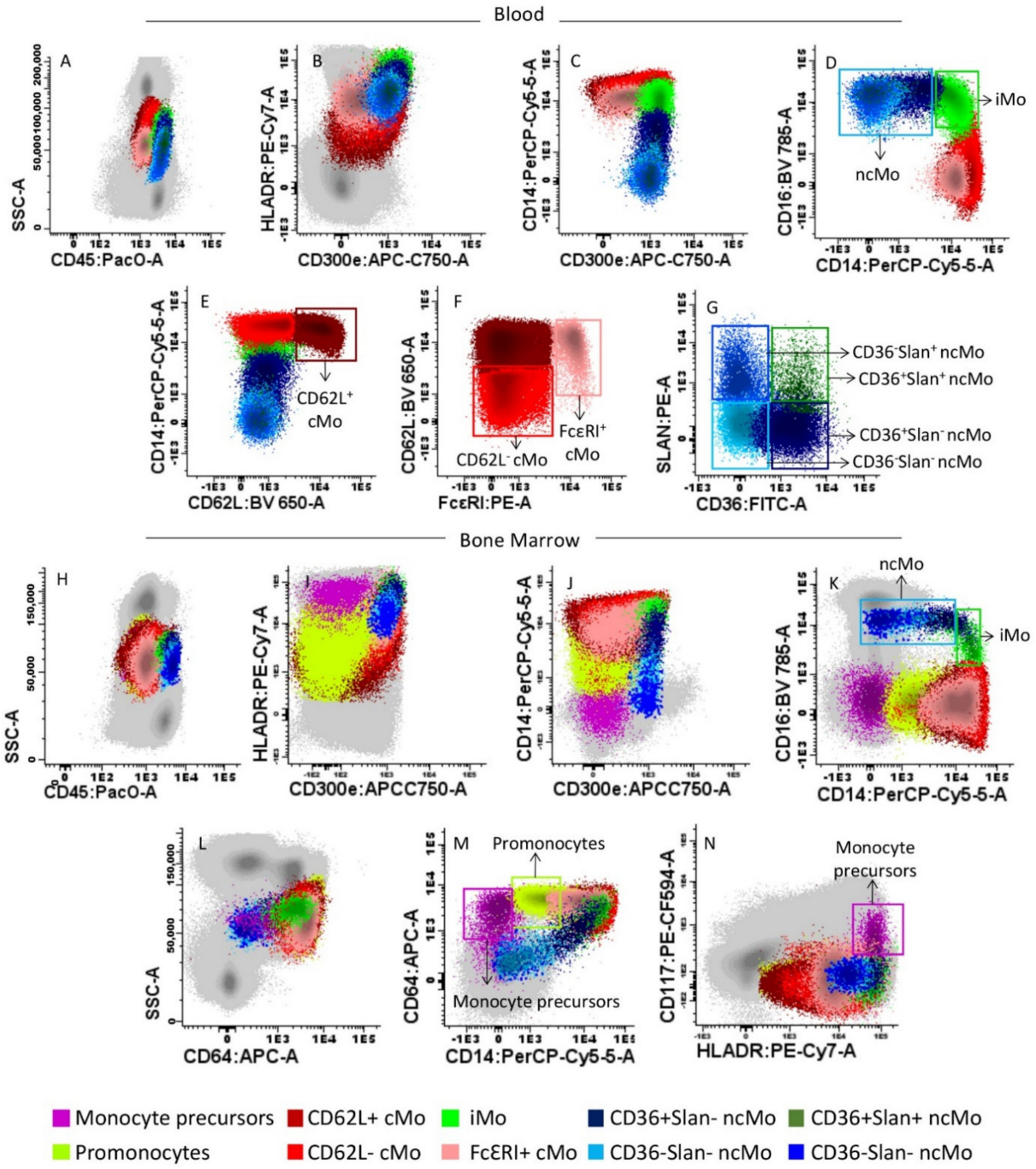

Figure 1. Gating strategy used for the identification of monocytes (Mo) and their different subsets in representative peripheral blood (PB) (upper panels **A**–**G**) and bone marrow (BM) (lower panels **H**–**N**) samples from a healthy donor (HD). The distinct blood (panels **A**–**G**) and BM (panels **H**–**N**) subsets of Mo identified are represented as colored events, after they had been gated and discriminated from all other PB and BM leucocytes (gray dots) based on their typical SSC/CD45 profile (panels **A**,**H**), and their unique positivity for CD300e, HLADR, CD64 and CD14 (panels **B**,**C**,**I**,**J**,**M**). Within total (mature) Mo, the following subsets were identified based on their distinct expression profile for CD14 and CD16 (panels **D**,**K**) plus FcεRI (panel **F**): $CD14^{hi}CD16^{-}$ classical (c)Mo, $CD14^{hi}CD16^{+}$ intermediate (i) Mo, and $CD14^{-/lo}CD16^{+}$ non-classical (nc)Mo and the subset of $CD14^{+}$ $CD16^{-}$ $Fc\varepsilon RI^{+}$ cMo. Within cMo, the $CD62L^{+}$ and $CD62L^{-}$ subsets were further identified (panel **E**), while ncMo were further separated into $CD36^{+}Slan^{-}$, $CD36^{-}Slan^{-}$, $CD36^{+}Slan^{+}$ and $CD36^{-}Slan$+ ncMo (Panel **G**). In BM samples, monoblasts and promonocytes were additionally identified, based on their phenotypic profile (positivity) for both CD117, HLADR (panels **N**) and expression of CD64 (panel **L**,**M**).

2.4. ELISA (Enzyme-Linked Immunosorbent Assay) Quantitation of Bone-Derived Markers in Plasma

Quantitation of the soluble levels of BALP, RANKL and OPG was performed in a total of 40 plasma samples from an identical number of subjects from the same patient cohort using commercially available ELISA kits, according to the instructions of the manufacturer (Cusabio Biotech, Wuhan, China). After sample preparation, optical densities were read at 450 nm using a Tecan Spectra Fluor Plus™ microplate reader, and data were analyzed using the Curve Expert 1.4 software (Cusabio Biotech).

2.5. Statistical Methods

The Kolmogorov–Smirnov (KS) test was used to test the normality of data. Since data were not normally distributed, the non-parametric statistical Wilcoxon or the Friedman tests and the Mann–Whitney U or the Kruskal–Wallis tests were subsequently used to assess the (two-sided) statistical significance of differences observed between two or more than two groups for paired and unpaired variables, respectively. The Chi-square test was used to compare frequencies of cases between different groups. For multivariate analysis, the T-distributed stochastic neighbor embedding analysis (T-SNE) was used (Infinicyt™ software). Statistical significance was set at p-values ≤ 0.05.

3. Results

3.1. Distribution of Monocytic Precursors, Mo, and Monocyte Subsets in BM

The distribution of monocytic precursors, as well as mature Mo and their subsets was first analyzed in BM aspirated samples of 19 MGUS, 13 SMM and 81 MM patients vs. 15 HD. Overall, a slightly increased percentage of monocytic precursors was observed in MGUS (vs. HD)-median (range) of 1.2% (0.83–2.3) vs. 0.9% (0.6–1.6) in HD ($p = 0.2$)-. In contrast, compared to MGUS cases, both SMM-median (range) of 0.9% (0.3–1.0; $p = 0.01$)- and MM-median (range) of 0.8% (0.4–1.7; $p = 0.04$)- showed lower counts of monocytic precursors in BM (Figure 2A) associated with significantly decreased percentages-median (range)- of monoblasts in BM of SMM vs. HD −0.08% (0.01–0.4) vs. 0.24% (0.01–0.6), respectively ($p = 0.03$)- (Figure 2B) and of promonocytes compared to MGUS −0.6% (0.1–3.7) vs. 1.1% (0.2–2.4), respectively ($p = 0.03$)- (Figure 2C). In line with these findings, the overall percentage-median (range)- of more mature Mo was significantly increased in BM of MGUS cases (vs. HD) −4.4% (2.2–7.8) vs. 3.5% (2.7–6.2) in HD ($p = 0.01$) but not of SMM −3.4% (1.3–6.2) vs. 4.4% (2.2–7.8) in MGUS ($p = 0.02$)- and MM cases −3.3% (1–6) vs. 4.4% (2.2–7.8) in MGUS ($p = 0.003$)- (Figure 2D). Such increased numbers of mature Mo in BM of MGUS was at the expense of the major compartment of recently produced cMo-median (range) of 3.6% (1.9–4.4) in MGUS vs. 2.4% (1.4–3.3) in HD ($p = 0.005$), 2.4% (1.2–3.4) in SMM ($p = 0.02$) and 2.2% (0.6–5) in MM ($p = 0.005$), respectively- (Figure 2E), altough no differences were observed among cMo subsets (Figure 2F–H). In contrast, MGUS, SMM and MM patients showed greater percentages -median (range)- in BM (vs HD) of both iMo −0.11% (0.03–0.41), 0.11% (0.04–0.2), and 0.11% (0.03–0.46) vs. 0.04% (0.0–1.1), respectively ($p \leq 0.02$)- (Figure 2I) and of ncMo −0.17% (0.02–0.59), 0.17% (0.09–0.39), and 0.19% (0.03–0.57) vs. 0.07% (0.03–0.13), respectively ($p \leq 0.03$)- (Figure 2J). The later increase in ncMo -median (range)- observed in BM of MGUS, SMM and MM was at the expense of the $CD36^{+}Slan^{-}$ subset of ncMo -0.08% (0.01–0.13), 0.06% (0.02–0.15) and 0.09% (0.02–0.42) vs. 0.03% (0–0.04) in HD, respectively ($p \leq 0.01$)- (Figure 2K) alone in MGUS or in combination with that of $CD36^{-}Slan^{-}$ −0.05% (0.02–0.13) vs. 0.02% (0–0.05) in HD ($p = 0.008$)- and $CD36^{-}Slan^{+}$ ncMo -0.04% (0.01–0.09) vs. 0.01% (0–0.05) in HD ($p = 0.03$)- in SMM or that of the $CD36^{-}Slan^{-}$ -0.05 (0.01–0.24) vs. 0.02% (0–0.05) in HD ($p = 0.005$)- and $CD36^{+}Slan^{+}$ subsets of ncMo -0.01% (0.0–0.09) vs. 0.0% (0.0–0.03) in HD ($p = 0.02$)- in MM (Figure 2L–N).

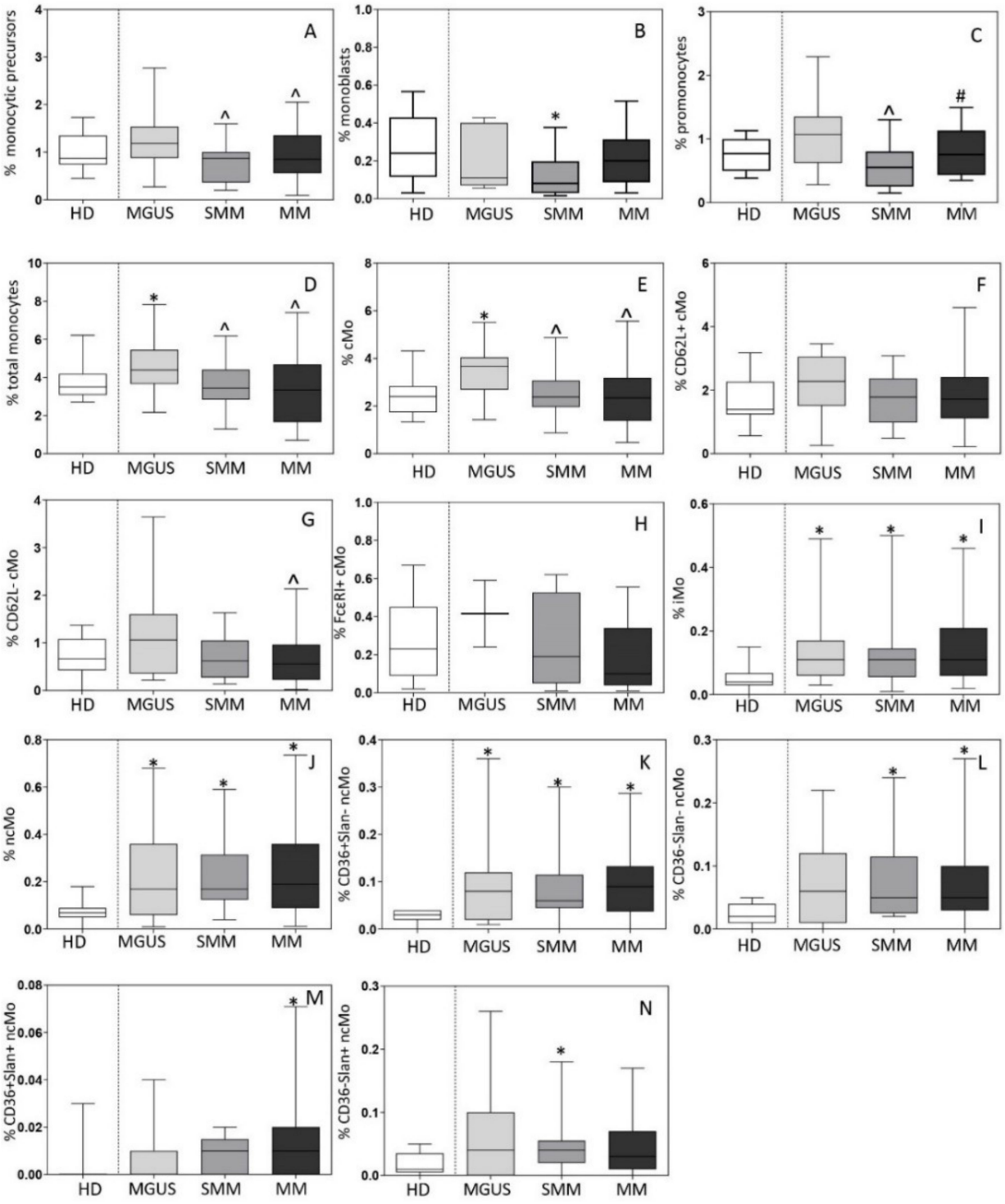

Figure 2. Distribution of monocytic precursors (total monocytic precursors, monoblasts and promonocytes; panels **A**–**C**), total monocytes (Mo) (panel **D**) and their subsets (panels **E**–**N**) in bone marrow (BM) of newly-diagnosed MGUS (n = 19), SMM (n = 13) and MM (n = 81) patients vs. age-matched HD (n = 15). In panels A-N, notched boxes extend from the 25th to the 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 5th and 95th percentiles, respectively. Statistical significant differences ($p \leq 0.05$) were observed vs. * HD, ˆ MGUS, and # SMM patients after applying the Mann–Whitney non-parametric test (as the KS normality test showed that data for these variables did not follow a normal distribution). cMo: classical monocytes; iMo: intermediate monocytes; ncMo: non-classical monocytes; HD: healthy donor; MGUS: monoclonal gammopathy of undetermined significance; SMM: smoldering multiple myeloma; MM: multiple myeloma.

Subsequently, the distribution of monocytic precursors and mature Mo and their subsets was investigated in active MM cases divided according to the international staging system (ISS) and the revised ISS (RISS). Thus, a decreased percentage -median (range)- of monoblasts was observed in BM of ISS-III and RISS-III patients -0.09% (0.01–0.5%) and 0.09% (0.01–0.3%)- vs. ISS-I and RISS-I cases -0.2% (0.03–0.6%) and 0.2% (0.01–0.6%), respectively ($p \leq 0.05$)-. In turn, $CD36^{+}Slan^{+}$ ncMo were significantly increased in ISS-III vs. ISS-I patients -median (range) of 0.01% (<0.01–0.2%) vs. 0.01% (<0.01–0.02%; $p = 0.05$)- and $Fc\varepsilon RI^{+}$ cMo were found at higher median (range) counts among RISS-III vs. RISS-II patients −0.4% (0.2–0.6%) vs. 0.05% (0.01–0.4%) $p = 0.02$-.

3.2. Distribution of Mo and Their Subsets in Blood

The overall distribution of Mo and their distinct subsets was subsequently investigated in blood of 22 MGUS, 13 SMM, and 88 MM patients, in parallel to 97 age-matched HD. Both SMM and MM patients displayed decreased -median (range)- counts in blood (vs HD) of total Mo −260 (98–950) cells/μL and 267 (71–1772) cells/μL vs. 388 (112–1131) cells/μL, respectively ($p \leq 0.05$)- and of cMo −194 (71–756) cells/μL and 199 (32–1271) cells/μL vs. 297 (95–1052) cMo/μL, respectively ($p \leq 0.05$)- (Figure 3A,B). In contrast to SMM and MM patients, MGUS cases displayed overall normal total Mo (and monocyte subsets) counts in blood (Figure 3). Of note, while decreased -median (range)- counts in blood of cMo in SMM were (exclusively) at the expense of $CD62L^{+}$ cMo −141 (53–518) cells/μL vs. 230 (69–732) cells/μL in HD ($p = 0.004$)-, in symptomatic MM all subsets of cMo were significantly decreased vs. HD, including $CD62L^{+}$ cMo −153 (59–476) cells/μL vs. 230 (69–732) cells/μL, respectively ($p = 0.02$)-, $CD62L^{-}$ cMo −32 (4–131) cells/μL vs. 51 (9–189) cells/μL, respectively ($p = 0.02$)- and particularly, $Fc\varepsilon RI^{+}$ cMo −1.4 (0.4–33) cells/μL vs. 30 (3–125) cells/μL, respectively ($p = 0.001$)- (Figure 3C–E).

In contrast to cMo, no significant differences were observed between the different patient groups and HD as regards both iMo and ncMo counts in blood (Figure 3F–K), except for decreased ncMo -median (range)- levels in MM -23 (4–71) cells/μL vs. 37 (11–84) cells/μL ($p = 0.01$)- (Figure 3G), at the expense of the $CD36^{-}Slan^{-}$, $CD36^{+}Slan^{+}$ and $CD36^{-}Slan^{+}$ subsets of ncMo −7 (1–31) cells/μL, 0.6 (0–5) cells/μL and 3 (0–18) cells/μL vs. 11 (2–43) cells/μL, 1.8 (0.3–5) cells/μL and 9 (3–24) cells/μL in HD, respectively ($p \leq 0.05$)-. No statistically significant differences were observed in the distribution of $CD36^{+}Slan^{-}$ ncMo in blood among the different groups of patients (Figure 2H–K).

Among active MM patients, ISS-III and ISS-II cases showed higher median (range) counts of iMo and $CD36^{-}Slan^{+}$ ncMo −16 (0.8–232) and 13 (0–80) cells/μL- than ISS-I MM patients −8 (3–40) and 8 (0–46) cells/μL, respectively ($p \leq 0.02$)-. In turn, RISS-III patients showed significantly higher counts of ncMo -median (range) of 50 (0.05–100) cells/μL- in blood, at the expense of $CD36^{-}Slan^{-}$ ncMo -median (range) of 17 (0.0–46) cells/μL-, compared to RISS-II cases -median (range) of 18 (0.0–195) and 9 (0.0–56) cells/μL, respectively ($p \leq 0.03$)-.

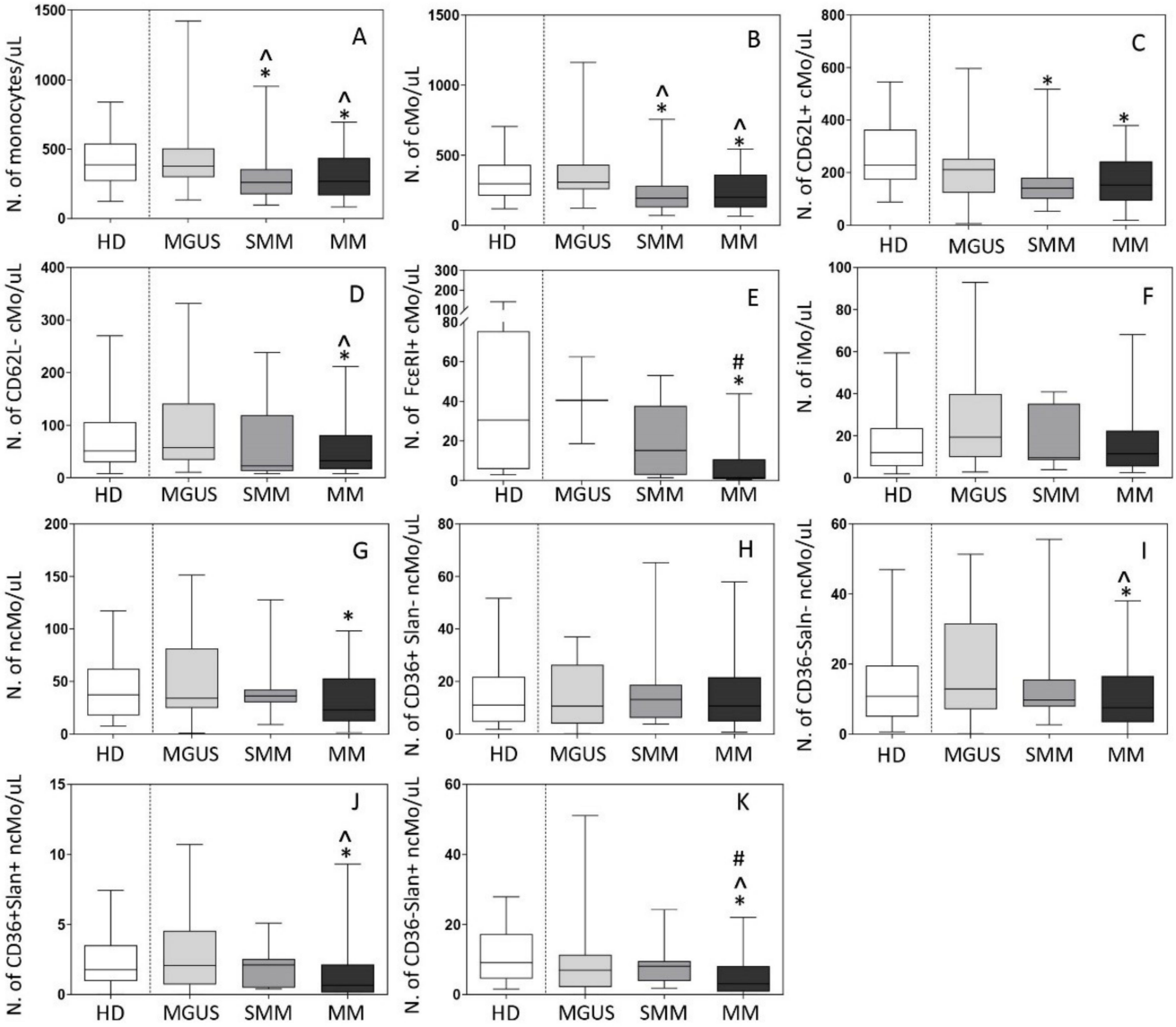

Figure 3. Distribution of total monocytes (Mo) (panel **A**) and monocyte subsets (panels **B**–**K**) in blood of newly-diagnosed MGUS (n = 22), SMM (n = 13) and MM (n = 88) patients vs. age-matched HD (n = 97). In (panels **A**–**K**), notched boxes extend from the 25th to the 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 5th and 95th percentiles, respectively. Statistical significant differences ($p \leq 0.05$) were observed vs. * HD, ^ MGUS, and # SMM patients after applying the Mann–Whitney non-parametric test (as the KS normality test showed that data for these variables did not follow a normal distribution). cMo: classical monocytes; iMo: intermediate monocytes; ncMo: non-classical monocytes; HD: healthy donor; MGUS: monoclonal gammopathy of undetermined significance; SMM: smoldering multiple myeloma; MM: multiple myeloma.

3.3. Inflammatory Cytokine and Bone-Derived Marker Levels in Plasma

Overall, distinct immune/bone marker profiles were observed in the plasma of MGUS (n = 12), SMM (n = 7) and MM (n = 18) patients investigated (Figure 4). Thus, MGUS and SMM patients showed increased IL1β levels -median (range)- in plasma −2.3 (0.0–14) and 4.1 (0.0–9) pg/mL vs. undetectable levels in HD, (p = 0.007 and p = 0.01), respectively-, while these were within the normal range (e.g., usually undetected) in MM −0.0 (0.0–21) pg/mL, vs. HD p = 0.3-. In contrast, greater IL8 serum levels were observed in both SMM and MM, -median (range) of 115 (13–2013) and 54 (0.0–1071) vs. 14 (5–29) pg/mL in HD, respectively ($p \leq 0.01$), but not in MGUS (p = 0.13). Interestingly, while MM patients showed increased -median (range)- IL6 −21 (0–133) pg/mL-, BALP −484 (200–1352) ng/mL- and RANKL

−985 (498–4246) pg/mL- levels in plasma, these three later markers were within the normal range in plasma of both SMM −1.2 (0.08–9) pg/mL, 159 (29–366) ng/mL and 453 (109–858) pg/mL vs. 2.5 (0–22) pg/mL, 173 (120–316) ng/mL and 580 (547–1082) pg/mL in HD, respectively ($p > 0.05$)- and MGUS (Figure 4A–G). Interestingly, when we compared MM patients with and without osteolytic lesions at diagnosis, significantly higher levels of IL6 -median (range) of 48 (0–32) vs. 13 (13–400) pg/mL, respectively ($p = 0.001$)- and BALP -median (range) of 594 (211–4201) vs. 387 (197–739) pg/mL, respectively ($p = 0.05$)- were detected among MM patients that had osteolytic lesions. Either normal (i.e., OPG) or undetectable (i.e., IL10, IL12p70 and TNFα) levels were found in the plasma of MGUS, SMM and MM patients for the other cytokines and bone-associated markers investigated (Figure 4F). Despite this, an increased RANKL/OPG ratio was observed in MM (either in presence or absence of bone osteolytic lesions) vs. SMM patients -median (range) of 3 (0.8–4) vs. 1 (0.2–2), $p \leq 0.01$- (Figure 4G). Of note, no significant correlation was observed between the Mo subset counts and inflammatory cytokine or bone-derived marker levels in plasma which might be due to the relatively small number of samples in which both setoff markers were analyzed.

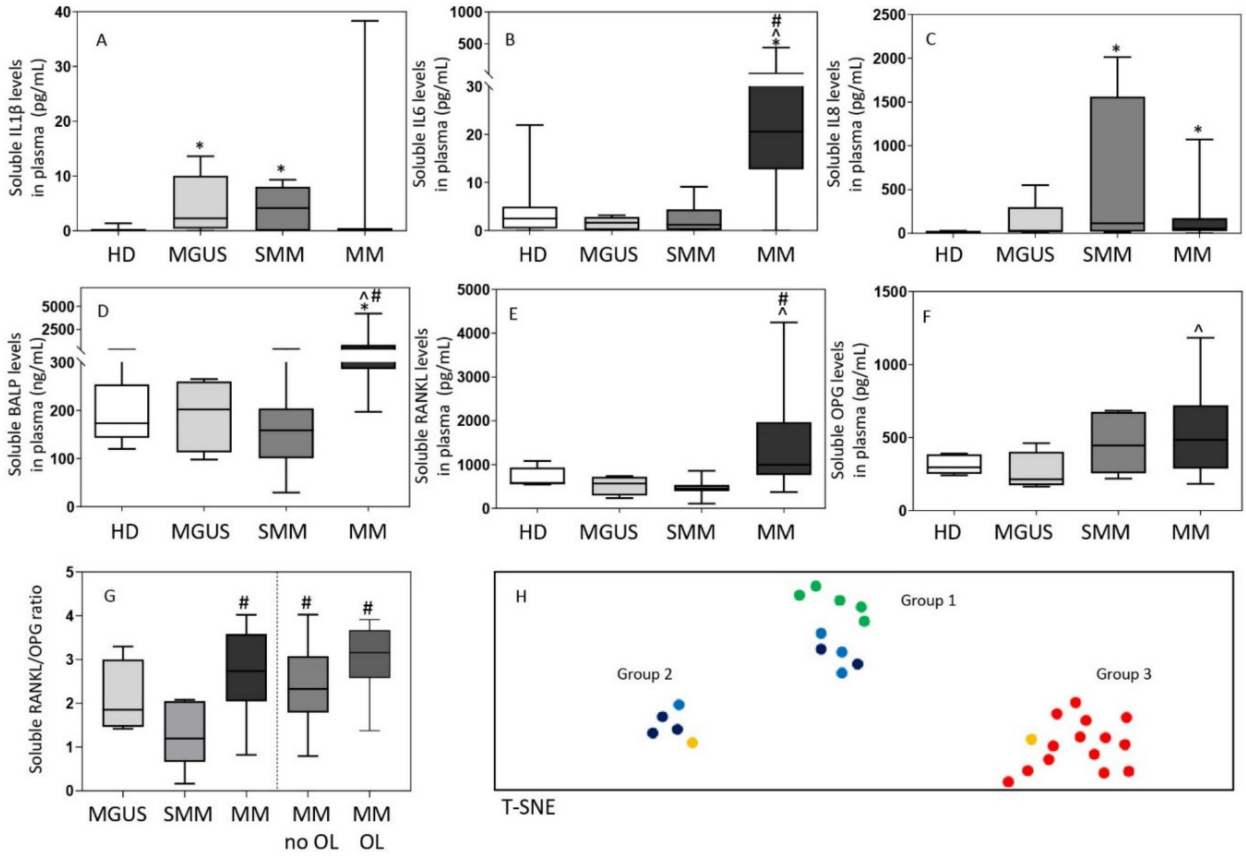

Figure 4. Soluble levels of cytokines (panels **A**–**C**) and bone-associated markers (panels **D**–**G**) and the overall immune/bone profiles (panel **H**) identified in plasma of MGUS ($n = 12$), SMM ($n = 7$), MM ($n = 18$) and HD ($n = 13$). In panels A-G, notched boxes extend from the 25th to the 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 50th and 95th percentiles, respectively. In panel H, HD are depicted as green dots, while MGUS, SMM and MM are colored as light blue, dark blue, and either yellow (no osteolytic lesions at diagnosis) or red (osteolytic lesions) dots, respectively. Statistical significant differences ($p \leq 0.05$) were observed vs. * HD, ^ MGUS, and # SMM patients after applying the Mann–Whitney non-parametric test (as the KS normality test showed that data for these variables did not follow a normal distribution). IL: interleukin; BALP: bone alkaline phosphatase; RANKL: receptor activator of nuclear factor-kB ligand; OPG: osteoprotegerin; HD: healthy donors; MGUS: monoclonal gammopathy of undetermined significance; SMM: smoldering myeloma; MM: multiple myeloma; OL: osteolytic lesions; T-SNE: T-distributed stochastic neighbor embedding.

Based on these findings, multivariate T-SNE analysis showed three clearly different patient clusters/groups ($p = 0.0001$) (Figure 4H). A first cluster/group consisted of all HD (50%) and most MGUS (30%) patients together with a smaller fraction of (all low-risk) SMM cases (20%). In a second group, SMM (including one-third of high-risk SMM cases) predominated (60%), together with a small fraction of MGUS (20%) and MM (20%) patients who showed no osteolytic lesions. Finally, the third group exclusively consisted of MM patients (100%), most of whom (97%) had osteolytic lesions at diagnosis.

Of note, all patients in group 1 showed normal serum levels of the different cytokine and bone markers investigated (Figure 5). In contrast, group 3 (MM) cases systematically showed increased -median (range)- levels of IL6 −41 (13–444) pg/mL-, BALP −656 (367–4201) ng/mL- and RANKL −1497 (841–4246) pg/mL- when compared with both group 1 −2.3 (0–22) pg/mL ($p = 0.0001$), 170 (29–316) ng/mL ($p = 0.0001$) and 533 (109–1082) pg/mL ($p = 0.0001$), respectively- and group 2 cases −3 (0.1–12) pg/mL ($p = 0.0007$), 245 (159–408) ng/mL ($p = 0.005$) and 737 (434–858) pg/mL ($p = 0.001$), respectively- (Figure 5). In turn, patients in group 2 showed greater levels -median (range)- in plasma of IL1β −6 (2–9) vs. 0.0 (0.0–4) and 0.0 (0.0–11) pg/mL in group 1 ($p = 0.0003$) and group 3 ($p = 0.002$), respectively- and IL8 −1071 (152–2013) vs. 21 (4–72) and 62 (44–203) pg/mL in group 1 ($p = 0.0002$) and in group 3 ($p = 0.005$), respectively- (Figure 5). Of note, MM patients included in group 3 also showed significantly lower counts -median (range)- of classical FcεRI$^+$ Mo in blood −6.7 (0.5–16) vs. 49 (15–97) cells/μL in group 1 ($p = 0.01$) and 30 (3–62) cells/μL in group 2 cases ($p = 0.2$)- (Figure 5G) while a similar distribution was observed in blood and BM among the three groups for all other subsets of mature Mo investigated (Figures S1 and S2). Despite this, group 2 patients showed lower percentages of monocytic precursors −0.36% (0.1–0.9) vs. 1.0% (0.6–4) and 0.8% (0.4–1.2) in group 1 ($p = 0.02$) and group 3 ($p = 0.1$), respectively-, including both lower counts of monoblasts −0.04% (0.01–0.2) vs. 0.1% (0.07–0.4) and 0.09% (0.06–0.3) in group 1 ($p = 0.05$) and 3 ($p = 0.04$), respectively- and promonocytes −0.32% (0.1–0.7) vs. 0.8% (0.5–4) and 0.7% (0.3–0.9) in group 1 ($p = 0.01$) and 3 ($p = 0.2$), respectively- (Figure S2A–C).

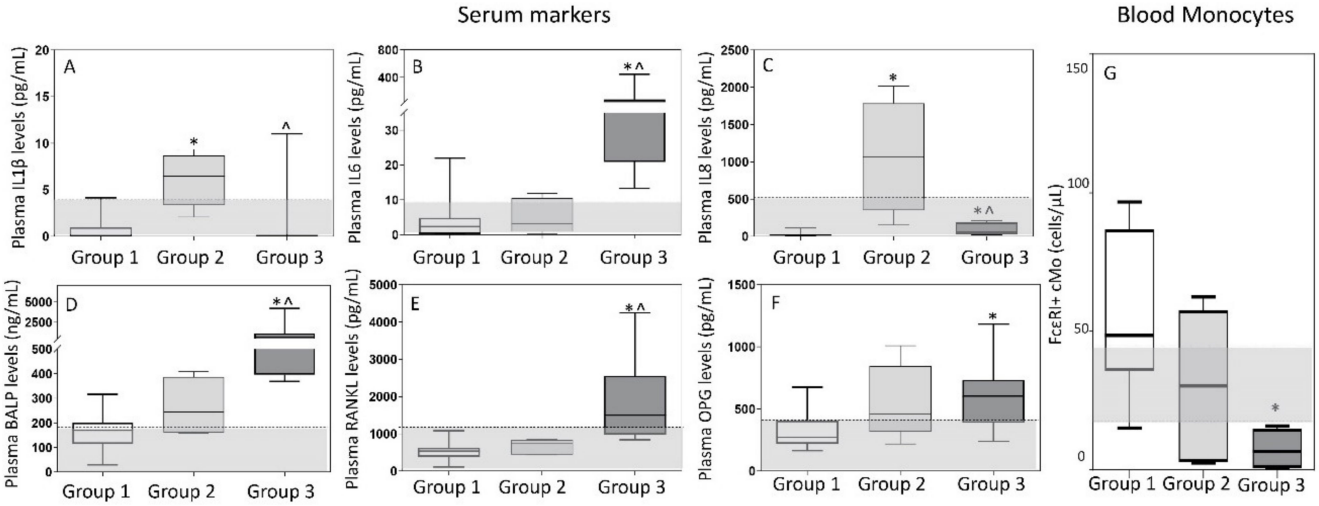

Figure 5. Soluble levels of cytokines (panels **A**–**C**) and bone-associated markers (panels **D**–**F**) in plasma and absolute FcεRI$^+$ cMo counts in blood (panel **G**) of MGUS, SMM, MM and HD classified by multivariate analysis into the T-distributed stochastic neighbor embedding (T-SNE)-defined groups 1, 2 and 3. In (panels **A**–**G**), notched boxes extend from the 25th to the 75th percentile values; the lines in the middle and vertical lines correspond to median values and the 5th and 95th percentiles, respectively. Normal range is represented by a gray area limited by a horizontal black dotted line. Statistical significant differences ($p \leq 0.05$) were observed vs. * group 1 or ^ group 2 after applying the Mann–Whitney non-parametric test (as the KS normality test showed that data for these variables did not follow a normal distribution). IL: interleukin; BALP: bone alkaline phosphatase; RANKL: receptor activator of the nuclear factor-kB ligand; OPG: osteoprotegerin; cMo: classical monocytes.

4. Discussion

At present, it is well-established that both the immune and bone microenvironment play a key role in the pathogenesis of PC neoplasms [4,5]. Among other immune cells [30–45], Mo [25,26,50] and macrophages [6,7,9] have emerged as key players in disrupting normal BM and bone homeostasis. This translates into increased levels in serum of multiple inflammatory cytokines [27,28] and markers [11–18,21,22] associated with increased bone resorption, particularly in MM [4,5]. In parallel, important advances have been achieved in the understanding of the maturation pathways leading to the production of Mo in BM and their heterogeneity in blood and BM, including the functional role of the many subsets of Mo identified so far [48,51–58]. Despite this, at present, there are limited data about the distribution of the distinct subsets of Mo in blood and BM of MGUS, SMM and MM patients, and the potential relationship with serum inflammatory and bone-associated biomarkers, known to be altered in these patients [11–18,21,22,27,28]. Here, we first investigated the distribution of Mo and monocyte subsets in blood and BM of a large cohort of MGUS, SMM and MM patients compared with age-matched HD. Subsequently, we determined the potential associations between the alterations identified in the blood and BM monocyte compartment and serum inflammatory and bone-associated biomarker profiles in a subset of the patients.

Overall, our results showed a progressively decreased production of monocytic precursors in both SMM and MM, but not MGUS. This translated into decreased counts of cMo in BM associated with lower counts of either recently produced CD62L$^+$ cMo in the blood of SMM and of all subsets of (CD62L$^+$, CD62L$^-$ and FcεRI$^+$) cMo in blood of MM, while MGUS cases showed a normal (or even slightly increased) production of cMo. In contrast, the more mature/differentiated iMo and end-stage ncMo were significantly increased in BM of both SMM and MM as well as MGUS patients, while their counts in blood were within the normal range (MGUS and SMM) or decreased (MM). Of note, among MM patients, cases at more advanced stages of the disease (e.g., ISS-III and RISS-III) showed lower percentages of monoblasts than ISS-I and RISS-I MM patients, together with higher percentages of CD36$^+$Slan$^+$ ncMo in BM. Interestingly, FcεRI$^+$ cMo were significantly more represented in BM of RISS-III vs. RISS-II. In addition, RISS-III cases also showed higher absolute counts of ncMo (at the expense of CD36$^-$Slan$^-$ ncMo). In blood, ISS-III or ISS-II MM cases had higher counts of blood circulating iMo and CD36$^-$Slan$^+$ ncMo vs. ISS-I MM cases.

Altogether, the above alterations might reflect early activation and increased production of Mo in MGUS, followed by chronic inflammation with a local increase in BM of more mature iMo and functionally exhausted ncMo. Subsequently, disruption of monocytic production in BM of SMM and MM patients would lead to a deficient production and release of recently formed CD62L$^+$ cMo into blood and thereby, decreased numbers of circulating cMo. Growth of the tumor PC compartment in BM would progressively favor a sustained increase of iMo and end-stage (functionally exhausted) inflammatory Slan$^+$ ncMo, together with inhibition in symptomatic MM, of the generation and/or accumulation of other subsets of CD62L$^-$, and particularly FcεRI$^+$ cMo. These results confirm and extend on previous observations that revealed quantitative alterations of cMo, iMo and ncMo, as well as of end-stage (Slan$^+$) ncMo in blood and BM of MGUS and MM [19,50]. In line with these findings, previous studies have shown increased numbers of iMo in BM of MM and demonstrated their pro-osteoclastogenic potential in ex vivo cultures. Altogether these findings highlight the potential relevance of iMo in the pathogenesis of bone disease in MM [19,59]. Despite this, these findings should be taken with caution and deserve further confirmation, as BM analyses were conducted in BM aspirated samples and not directly in core (biopsy) BM tissue specimens.

In order to better understand the functional implications of these (altered) patterns of monocyte production and maturation kinetics in the different BM and blood compartments, we further investigated the levels of several inflammatory cytokines in the serum of our patients. Overall, our results showed increased levels of IL1β, IL6, and/or IL8 in a fraction

of MGUS and all SMM and MM patients. In line with these findings, Martín-Ayuso et al. [28] have previously shown enhanced ex vivo secretion (vs HD) of proinflammatory cytokines, such as IL6, IL8, IL12, TNFα and IL15 by DC and ncMo in MGUS and MM patients in the presence of undetectable IL1β serum levels. Similarly, Bosseboeuf et al. [60] found increased levels (vs HD) of serum IL1β, IL6 and IL8 (out of 40 cytokines investigated) in a pooled series of 64 MGUS plus MM patients. More interestingly, when we considered MGUS, SMM and MM patients separately, increased serum IL1β was specifically found among MGUS and SMM patients, while increased serum IL8 levels were typically detected in SMM and MM cases, and elevated serum IL6 was almost restricted to MM, supporting the existence of different patterns of secretion of inflammatory cytokines in MGUS, SMM and MM. Of note, IL1β and IL8 have both been linked to tumor cell- and immune cell-associated senescence, and acquisition of a pro-inflammatory secretory phenotype (SASP), which would favor further genetic and chromosomal instability, with relatively limited effects on bone metabolism [61]. In contrast, previous studies have shown that IL6 is a proinflammatory cytokine also involved in bone remodeling [20], that once released to the bone matrix inhibits osteoblastic activity and induces production of RANKL, parathormone-related protein and prostaglandin E2, with a (synergistic) pro-osteoclastic effect with IL6 in promoting bone resorption [20]. Local production of parathormone and vitamin D further stimulate osteoblasts to produce more IL6 and RANKL in a positive feedback loop that further enhances osteoclastogenesis [20]. In line with these findings, our results showed a parallel increase in serum IL6, RANKL and BALP [17,18] restricted to MM, particularly to those MM patients who showed osteolytic lesions at diagnosis. However, in contrast with several previous studies, we could not confirm a simultaneous decrease in OPG serum levels in MM [14,15]. Despite this, the RANKL/OPG ratio was significantly increased in MM vs. both MGUS and SMM. Of note, despite the macrophage inflammatory protein (MIP)-1α also plays an important role in osteoclast formation with a potential for further enhancing RANKL and IL6 effects on osteoclasts, in this study we did not analyze MIP 1α levels in plasma [62]. In any case, based on the plasma levels of the IL1β, IL8 and IL6 cytokines and the BALP and RANKL bone-related markers, three clearly different profiles were identified among HD, and MGUS, SMM and MM patients. Thus, HD, most MGUS cases and a minority of SMM (low-risk)showed a normal cytokine and bone-marker serum profile, while a senescent-like pattern was observed among the remaining MGUS cases, most SMM patients and a minority of MM who showed no bone lesions, and a pro-inflammatory profile was restricted to MM patients who typically had osteolytic lesions. Of note, the later profile was associated with significantly decreased numbers of the FcεRI$^+$ subset of cMo in blood. In this regard, recent studies have shown that FcεRI$^+$ cMo are a functionally different subset of Mo with a unique immunomodulatory role, which act as the regulator of (allergic and potentially also other types of) inflammation [63,64]. If this holds true, an impaired function of this specific subset of immunomodulatory Mo might facilitate the transition in MM between a senescent-like and a more prominent pro-inflammatory (IL6-associated) microenvironment, associated with progressive emergence of bone disease [65]. However, further studies are required to establish the actual functional role of these cells in humans, and their potentially immunomodulatory effect in MGUS and SMM vs. MM.

5. Conclusions

Our results show an altered distribution of Mo subsets in BM of MGUS, SMM and MM, associated with a progressively decreased production of CD62L$^+$ cMo in SMM (blood), in addition to CD62L$^-$ (blood and BM) and FcεRI$^+$ cMo in MM (blood).

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6694/13/6/1454/s1, Figure S1: Distribution of total monocytes (panel A) and monocyte subsets (panels B-K) in blood of MGUS, SMM, MM and HD grouped by T-SNE (groups 1, 2 and 3), Figure S2: Distribution of monocytic precursors (total precursors, monoblasts and promonocytes) (panels A–C), total monocytes (panel D) and their subsets (panels E–N) in bone marrow of MGUS, SMM, MM and

HD grouped by T-SNE (groups 1, 2 and 3), Table S1: Clinical characteristics of MGUS, SMM and MM patients and HD.

Author Contributions: A.O., J.J.M.v.D. and B.D. designed the study; A.O., D.D., C.T., J.A. and W.B.L.v.d.B. designed the methodology to be applied; D.D., C.T., A.M., A.P.-P. and P.A. performed the experiments; N.P., B.P., F.S., A.R. and S.M. collected informed consents, samples and patient data; D.D., L.S.-F., J.F.-M. and N.P. reviewed all data; D.D. analyzed the data and A.O. revised the results of data analysis; D.D. and A.O wrote the manuscript. All authors have revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Biomedical Research Networking Center Consortium-CIBER-CIBERONC (CB16/12/00400, CB16/12/00369 and CB16/12/00233-FEDER), PI13/01412-FEDER, from the Instituto de Salud Carlos III (ISCIII), Ministerio de Economía y Competitividad, Madrid, Spain; the European Research Council (ERC) under the European Union's Horizon 2020 Research and Innovation Programme (grant agreement ERC-2015-AdG 695655 (TiMaScan); and the Black Swan Research Initiative of the International Myeloma Foundation (Los Angeles, CA, USA), (grant IMF13/IMF16).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved (on the 26th of February 2007) by the Ethics Committee of the University Hospital of Salamanca/IBSAL (Health Area of Salamanca, Salamanca, Spain)".

Informed Consent Statement: All participants gave their informed consent to participate in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The Authors thank Quentin Lecrevisse for support with Infinicyt™ tools.

Conflicts of Interest: J.J.M.v.D., and A.O. report being the inventors on patent PCT/NL2012/050132 (Methods and means for monitoring disruption of tissue homeostasis in the total body). D.D., J.A., C.T., W.B.L.v.d.B., J.J.M.v.D. and A.O. report being the inventors on the patent PCT/NL2020/050688 (Means and methods for multiparameter cytometry-based leukocyte subsetting). J.J.M.v.D. and A.O. report to be chairmen of the EuroFlow scientific foundation, which receives royalties from licensed patents, which are collectively owned by the participants of the EuroFlow Foundation. In addition, J.J.M.v.D. and A.O. report an Educational Services Agreement between BD Biosciences and their universities. The other authors declare that they have no relevant conflicts of interest.

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Discussion

Over the past few decades, significant efforts have been made to investigate and better understand the role of DCs and Mo in the immune system in general, and the immune response, in particular. For this purpose, important efforts have been made based on multiple integrated techniques to better identify, define and characterize the different types of the above cells include, leading to a deeper knowledge about both their functions and immunophenotypic profiles. Thus, recent studies have revealed that both DCs and Mo, include highly diverse cell populations (8, 27, 46, 57, 62, 67-69, 87), while the specific functional consequences at the level of the molecular and biological mechanisms responsible for such heterogeneity, still remaining to be fully understood.

The general objective of the research performed in this doctoral thesis focused on achieving a more comprehensive and rational understanding of the role of the different populations of DCs and Mo in healthy individuals (throughout life), as a frame of reference to better identify their alteration in different diagnostic subtypes of PC neoplasms.

DCs are long known for their ability to capture and present antigens to T cells and initiate adaptative immune responses, while blood Mo are viewed as BM-derived precursors of both tissue Mϕ and DCs, which are key cells in immune surveillance, inflammation and tissue repair (13-17). By investigating the immunophenotypic heterogeneity of DCs and Mo, we aim at shedding light on the specific subsets those two cell populations include (particularly in blood), identify marker combinations for their clearcut identification, and provide some clues about their potentially unique functional differences, for a better understanding of their role in normal homeostatic and various disease conditions, such as autoimmune disorders, infectious diseases, and inflammatory conditions and cancer.

For this purpose, in this study we first investigated (more in depth) the immunophenotypic profiles of DCs and Mo, to subsequently establish the normal phenotypic features and ranges for these cells in blood throughout human life, and their specific patterns of alteration present in patients diagnosed with different PC neoplasm subtypes.

Immunophenotypic characteristics of normal DCs and Mo and their (sub)populations

In order to investigate in depth the immunophenotype of different populations of blood DCs and Mo, we tested a large panel of 72 monoclonal antibodies that had been submitted to the DC section of the 10th Human Leucocyte Differentiation Workshop (HLDA10-DC-task), based on samples from a cohort of healthy adults.

Overall, our results revealed distinct expression profiles for the different molecules investigated across different subsets of PB DCs and Mo, in parallel to some common expression profiles, depending to a certain extent, from the functional group of the molecules investigated and/or the specific subset of antigen-presenting cells analyzed. A particular focus was placed on the C-type lectin-like receptors (CLRs), a group of more than a thousand receptors which are predominantly expressed on phagocytes (200, 201). These receptors are critical for antigen capture and subsequent activation of intracellular signaling cascades, as they trigger various cellular and immunological responses required for maintaining the normal homeostasis of the immune system and regulating the immune responses during infection (201, 202). In addition, CLRs are also known to play important roles in autoimmune diseases, allergy, and cancer, showing a high efficacy as PRRs due to their ability to bind specific carbohydrates associated with human pathogens (201, 202). Upon encountering their ligands derived from "non-self" (pathogen-associated molecular patterns - PAMPs), "damaged self" (damage-associated molecular patterns - DAMPs), or "altered self" (tumor-associated

molecular patterns -TAMPs), CLRs activate and/or modulate specific downstream immune functions (201, 202).

Interestingly, pDCs showed lack of expression of any of the CLRs evaluated, except for partial and low expression levels of the inhibitory molecules CD367 (CLEC4A/DCIR) and CD371 (CLC12A/MICL/CCL-1), respectively. At present it is well-established that both CD367 and CD371 play a crucial role in the interaction between innate and adaptive immunity during tolerogenic immune responses (201, 203). The former molecule (CD367) also facilitates antigen cross-presentation by DCs, while CD371 senses dead cells and regulates inflammation in response to cell death (201, 203). In contrast to pDCs, cDCs expressed both CD367 and high levels of CD371, together with partial and low expression of CD302. This later molecule has recently attracted attention as a potential immunotherapeutic target, particularly in acute myeloid leukemia, due to its involvement in immune regulation and its expression on antigen-presenting cells (204). Regarding normal blood Mo, expression of these molecules by various subsets was observed, but with distinct immunophenotypic profiles. For example some Mo subsets (cMo and iMo) showed a relatively high reactivity for CD367, whereas ncMo, and particularly $Slan^{+}$ ncMo, displayed lower levels of this tolerogenic marker. This later subset of ncMo has been associated with the induction of potent pro-inflammatory Th1 and Th17 immune responses, in agreement with the low expression levels of CD367, suggesting a more mature phenotype for this subset of Mo (56), since CD367 expression on DCs has been shown to decrease following maturation (205).

Like for the above tolerogenic CLRs, heterogeneous immunophenotypic profiles were also found in normal blood Mo and DC for CLRs which act as activation-related molecules, like CD368, CD369 and CLEC5A. Thus, expression of CD368 (CLEC4D/Dectin-3), CD369 (CLEC7A/Dectin-1) and CLEC5A was restricted to Mo, while absent in DCs, higher levels being

observed in cMo and iMo compared to ncMo. These findings are in line with previous observations (206-209) that show that all these activating receptors are associated with specific Mo-dependent immunological responses. Thus, CD368 acts as a receptor for mycobacteria (210), CD369 functions as a receptor for beta-glucans from fungal cell walls (211) and CLEC5A has been proven to be a receptor for the Dengue virus (207). Stimulation of these Syk-coupled CLRs leads to signaling cascades that trigger Mo-related functions such as phagocytosis, production of ROS and secretion of proinflammatory chemokines and cytokines (206).

The phagocytic-related proteins FPR1 and FPRL1/FPRL2, as well as the myeloid-cell-associated molecules MAIR II and TREM2, together with the DC-related marker CD85h, all showed restricted expression to Mo and, to a lesser extent, cDCs, with consistently lower levels of expression in normal blood ncMo compared to both cMo and iMo. Altogether, these finding suggests that ncMo may represent a final stage of maturation of monocytic cells where antigen presentation and phagocytosis events mediated via CLRs, Ig-like V-type receptors (e.g., TREM-2) or G protein-coupled receptors (e.g., FPR1) are no longer feasible. However, these data might also reflect that ncMo are more prone to undergo apoptosis and cell death. Of note, selective expression of CD85g was observed only on pDCs, where this receptor acts as a negative modulator of IFN-α production, potentially as a homeostatic regulatory mechanism on (relatively immature) circulating blood pDCs (212).

In our study we also investigated the pattern of expression of normal blood DCs and Mo of T-cell immunoglobulin mucin receptors (TIMs), particularly TIM-1 and TIM-3 (CD365 and CD366 respectively, according the CD code nomenclature) which are typically expressed on activated/effector and exhausted T cells (213, 214). While CD365 can associate with the T-cell receptor complex (TCR/CD3) and mediate T-cell activation signals (215), CD366 inhibits

Th1-mediated autoimmune and alloimmune responses, at the same time it promotes immune tolerance (213). Additionally, these TIM receptors (TIM-1 and TIM-3) have been implicated in phagocytosis of apoptotic cells by antigen-presenting cells (216), but none of the DC and Mo populations present in blood of healthy adults expressed CD365. In contrast, cDCs and Mo, (but not normal pDCs) expressed CD366 together with other T-cell inhibitory molecules such as CD101 and ULBP-3. Of note, the levels of expression of these later three molecules were significantly higher in cDCs than in Mo, suggesting a more pronounced inhibitory receptor molecule-associated profile for cDCs *vs* the Mo.

Among the cell surface molecules investigated, we also explored the expression of the chemokine/cytokine receptors CD135 (Flt3) and CD195 (CCR5). Our results showed that CD135 which plays a critical role in cell proliferation and differentiation (217), was strongly expressed on cDCs and dimly positive on Mo, while CD195 was found on cDCs and pDCs, and at lower expression levels also on Mo. These observations suggest that CD135 and/or CD195 may have preferential roles in immune-dependent responses mediated by cDCs and/or pDCs than those where Mo are involved. Finally, the purinergic receptor P2X7, which mediates cellular responses to extracellular ATP and triggers downstream events leading to cell death by apoptosis(218), was a highly expressed marker on iMo and Slan$^-$ ncMo, further supporting the notion that ncMo could represent an end-stage maturation subset of Mo more prone to undergo apoptosis. Interestingly, slight differences were also observed within the subset of ncMo based on the expression of Slan which, along with the divergent GEPs and immunophenotypes of Slan$^-$ and Slan$^+$ ncMo (68), suggest potential functional differences between these two populations of ncMo.

Altogether, our findings contribute to the identification of new markers associated with specific populations of normal blood DCs and Mo, pointing out also to the different functional behavior and role of these distinct populations of antigen-presenting cells.

Distribution of different subsets of circulating Mo in CB and PB through human life and their (lymphoid) tissue counterparts

Based on all the above, and additional data from the literature, at present it is well established that Mo represent a heterogeneous phenotypic and functional cell population, both in PB and in the BM, which undergoes changes in various disease conditions such as infection, autoimmunity, respiratory and cardiovascular diseases, and inflammatory disorders (56, 219-221). Despite this, at the moment of starting this doctoral work, no systematic study had been reported in the literature in which normal reference ranges for the different subsets of blood Mo had been defined throughout human life, from CB and newborns to the elderly. The second objective of our study address this gap, by determining the numerical distribution of six different subsets of Mo in blood across different ages, and thereby defining a frame of reference for subsequent (future) identification of potential disease-associated (altered) profiles. In order to gain further insight into the potential tissue relationship between cMo, iMo and ncMo, we also analyzed the distribution of these subsets of Mo in normal human BM, LN and spleen, for better understanding of the trafficking of these cells throughout the human body.

Overall, our results demonstrated that the distribution of circulating Mo subsets varies significantly throughout life, with a relevant peak after birth for the first six months of life, followed by a decrease until the age of 8 to 13. These fluctuations likely reflect the greater production and release of $CD62L^+$ cMo from the BM into the bloodstream (52), which is necessary

to populate the various tissues throughout the human body, similarly to what has been previously reported for B cells (222). Interestingly, a second peak of $CD62L^+$ cMo was observed during adolescence and in younger adults, followed by a decline until the age of 30 to 50 years, which might be related to the changes that occur in parallel to growth during puberty and adolescence. Generally, these changes and peaks might just reflect the early-in-life requirement for sufficient numbers of Mo to fill the distinct (growing) tissues throughout the body. In turn, iMo and ncMo exhibited different kinetics in blood than those of cMo, peaking at later stages in life. Thus, iMo reached their maximum levels in newborns, while ncMo, particularly $Slan^+$ ncMo, progressively increased from birth (e.g., CB) until six months of age, prior to their progressive decline until the age of 30-50 years, following a pattern similar to $CD62L^+$ cMo. Beyond this age range, all Mo subsets showed an increase in blood. Overall, these results confirm and extend on previous observations, which focused on CB and adult blood samples from subjects within specific age ranges (52, 53, 55), but did not provide detailed subsetting of cMo and ncMo into their $CD62L^+$ and $CD62L^-$ or $Slan^-$ and $Slan^+$ cells, respectively. The increase in $CD16^+$ iMo and ncMo observed in the earlier periods of life and after the age of 50-60 years, is also consistent with previous data from the literature (53, 55), and might reflect an increased tissue turnover, apoptosis (223) and immunosenescence (54) during those periods. Of note, in a subset of samples we also investigated the presence and numerical distribution of $FcεRI^+$ Mo which had been first identified in blood of atopic subjects (224). Here, we confirm the distinct and unique $CD14^{hi}CD16^-$ phenotype of these cells, and their systematic presence in every blood sample analyzed from healthy subjects.

Previous reports suggested that statistically significant lower numbers of blood iMo might be found in women *vs* men, but we did not find consistent sex differences in the distribution of this population and other populations of blood Mo, except for adults aged 51-60 years (225).

Based on the overall distribution pattern of Mo subsets in blood throughout life, the findings support the notion that iMo and ncMo might represent more advanced stages of maturation of Mo, compared to cMo that peaked earlier in life, in line also with their senescent phenotype. Overall, these results are in agreement with previous data demonstrating a direct relationship between these (major) subsets of blood Mo. Actually, it has been recently shown -after analyzing the fate of isolated cMo from HD grafted into humanized mice- that human cMo do have the potential to give rise (e.g., differentiate) to iMo and ncMo (48). Whether such maturation steps occur in blood or outside the bloodstream was recently investigated *in vivo* with 6,6-$^{2}H_{2}$ glucose infused to healthy individuals and subsequently monitored in blood-purified cMo, iMo, and ncMo (47). These studies revealed that ^{2}H-enriched DNA peaks in cMo at day +3-4, while for iMo and ncMo it reached its maximum levels at day +4 and day +8 post-infusion, respectively (48). These findings support the notion that the differentiation of cMo into intermediate and ncMo most likely occurs outside the blood compartment, rather than inside bloodstream.

To investigated the potential tissue relationship between the three major compartments of blood cMo, iMo and ncMo, we further investigated their presence and distribution in secondary lymphoid tissues. Our results confirmed the presence of all major blood Mo subsets in BM, LN and spleen. In addition, monocytic precursor cells were also found at relatively high percentages in normal BM. Interestingly, the relative distribution of the two more mature Mo subsets varied significantly in LN and spleen compared to both blood and BM. Thus, $CD62L^{+}$ cMo were the predominant subset in blood and BM, while $CD62L^{-}$ cMo predominated in LN and spleen, where higher percentages of iMo compared to blood and BM, were also found.

Recruitment of blood Mo to distinct tissues throughout the body is primarily controlled by adhesion to the vascular endothelium (80). Among other molecules, CD62L has been shown to play an important role in early Mo adhesion and diapedesis (226), accounting for 90% of the leucocyte forces involved in rolling on the endothelial wall *in vivo* (227). Upon adhesion to the endothelium and diapedesis, CD62L expression is down-regulated on cMo (228). Therefore, the predominance of $CD62L^-$ cMo in LN and spleen *vs* blood and BM, where $CD62L^+$ cMo were by far the most represented subset of mature Mo, most likely reflects the more mature nature of cMo in the former lymphoid tissues as compared to BM and blood. Similarly, the greater proportion of iMo in LN and spleen, might also reflect a greater rate of production of these cells outside the blood compartment (e.g. in lymphoid tissues), further supporting the notion that maturation of cMo into iMo might preferentially occur outside the bloodstream. Of note, iMo have been reported to be the most efficient monocytic subset with respect to antigen processing and presentation (45, 56). Furthermore, iMo express high levels of CD192 (CCR2) which would favor its greater representation in lymphoid tissues (*vs* blood and BM), to where they would be actively recruited for triggering early T-cell responses (219). Altogether, these observations support a major functional role for iMo in LN and spleen, rather than in blood.

In contrast, ncMo were found to be more prevalent in blood and spleen, while in BM and LN they were minimally represented. Notably, $Slan^+$ ncMo were absent or present at very low numbers in LN. These findings suggest that iMo generated in (distinct) tissues might migrate through the LN to blood, where they generate ncMo. In line with this hypothesis, ncMo have been considered end-stage monocytic cells with decreased expression of phagocytic receptors, except CD16, a molecule that has been recently involved in removal of aged (i.e. IgG-coated) erythrocytes (229). If this holds true, the higher frequency of end-stage ncMo observed in spleen

vs LN might suggest that spleen (via blood) could also be the final destination for these cells before they die. In line with this hypothesis, recent GEP studies have shown that Slan^{+} ncMo display higher levels of expression of the ubiquitin C transcript, which, among other cellular functions, is associated with cell-signaling, transcription and activation of apoptosis (220). In addition, this subset of ncMo also expresses high levels of TNFα and CX3CR1, together with lower amounts of CD192, which contrasts with the expression pattern of these molecules in Slan^{-} ncMo (220). Altogether, these findings suggest that cMo might migrate from BM to blood and from there to distinct lymphoid (and potentially also other) tissues throughout the body, where they lose CD62L and differentiate into iMo; iMo would subsequently recirculate via the lymph system and sequentially give rise to Slan^{-} and Slan^{+} ncMo, which re-enter the circulation, and reach the spleen. This is consistent with the in vivo 6,6-2H2 glucose monocyte tracking studies (47), which demonstrated that cMo are the first monocytic subset to appear in PB, followed by iMo and ncMo, supporting the notion that, at least in part, the differentiation steps between these subsets of Mo might occur outside blood.

Altogether, our results demonstrate that the number of circulating blood Mo and their subsets varies significantly throughout life, providing a frame of reference for studies in distinct disease conditions. The differential distribution of the distinct subsets of Mo evaluated in different human (lymphoid) tissues, highlights the highly-dynamic trafficking of these cells throughout the body.

Distribution of different populations of Mo in PB and BM of MGUS, SMM and MM patients and its relationship with immune and bone serum marker profiles.

PC neoplasms, from MGUS to MM, share a common origin in a neoplastic PC that leads to a heterogeneous clinical behavior associated with a progressively higher severity of disease reflected by the functional impairment of different tissues such as the BM (hematopoiesis), the kidney and the bone, among other tissues. (142). One major contributing factor to disease progression in PC neoplasms relies on the enhanced osteolytic activity associated with bone destruction observed in the more advanced and malignant disease subtypes (i.e., MM) (230). At present it is well-established that both the bone microenvironment and immune cells such as Mo (181, 198, 231) and Mϕ (179, 232, 233), as well as soluble components like cytokines (182, 199), bone-derived proteins/products (156-163, 166, 167), and CXCR4 (142, 231), play an important role in the pathogenesis of PC neoplasms. Despite all the above, at the moment of starting this work, the specific distribution of Mo subsets in blood and BM of MGUS, SMM, and MM, and their potential association with the altered immune and bone profiles observed in these patients, had not been thoroughly investigated. Here, we analyzed the distribution of different subsets of Mo in blood and BM of patients with different diagnostic subtypes of PC neoplasms and correlated their alterations with the serum immune and bone biomarker signatures.

Overall, our results revealed differences in the distribution of blood Mo subsets among patients with distinct diagnostic subtypes of PC neoplasms, particularly as regards cMo. Thus, significantly decreased levels of $CD62L^+$ cMo and of both $CD62L^+$ and $CD62L^-$ cMo, as well as $Fc\varepsilon RI^+$ cMo were found among SMM and MM patients (compared to HD and to MGUS or both MGUS and SMM cases), respectively. In contrast, MGUS patients exhibited normal levels of all

cMo subsets (similar to those of HD). In addition, SMM and MM patients also showed lower percentages of monocytic precursors in the BM (once compared to MGUS cases), together with decreased percentages of monoblasts and promonocytes (*vs* MGUS and SMM patients) in the BM. Interestingly, the overall percentage of the more mature Mo was significantly increased in BM of MGUS cases compared to HD, but not in SMM or MM patients. These alterations may reflect an attempt to control (tumor-associated) chronic inflammation, linked to a higher production of cMo in the earlier stages of the disease (MGUS), followed by a deficient production and release of monocytic precursors and $CD62L^+$ cMo from BM to blood in SMM, that in active MM would also be extended to the $CD62L^-$ and $FcεRI^+$ subsets of cMo. Of note, a similar pattern was observed in blood for the more advanced stages of maturation of Mo, with decreased ncMo counts in MM, particularly at the expense of the $CD36^-Slan^-$ and $CD36^+Slan^+$ subsets, (once compared to HD and MGUS) in combination with the $CD36^-Slan^+$ subset (when compared to SMM patients). In contrast, BM iMo and ncMo were increased in all patient groups (MGUS, SMM, and MM) once compared to HD. These later findings would support the existence of an increased migration of ncMo (along with iMo) towards the BM via the CXCR4-CXCL12 axis-(94, 142, 231), suggesting the involvement of these two subsets of Mo in the pathophysiology of SMM, MM, and to a lesser extent also, MGUS. Of note, these results are consistent with previous observations about the quantitative alterations in total cMo and ncMo (including $Slan^+$ ncMo) that exist in the blood and BM of MGUS and MM patients (231), and they extend on those findings by providing more detailed subset analysis, including new data about SMM patients.

In previous studies by other groups, an association between the altered distribution of Mo subsets in blood and alterations in the cytokine network detectable in the serum, had already emerged among patients with monoclonal gammopathies (182, 231). Thus, increased

ex vivo secretion of proinflammatory cytokines (e.g., as IL-6, IL-8, IL-12, TNFα, and IL-15) by ncMo, in the absence of detectable IL-1β had already been reported in MGUS and MM compared to HD (182). In parallel, contradictory increased serum levels of IL-1β, in addition to higher amounts of IL-6, and IL-8 have also been found in both MGUS and active MM patients compared to HD (234). Our results confirm and expand on these findings by demonstrating increased (*vs* HD) serum IL-1β and IL-8 levels in MGUS and SMM patients and in SMM and MM patients, respectively. Furthermore, we observed significantly elevated serum IL-6 levels in MM patients compared to all other groups, including HD, MGUS, and SMM, all of which showed normal levels of this cytokine. More than a proinflammatory cytokine, IL-6 also plays a role in bone remodeling as a pro-osteoclastic factor, promoting bone resorption and inhibiting osteoblastic activity (165). Consistent with this, our results showed increased serum levels of RANKL (a mediator of osteoclastogenesis) in parallel to IL-6 in MM patients. In addition, in MM we also found elevated serum levels of markers associated with bone formation, such as BALP, in line with previous findings by others (162, 163). Interestingly, once compared to MGUS cases, MM patients also displayed elevated levels of OPG (osteoprotegerin) which is in contrast with previous studies reporting decreased serum OPG levels in MM (156-160). Such discrepancy might be due to the inclusion of MM patients that presented with and without osteolytic lesions. Finally, patients with different PC neoplasms also showed elevated serum levels of CXCR4, a chemokine receptor known to be expressed by both $Slan^+$ ncMo (94) and migrating tumor PC (165, 231). Of note however, no significant differences were observed between MGUS, SMM and active MM patients for this later marker.

Multivariate analysis (TSNE: t-distributed stochastic neighbor embedding) based on the soluble levels of IL-1β, IL-6, IL-8, BALP, RANKL, OPG, and CXCR4 levels in the serum,

confirmed the existence of a great heterogeneity both among and within the MGUS, SMM and MM patient groups, pointing out the existence of different clusters, independently of the initial diagnose. Interestingly, when we repeated the multivariate (i.e., TSNE) analysis including the counts for each specific subset of Mo in blood and BM, together with the serum levels of the different cytokines, CXCR4 and bone markers investigated, three clearly distinct groups of subjects emerged which included: 1) HD and most MGUS cases; 2) SMM and MM patients who showed no osteolytic lesions; and, 3) MM patients with osteolytic lesions. Further validation studies in larger series of patients are needed to determine the actual clinical impact of these preliminary results.

Altogether, our findings provide novel insight into the pathophysiology of monoclonal gammopathies, by revealing significant differences in the distribution and kinetics of Mo subsets in blood and BM of MGUS, SMM, and MM patients, in association with unique immune and bone marker profiles in the serum. Further validation studies in larger patient cohorts of patients are warranted to fully understand the clinical implications of these preliminary data.

Conclusions

Regarding the first objective of this study, focused on the in-depth characterization of the immunophenotype of different major populations of blood DCs and Mo, based on new DC-associated markers submitted to the DC section of the Tenth Human Leucocyte Differentiation Antigen Workshop:

1. Blood DCs, as well as cMo and iMo, showed higher expression of both the inhibitory CLRs CD367 and CD371 and the activation CLRs CD368, CD369 and CLEC5A, when compared to blood pDCs and ncMo (and particularly, Slan^{+} ncMo). These findings, together with the unique pattern of expression of (several) phagocytic- and myeloid-related markers restricted to cMo and iMo, and at lower levels, ncMo, suggest that the antigen presentation and phagocytosis functions are more closely related to DCs and to both cMo and iMo, respectively, while ncMo might represent a final stage of maturation of monocytes more prone to apoptosis, as further supported by their pattern of expression of the purinergic receptor P2X7.

2. None of the blood circulating populations of DCs and Mo expressed detectable levels of the T-cell immunoglobulin mucin receptor 1 protein CD365, whereas CD366 (the T-cell Ig mucin receptor 2) was strongly expressed on cDCs (but not normal pDCs) and Mo, suggesting more immature features associated with a more pronounced inhibitory receptor molecule-associated profile for blood cDCs and Mo, than pDC.

With respect to the second objective of our study, aimed at determining the normal distribution and phenotype of different subsets of human DCs and Mo in human blood throughout life (from CB and newborns to the elderly), and to investigate the relationship between these populations of blood Mo and their corresponding BM, LN and spleen counterparts:

3. In CB samples, higher counts of cMo were detected, while iMo/ncMo peaked later in newborns, with a clear predominance of immature $Slan^-$ ncMo over the more mature $Slan^+$ subset until the age of 5 years, and a subsequent decrease through childhood and young adults. The different kinetics observed for the distinct subsets of blood Mo, might reflect the greater early-in-life requirement of sufficient numbers of Mo to fill the distinct (growing) tissues throughout the body, associated with a progressively greater representation in blood of the two more mature populations of (potentially tissue-derived) Mo.

4. Significantly lower percentages of $CD62L^+$ cMo, at the expense of greater frequencies of $CD62L^-$ cMo and iMo, were found in LN and spleen, compared to blood and BM, where $CD62L^+$ cMo predominated. These observations might reflect the more mature nature of cMo that populate secondary lymphoid tissues *vs* blood and BM. Similarly, the greater proportion of iMo in LN and spleen (*vs* blood and BM), might also reflect a greater rate of differentiation of cMo toward iMo outside blood, further supporting the notion that maturation of cMo into iMo might preferentially occur outside the bloodstream.

5. Surprisingly, Slan^{+} ncMo were highly represented in blood and the spleen, while virtually absent in LN and present at minimal levels in BM. Altogether, these results suggest that iMo generated in different tissues throughout the body might migrate through the LN to blood, where they generate ncMo that might specifically home in the spleen as end-stage Mo.

6. Blood Mo expressing FcƐRI^{+} represented a minor population of all blood Mo with a distinct and unique CD14^{hi}CD16^{-} cMo-associated phenotype, which was systematically present in CB and peripheral blood samples of all individuals included in this study, while absent in normal LN. The specific functional role of this new minor subpopulation of classical Mo, remains to be elucidated.

Regarding the third objective, focused on the identification of potentially altered numerical and phenotypic patterns of the different populations of Mo in PB and BM of MGUS, SMM and MM patients, and its relationship with immune and bone serum marker profiles:

7. Patients diagnosed with SMM and MM exhibited significantly decreased levels of either CD62L^{+} cMo alone or both CD62L^{+} and CD62L^{-} cMo together with FcεRI^{+} cMo, respectively. In turn, MGUS patients, but not SMM and MM cases, showed increased percentages of cMo in BM. Altogether, these results point out to an increased production of cMo in BM of MGUS patients in an attempt to control the pre-established chronic inflammation microenvironment, followed by a decreased

production and release to blood of BM matured $CD62L^+$ cMo, and both of $CD62L^+$ cMo and $CD62L^-$ cMo as well as $Fc\varepsilon RI^+$ cMo, in SMM and active MM patients, respectively.

8. BM iMo and ncMo were found to be increased in all groups of patients diagnosed with PC neoplasms studied, compared to HD, supporting an increased migration of ncMo and iMo toward the BM, with a potentially relevant role for both subsets of Mo in the pathophysiology of SMM, MM and (to a lesser extent) MGUS, as part of the altered tumor PC microenvironment in the BM.

9. In parallel to the numerical alterations described above, blood and/or tissue monocytes/macrophages also appear to be functionally altered in patients presenting with distinct diagnostic subtypes of PC neoplasms. Thus, increased levels (*vs* HD) of serum IL1β alone, of both IL1β and IL8, and of IL1β, IL8, IL6, and the bone biomarkers RANKL, BALP and osteoprotegerin, were found in MGUS, SMM and MM, respectively, leading to a clear cut definition of three distinct groups of patients whose clinical significance deserves further studies in larger cohorts of patients: i) MGUS cases with a similar profile to HD; ii) SMM patients and MM cases who show no osteolytic lesions; and finally, c) MM patients presenting with typical osteolytic lesions.

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TÍTULO: Caracterización de las células dendríticas y del sistema monocito-macrófago a lo largo de la vida: alteraciones en las gammapatías monoclonales

INTRODUCCIÓN

Los monocitos (Mo) y los macrófagos (Mϕ), junto con las células dendríticas (CDs), forman parte del sistema mononuclear fagocítico, compuesto por células inmunes funcionalmente relacionadas entre sí ("fagocitos mononucleares"), que desempeñan un papel esencial como células presentadoras de antígenos (CPA) en la conexión entre la respuesta inmune innata y la adaptativa. Hoy se reconoce que tanto los Mo/Mϕ como las CDs están integradas por un número significativo de diversas subpoblaciones celulares, heterogéneas y con distintas funciones efectoras e inmunomoduladoras, que constituyen una red celular compleja capaz de integrar múltiples señales ambientales, generando inmunidad o tolerancia. Hasta la fecha se han identificado dos subpoblaciones mayoritarias de CDs humanas circulantes en sangre: las CDs plasmocitoides (CDp) y las CDs convencionales (CDc), también conocidas como CDs mieloides (CDm). Del mismo modo, se han descrito diversas subpoblaciones de Mo con perfiles fenotípicos y funciones diferentes en sangre humana, y que incluyen los Mo clásico (cMo), intermedios (iMo) y no clásicos (ncMo).

Aunque nuestro conocimiento sobre las CDs y los Mo ha aumentado considerablemente en los últimos años, todavía seguimos sin comprender completamente la posible relación madurativa que pueda existir entre las distintas poblaciones de CDs y de Mo detectables en sangre y otros tejidos, y las diferencias funcionales existentes entre ellas. Además, no disponemos de información acerca de la distribución de las diferentes subpoblaciones de Mo y CDs (y de sus fenotipos) en sangre a lo largo de la vida, desde el nacimiento a la vejez. La definición de los rangos normales de referencia de Mo y CDs en sangre, asociados a la edad, constituye una pieza de información esencial para poder identificar sus alteraciones y la naturaleza de las mismas en distintas enfermedades de base inmunológica, neoplásica o de otra índole. En este sentido, actualmente disponemos de información limitada disponible sobre la distribución de las diferentes poblaciones de Mo y CDs en sangre y médula ósea (MO) de sujetos sanos, así como en diferentes situaciones patológicas, incluidas aquellas en las que se ha referido que estas células inmunológicas desempeñan un papel relevante, como ocurre en las neoplasias de células plasmáticas (CP).

Las neoplasias de CP son un grupo heterogéneo de trastornos neoplásicos con diferentes niveles de infiltración de la MO, sangre y/o otros tejidos por CP tumorales, asociadas a un comportamiento clínico variable, que incluyen la gammapatía monoclonal de significado incierto (GMSI), el mieloma múltiple quiescente (MMQ) y el

MM activo; se ha visto que en estas enfermedades las células del sistema inmune innato, como los Mo/Mϕ y las CDs, desempeñan un papel importante en el crecimiento de las células tumorales, su plasticidad y los mecanismos de escape del tumor de la vigilancia inmunológica. El conocimiento preciso de las alteraciones específicas de las diferentes poblaciones de Mo de sangre y MO de pacientes diagnosticados de GMSI, MMQ y MM, y la posible relación existente entre estas alteraciones y otros marcadores séricos relacionados con el entorno tumoral y el sistema inmunológico, podrían contribuir a una mejor comprensión de las diferencias clínicas y biológicas observadas entre los pacientes con diferentes subtipos diagnósticos de neoplasias de CP.

OBJETIVOS

Partiendo de lo anteriormente expuesto, en esta tesis doctoral nos propusimos como objetivo general, investigar en mayor profundidad el fenotipo de las CDs y Mo humanos y la cinética a lo largo de la vida de sus diferentes poblaciones en sangre respecto a MO, ganglio linfático y bazo, con el fin de poder identificar mejor sus alteraciones en pacientes con diferentes neoplasias de CP (i.e., GMSI, MMQ y MM). Para lograr este objetivo general, abordamos tres objetivos específicos:

1. Analizar en detalle el fenotipo de diferentes poblaciones de CDs y Mo humanos circulantes en sangre, empleando un amplio panel de (nuevos) anticuerpos monoclonales sometidos a evaluación por parte del *"Tenth Human Leucocyte Differentiation Antigen Workshop* en la sección de CDs *(HLDA10-DC task)".*

2. Investigar la distribución de diferentes poblaciones de Mo circulantes en sangre a lo largo de la vida, desde el cordón umbilical y el recién nacido hasta la edad adulta y los ancianos, con el fin de establecer rangos de referencia normales de acuerdo con la edad y el sexo, y comprender mejor la relación existente entre los compartimentos celulares de Mo circulantes en sangre y los Mo/Mϕ presentes en MO, ganglio linfático y bazo.

3. Identificar alteraciones en la distribución y fenotipo de las diferentes poblaciones de Mo presentes en la MO y la sangre periférica (SP) de pacientes con distintas categorías diagnósticas de neoplasias de CP (i.e., GMSI, MMQ y MM), y su posible contribución, junto a un amplio panel de citocinas séricas y de otros marcadores óseos, para definir distintos perfiles de alteración de la interacción entre la CP neoplásica, el sistema inmune, y el metabolismo óseo, en cada entidad diagnóstica estudiada.

MATERIAL, MÉTODOS Y RESULTADOS

Características fenotípicas de las células dendríticas y monocitos en sangre humana

Si bien en los últimos años hemos avanzado considerablemente en la comprensión del papel funcional de las diferentes poblaciones de CDs y de Mo, también se ha puesto de manifiesto que hay una gran heterogeneidad dentro de ambos compartimientos de CPA; por tanto se requieren estudios adicionales para poder definir mejor su función. En este trabajo realizamos una caracterización inmunofenotípica detallada del compartimento de CPA (CDs y Mo) circulantes; para ello, estudiamos muestras de SP de cinco adultos (voluntarios) sanos mediante citometría de flujo de ocho colores, empleando un panel amplio de 72 anticuerpos monoclonales, clasificados globalmente en siete nuevos *clusters* de diferenciación (CD), siguiendo las recomendaciones del *10th Human Leucocyte Differentiation Antigen Workshop* (sección de CDs). Nuestros resultados mostraron que las CDp constituían la única población de CPA de SP que expresaba CD85g y CD195, mientras que carecían del resto de las moléculas investigadas. Por el contrario, las CDm expresaban receptores inhibidores de lectinas de tipo C y otras moléculas asociadas a respuestas inhibidoras, mientras que los Mo expresaban tanto receptores inhibidores como activadores de lectinas de tipo C, junto con otros receptores asociados a fagocitosis. En general, en los Mo de SP se

observaron niveles de expresión progresivamente inferiores de los marcadores explorados, desde los cMo hasta los ncMo Slan^{-} y Slan^{+}, excepto para el receptor endocítico CD368, presente de forma exclusiva en los cMo, junto con los receptores moduladores/señalizadores CD369 y CD371. Asimismo, la molécula (inhibidora) CD101 se expresaba de forma más intensa en los ncMo Slan^{+}, respecto a los ncMo Slan^{-}. En resumen, nuestros resultados muestran que el patrón de expresión de las diferentes moléculas señalizadoras y receptores analizados en este trabajo, varía en las diferentes poblaciones de CPA de SP, aunque con perfiles de expresión similares para aquellas moléculas incluidas dentro de un mismo grupo funcional. Estos hallazgos sugieren que cada población de CPA tendría capacidad de reconocer patrones moleculares diferentes, y por tanto de generar distintas señales en respuesta a cada patrón molecular y, en consecuencia, tendrían un papel funcional distinto entre ellas.

Distribución de distintas poblaciones de monocitos en sangre a lo largo de la vida

Los Mo presentes en SP constituyen una población heterogénea de células que incluyen, entre otras, los cMo CD14^{hi} CD16^{-}, los iMo CD14^{hi} CD16^{+} y los ncMo CD14$^{-/lo}$ CD16^{+}. Aunque en los últimos años se ha incrementado la información existente en la bibliografía sobre los distintos compartimentos de Mo normales en sangre, seguimos sin disponer de datos fiables acerca de su cinética a lo largo de la vida, y su relación con las

células del sistema Mo/Mϕ de MO o de los órganos linfoides secundarios. Por ello, en el presente trabajo nos planteamos analizar mediante citometría de flujo la distribución de las distintas poblaciones de Mo en sangre humana, a lo largo de la vida, desde la sangre de cordón umbilical y el recién nacido hasta la edad adulta y los ancianos (n=188), comparándola con la observada en diferentes tejidos linfoides de individuos adultos sanos, incluyendo MO (n=9), ganglio linfático (n=11) y bazo (n=11).

En conjunto, identificamos 6 poblaciones de Mo en SP normal, cuya distribución variaba de manera significativa a lo largo de la vida, especialmente durante los primeros 6 meses tras el nacimiento. En concreto, los cMo alcanzaban su pico en sangre de cordón umbilical, mientras que los números más elevados de iMo y ncMo se observaban en recién nacidos, disminuyendo sus cifras posteriormente durante la infancia y la edad adulta hasta los 50 años. Cabe destacar que todas las (seis) poblaciones de Mo identificadas estaban presentes de forma sistemática, aunque en número variable, en todos los tejidos analizados, excepto en el ganglio linfático, donde los cMo FcεRI^{+} y los ncMo Slan^{+} estaban prácticamente ausentes. En general, encontramos cifras significativamente inferiores de cMo CD62L^{+} en el ganglio linfático y en el bazo, en comparación con la MO ($p<0.01$) y la SP (<0.01), a expensas de un porcentaje más elevado de iMo; por el contrario, los ncMo estaban especialmente más representados en la sangre y en el bazo.

En resumen, nuestros resultados muestran que la cantidad de Mo normales circulantes en sangre y sus distintas subpoblaciones varía significativamente a lo largo de la vida, y en este sentido constituyen un marco de referencia de la normalidad (por rango de edad) para la identificación de perfiles potencialmente alterados en distintas enfermedades. La distribución diferencial de las diversas poblaciones de Mo en distintos tejidos linfoides podría contribuir a una mejor comprensión de su cinética y perfil de migración y maduración tisular en el organismo a lo largo de la vida.

Perfiles de distribución de distintas poblaciones de monocitos en sangre y de marcadores inflamatorios y óseos en suero en pacientes con neoplasias de CP

Estudios previos han demostrado la existencia de alteraciones significativas en los Mo/Mϕ de pacientes con distintos tipos de neoplasias de CP, como la GMSI, el MMQ y el MM sintomático, con un impacto en la alteración de la homeostasis normal del microambiente de la MO. En este trabajo analizamos la distribución de diferentes poblaciones de Mo en sangre y MO de pacientes recién diagnosticados de GMSI (n=23), MMQ (n=14) y MM (n=99), en comparación con la distribución en sangre y MO de donantes sanos (DS; n=107). En paralelo, cuantificamos los niveles en suero de un amplio panel de citocinas y biomarcadores asociados al metabolismo óseo. Nuestros resultados mostraron que mientras que en pacientes con GMSI existía una producción

normal de (precursores) de Mo, incluyendo cMo, ambos estaban significativamente disminuidos en la MO de pacientes con MMQ y MM ($p \leq 0.02$). Esto se traducía en recuentos significativamente más bajos de cMo $CD62L^+$ recién producidos en la SP de los pacientes con MMQ ($p=0.004$), y de todas las subpoblaciones de cMo ($CD62L^+$, $CD62L^-$ y $Fc\varepsilon RI^+$) en los pacientes con MM ($p \leq 0.02$). Por el contrario, en la MO de los sujetos con GMSI ($p \leq 0.03$), MMQ ($p \leq 0.03$) y MM ($p \leq 0.002$) existía un incremento significativo de iMo y ncMo, si bien en la sangre ambas poblaciones de Mo estaban dentro de los límites de la normalidad (en la GMSI y en el MMQ) o disminuidos (en el MM; $p=0.01$). Paralelamente a estas alteraciones, observamos niveles séricos elevados de interleucina (IL)1β en la GMSI ($p=0.007$) y en el MMQ ($p=0.01$), junto a concentraciones superiores de IL8 en el MMQ ($p=0.01$) y en el MM ($p=0.002$), así como niveles más elevados de IL6 ($p=0.002$), RANKL ($p=0.01$) y fosfatasa alcalina (FA) ósea ($p=0.01$) (asociado a un recuento disminuido de cMo $Fc\varepsilon RI^+$) en los pacientes con MM que presentaban lesiones osteolíticas. En su conjunto, estas alteraciones se traducían en tres perfiles inmunes/óseos distintos: 1) normal, presente en DS y en la mayoría de las GMSI); 2) senescente, asociado a un aumento de IL1β y/o IL8, presente en una minoría de las GMSI, la mayoría de los MMQ y algunos pacientes con MM sin lesión ósea; y 3) proinflamatorio, con niveles séricos elevados de IL6, RANKL y FA ósea y recuentos significativamente disminuidos de cMo $Fc\varepsilon RI^+$ inmunomoduladores ($p=0.01$),

característico de los pacientes con MM con lesión ósea asociada. Estos resultados aportan nuevos datos sobre la patogenia de las neoplasias de CP y el posible papel de los cMo FcεRI^{+} en la homeostasis del tejido óseo normal.

CONCLUSIONES

Con respecto al primer objetivo de este estudio, centrado en la caracterización detallada del inmunofenotipo de diferentes poblaciones de CDs y Mo de SP, basado en nuevos marcadores sometidos a la evaluación del *"10th Human Leucocyte Differentiation Antigen Workshop* en la sección de CDs *(HLDA10-DC task)":*

1. Las CDs de sangre, así como los cMo y los iMo, expresaban con mayor intensidad -respecto a las CDp y a los ncMo (especialmente los ncMo Slan^{+})-, tanto los receptores inhibidores tipo lectina C CD367 y CD371, como los receptores activadores tipo lectina C CD368, CD369 y CLEC5A. Estos hallazgos, junto con el patrón de expresión de (varios) marcadores mieloides y marcadores relacionados con la fagocitosis restringido a cMo e iMo, y en menor medida a ncMo, apoyan los hallazgos previos que indican que las funciones de presentación de antígenos y de fagocitosis están más relacionadas con las CDs y con los cMo e iMo, respectivamente, mientras que los ncMo podrían representar

una etapa final de maduración de Mo más propensos a la apoptosis, de acuerdo también con su patrón de expresión del receptor purinérgico P2X7.

2. Ninguna de las poblaciones de CDs y Mo circulantes en sangre expresaba niveles detectables de la proteína CD365, mientras que CD366 se expresaba de forma intensa en las CDc y los Mo (pero no en las CDp normales), lo cual sugiere características más inmaduras, asociadas con un perfil de expresión de receptores inhibidores más marcado en las CDc y los Mo, respecto a las CDp.

Con respecto al segundo objetivo de nuestro estudio, enfocado a determinar la distribución y el fenotipo de diferentes subpoblaciones de CDs y Mo normales en la SP a lo largo de la vida, e investigar la relación existente entre estas poblaciones de Mo de sangre y sus posibles contrapartidas de MO, ganglio linfático y bazo:

3. Los cMo alcanzaron sus cifras más elevadas en la sangre de cordón umbilical, mientras que el nivel más alto de iMo/ncMo se alcanzó en el recién nacido, con un claro predominio de los ncMo Slan^{-} más inmaduros (vs. los ncMo Slan^{+} más diferenciados) hasta los 5 años de edad, disminuyendo posteriormente el número de estas células en sangre a lo largo de la infancia y la edad adulta. La

diferente cinética observada para las distintas poblaciones de Mo de sangre podría reflejar una mayor necesidad de Mo en las etapas más tempranas de la vida, en las que habría que producir un número lo suficientemente elevado de estas células con la finalidad de "llenar" los distintos tejidos del organismo en las etapas de mayor crecimiento; esto iría asociado a una representación en sangre cada vez mayor de las dos poblaciones de Mo más maduros, presumiblemente derivados de los tejidos en los que habrían llevado a cabo sus funciones.

4. Respecto a la sangre y la MO, tejidos en los que predominan los cMo CD62L^{+}, en el ganglio linfático y en el bazo se observaron porcentajes significativamente inferiores de cMo CD62L^{+}, a expensas de un mayor predominio de cMo CD62L^{-} y de iMo. Estos hallazgos podrían reflejar la naturaleza más madura de los cMo presentes en los órganos linfoides secundarios respecto tanto a la SP como a la MO. Del mismo modo, la mayor proporción de iMo observada en el ganglio linfático y en el bazo (en comparación con la SP y la MO) podría ser también el reflejo de una mayor tasa de diferenciación de cMo hacia iMo en estos tejidos, lo cual apoyaría la hipótesis que sugiere que la maduración de cMo a iMo podría ocurrir de forma preferente fuera del torrente sanguíneo.

5. En términos generales, mientras que los ncMo Slan^{+} se encontraban altamente representados en sangre y bazo, ambas poblaciones estaban prácticamente ausentes en el ganglio linfático, y presentes en niveles mínimos en MO. En conjunto, estos resultados sugieren que los iMo generados en diferentes tejidos del organismo podrían migrar a través de los ganglios linfáticos a la sangre, donde generarían ncMo que podrían alojarse específicamente en el bazo, de acuerdo con las características preapoptóticas de esta población de Mo.

6. Los Mo de SP FcεRI^{+} mostraban un fenotipo de cMo CD14^{hi} CD16^{-} distinto y único, constituyendo una población minoritaria de Mo presente de forma sistemática tanto en sangre de cordón umbilical como en la SP de todos los individuos incluidos en este estudio; por el contrario, era una población celular indetectable en el ganglio linfático normal. Por el momento, seguimos sin conocer el posible significado e implicaciones funcionales de estos hallazgos.

Con respecto al tercer objetivo específico de esta tesis doctoral, centrado en la identificación de los posibles patrones de alteración de las diferentes poblaciones de Mo en sangre y MO de pacientes con GMSI, MMQ y MM, y su relación con los perfiles séricos de marcadores inflamatorios y óseos:

7. Los pacientes con MMQ y MM presentaban niveles significativamente disminuidos de cMo $CD62L^+$ y de cMo $CD62L^+$, cMo $CD62L^-$ y de cMo $Fc\varepsilon RI^+$, respectivamente. Además, a diferencia de lo observado en el MMQ y en el MM, en la GMSI encontramos porcentajes significativamente elevados de cMo en las muestras de MO. Globalmente, estos hallazgos podrían reflejar una mayor producción de cMo en la MO de pacientes con GMSI, con la finalidad de controlar el microambiente medular alterado en estos sujetos, seguido de una disminución de la producción y de la liberación a la sangre de cMo $CD62L^+$ maduros desde la MO y de cMo $CD62L^+$, $CD62L^-$, y $Fc\varepsilon RI^+$, en pacientes con MMQ y MM, respectivamente.

8. En términos generales, los pacientes con neoplasias de CP tenían cifras más elevadas en la MO de iMo y ncMo respecto a sujetos sanos, que apoyaría la existencia de una mayor tasa de migración de ncMo e iMo desde los tejidos a la sangre y de esta hacia la MO, asociado a un papel potencialmente relevante de ambas poblaciones de Mo en la fisiopatología del MMQ, MM y (en menor medida también) de la GMSI, como parte integrante de un microambiente medular alterado.

9. Paralelamente a las alteraciones numéricas anteriormente descritas, los Mo/Mϕ presentes en la SP y/o los tejidos linfoides de pacientes con diferentes categorías diagnósticas de neoplasias de CP parecen estar alterados también funcionalmente. En este sentido, observamos niveles séricos elevados (en comparación con los sujetos sanos) de IL-1β, de IL-1β e IL-8, y de IL-1β, IL8, IL6 y los biomarcadores óseos RANKL, FA ósea y osteoprotegerina en la GMSI, el MMQ y el MM, respectivamente. Estos patrones nos han permitido definir tres grupos de pacientes cuya relevancia clínica requiere de estudios en series más amplias de pacientes: i) pacientes con GMSI y un perfil similar al de los sujetos sanos; ii) pacientes con MMQ o MM sin lesión ósea; y, finalmente, c) pacientes con MM que presentaban lesiones osteolíticas típicas.