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.

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'Mononuclear phagocytes' (that is, dendritic cells (DCs), monocytes and macrophages) are functionally related immune cells that have an essential role as antigen-presenting cells (APC). 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. Despite these general roles, it is well known that both DCs and monocytes/macrophages are heterogeneous cell populations. 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 monocytes in healthy subjects; (a) CD14++/CD16− classical monocytes (cMo) that represent 90–95% of all PB monocytes in healthy adults; (b) CD14++/CD16+ or intermediate monocytes (iMo); and (c) CD14lo/CD16+ non-classical monocytes (ncMo). The latter two subsets represent ~ 5–10% of all circulating monocytes in healthy adults.

All PB subsets of DCs and monocytes are functionally related cells, which share antigen-presentation functions, among other roles.
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. 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](image-url) Immuno-phenotypical 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).
Interestingly, all mDCs from the 111F8.04 and 50C1 monoclonal antibodies (mAb), respectively (CLEC12A/MICL/CCL-1) were observed on the PB by using the low levels of expression of CD367 (CLEC4A/DCIR) and CD371 positive on the cell surface membrane. Actually, for pDCs, only pDCs, in which none of the CLRs analysed were found to be clearly subsets of PB APC investigated. However, this was not the case for normal human PB APCs.

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. 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 FPRL1 and MAIR II and monocytes expressed FPRL1/FPRL2 and TREM2 (in addition to FPRL1 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 selectively expressed by pDCs (Figure 1b). In contrast, CD85g, a molecule known to mediate signals that negatively modulate IFNγ production, was 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                     | mDCd+, cMo+, iMo−/+, ncMo−/+ |
|                                    | CD367           | FAB1748P| 10–13                     | mDC+++, cMo−, iMo−, ncMo−/+ |
|                                    | CD367           | 11F8.04 | 10–71                     | mDC++, pDC−, cMo+, iMo−, ncMo− |
|                                    | CD367           | 9E8     | 10–72                     | mDC+, cMo−, iMo−, ncMo−/+ |
|                                    | CD368           | FAB2806P| 10–21                     | cMo−/+, iMo− |
|                                    | CD368           | 9B9     | 10–78                     | cMo−, iMo− |
|                                    | CD369           | GE2     | 10–01                     | –          |
|                                    | CD369           | FAB1859P| 10–35                     | cMo−, iMo−, ncMo−/+ |
|                                    | CD369           | 15 E 2  | 10–79                     | cMo−, iMo−, ncMo− |
|                                    | CD370           | 8F9     | 10–02                     | –          |
|                                    | CD370           | 9A11    | 10–09                     | –          |
|                                    | CD370           | FAB6049P| 10–45                     | –          |
|                                    | CD371           | HB3     | 10–17                     | mDC−/+, cMo−, iMo−, ncMo−/+ |
|                                    | CD371           | FAB2946P| 10–51                     | mDC−/+, cMo−, iMo−, ncMo−/+ |
|                                    | CD371           | 50C1    | 10–73                     | mDC−/+, pDC−, cMo−, iMo−, ncMo−/+ |
|                                    | Clec2D          | FAB3480P| 10–06                     | –          |
|                                    | Clec5A          | FAB2238P| 10–28                     | cMo− |
|                                    | Clec5G          | 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           | ANCO812 | 10–61                     | –          |
|                                    | CD365           | FAB1750P| 10–14                     | –          |
|                                    | CD365           | 1012    | 10–67                     | –          |
|                                    | CD366           | FAB2365P| 10–24                     | mDC−/+, cMo−, iMo−, ncMo−/+ |
|                                    | CD366           | F38-2E2 | 10–75                     | mDC−/+, cMo−, iMo−, ncMo−/+ |
|                                    | ULBP-3          | FAB1517P| 10–52                     | –          |
|                                    | B7-H4           | M1H43   | 10–64                     | –          |
|                                    | TSLP-R          | 1B4     | 10–68                     | –          |
|                                    | TIM4            | 9F4     | 10–81                     | –          |
| DC-related markers                | CD1a            | 010e    | 10–03                     | –          |
|                                    | CD1a            | O619    | 10–10                     | –          |
|                                    | CD1b            | O249    | 10–18                     | –          |
|                                    | CD1c            | L161    | 10–26                     | –          |
|                                    | CD85g           | 17G10.2 | 10–66                     | pDC−/++ |
|                                    | CD85h           | 24      | 10–74                     | mDC−+, cMo−, iMo−, ncMo− |
|                                    | CD209           | 11B8A8.05 | 10–83                  | –          |
| Phagocytic-related molecules       | FPRL1/FPRL2     | FAB3479P| 10–30                     | cMo−/+, iMo−/+ |
|                                    | FPR1           | FAB3744P| 10–47                     | mDC−/+, cMo−/+, iMo−/+, SLAN−ncMo−/+ |
|                                    | 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−/+, cMo−, iMo−, ncMo−/+ |
|                                    | TREM2           | FAB17291P| 10–07                 | cMo−, iMo−, SLAN−ncMo−/+ |
|                                    | FDF03           | 3G2H   | 10–84                     | –          |
| Chemokine and cytokine receptors   | CD135           | FAB812P | 10–15                     | mDC−/++ |
|                                    | CD195           | HEK1/85 | 10–76                     | mDC−/++, pDC−/++, iMo−/+ |
|                                    | 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                 | –          |
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.

Table 1 (Continued)

| Specificity | Target molecule | Clone | Antibody (workshop codes) | Reactivity |
|-------------|----------------|-------|---------------------------|------------|
| Other molecules (miscellaneous) | 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 | ANC809 | 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); + (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.

CLR receptors comprise >1000 receptors, which are typically found on phagocytes. These receptors are essential for antigen capturing, 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, CLR receptors also have an important role in diseases and conditions such as autoimmunity, allergy and cancer. 10 CLR receptors are associated with carbohydrate binding (that is, mannose, N-acetylgalcosamine, 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 CLR receptors 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 CLR receptors 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, CD67 (CLECA4/DCIR) and CD371 (C12A/MICL/CCL-1) contain ITIMs (immunoreceptor tyrosine-based inhibitory motifs) in their cytoplasmic tails and both act as inhibitory receptors, having 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 receptors 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 CLECA4/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 (SSCint) 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++ intermediate monocytes (iMo) and CD14lo/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 effects17) 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 inflammatory 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 wall19 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/cytokines. 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 FPR1L/FPR2L), 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 homeostatic 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/effecter T cells.36,27 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 –CICIBMC– 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 ~5 x 10^5 cells in 100 µl per test. Seven common markers (barcode) 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-HLAD-DR-Pacific Blue (PaB)- (Clone L243); CD45-Pacific Orange (PaO)– (Clone H130); CD33– peridinin chlorophyll-protein-cyanin 5.5 (PerCP-Cy5.5)– (Clone P67.6); CD123–allophycocyanin (APC)- (Clone AC145); CD16–phycoerythrin-cyanine 7 (PECy7)– (Clone 3G8); CD14–APC-Hilite 7 (APC-H7)– (Clone MapP9) 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 mAbs, 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-HLAD-DR-PaB (Biolegend, San Diego, CA, USA), all purchased from Becton Dickinson Biosciences (BD; San Jose, CA, USA), two washing steps in phosphate buffered saline (PBS; pH 7.6) to remove the

**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.

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