



Minireview

Profiling CD antigens on leukaemias with an antibody microarray

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ABSTRACT

Cluster of differentiation (CD) antigens are defined when a surface molecule found on some members of a standard panel of human cells reacts with at least one novel antibody, and there is good accompanying molecular data. Monoclonal antibodies to surface CD antigens on leukocytes have been used for flow cytometry, and more recently to construct microarrays that capture live cells. These DotScan™ microarrays enable the rapid and highly parallel characterization of repertoires of CD antigens whose expression patterns may be correlated with discrete leukaemia subtypes, or used to define biomarker 'signatures' for non-hematological diseases. DotScan™ with fluorescence multiplexing enables profiling of CD antigens for minor subsets of cells, such as colorectal cancer cells and tumour-infiltrating lymphocytes from a surgical sample.

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1. Surface profiling of leukaemias using DotScan™

Human leukaemias may be classified as acute or chronic and are derived from lymphoid or myeloid precursor cells. They are currently classified using multiple criteria including morphology, karyotype, cytochemistry and a limited immunophenotype of 10–15 Cluster of differentiation (CD) antigens [1]. While flow cytometry is considered the 'gold standard' for identification of CD antigens expressed on the surface of leukocytes, only a limited number of antigens can be identified in a single analysis. An alternative procedure involving capture of live cells by a microarray of CD antibodies on a nitrocellulose film (DotScan™) has been developed that enables concurrent identification of many CD antigens in parallel (currently 82–147) [2]. In a clinical analysis involving 796 leukaemia patients and healthy control subjects, Belov et al. [3]

demonstrated that surface profiles of multiple CD antigens obtained from a DotScan™ microarray consisting of 82 monoclonal antibodies with specificities directed against a range of different CD antigens are sufficient to classify leukaemias from samples of peripheral blood (PB) or bone marrow aspirates (BMA). The classification of PB samples using DotScan™ showed a 93.9% correlation with established criteria, and a 96.7% correlation with BMA, indicating that an extensive immunophenotype alone is sufficient to classify the disease when a leukaemic clone dominates the cell population. The extensive CD antigen immunophenotypes obtained using highly parallel proteomic analysis on DotScan™ may also have utility both as prognostic indicators and as predictive biomarker signatures of clinical response to a specific therapeutic intervention.

2. Classification of leukaemias

Flow cytometry using a limited surface immunophenotype (10–15 CD antigens) comprises one of multiple criteria for the diagnosis of human leukaemias. Cell surface profiling of CD antigens is especially important in the diagnosis of acute lymphoblastic leukaemia (ALL), helping to indicate the subtype of cell using lineage-specific antigens and to distinguish undifferentiated acute myeloid leukaemia (AML) from ALL [1]. Several groups have investigated the possibility of diagnosing leukaemias based solely upon immunophenotype [4,5]. Flow cytometry was used to

Abbreviations: ALL, acute lymphocytic leukaemia; AML, acute myeloid leukaemia; APL, acute promyelocytic leukaemia; ATRA, *all-trans* retinoic acid; BMA, bone marrow aspirate; CD antigen, cluster of differentiation antigen; CEA, chorio-embryonic antigen; CLL, chronic lymphocytic leukaemia; 1,25D₃, 1- α ,25-dihydroxyvitamin D₃; CRC, colorectal cancer; EpCAM, epithelial cell adhesion molecule; HLDA, human leukocyte differentiation antigens; TILs, tumour-infiltrating lymphocytes; PB, peripheral blood; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate

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identify a limited profile of CD antigens for each clinical condition. Flow cytometry is labour-intensive, expensive and enables parallel measurement of only a small number of antigens. By contrast, DotScan™ enables simultaneous and highly parallel screening of many (currently 82–147) CD antigens, and this extensive surface immuno-profiling appears sufficient to classify most human leukaemias. The CD antigens identified by the microarray correspond well with those identified by flow cytometry [2,3] although different parameters are measured. In the case of flow cytometry, the fluorescence intensity per cell is assessed on a gated population of cells and compared with the level of fluorescence for control antibodies. For cell capture on an antibody microarray, there is a threshold density of surface antigens sufficient to provide stable cell capture and the number of cells expressing a surface antigen above that threshold density are quantified as the average density of cells on each immobilized antibody dot. Monoclonal antibodies immobilised on nitrocellulose may have reduced access to some CD antigens expressed on the surface of live cells as compared with the equivalent fluorescently-labelled antibodies in free solution in flow cytometry.

We have used DotScan™ to compile a database of mononuclear leukocyte immunophenotypes from peripheral blood or bone marrow aspirates derived from patients with a range of different leukaemias [3]. AML comprises a number of diseases and other groups have attempted to define AML sub-types using surface immuno-profiling. The best characterized subtype of AML is t(15;17) or acute promyelocytic leukaemia (APL), with low or absent expression of CD11a, CD11b, CD18 and HLA-DR, with expression of CD9 and CD117 [6,7], consistent with the consensus profile for APL obtained using DotScan™ [3]. With more samples profiled, DotScan™ may provide a new classification system for AML and other leukaemias based solely on extensive immunophenotypes.

Fig. 1 shows dot patterns obtained from cell capture on DotScan™ microarrays for normal mononuclear leukocytes from peripheral blood, and a sample from a patient with AML in which the AML cells are the predominant population. These dot patterns may be transformed into cell surface CD antigen expression profiles using software that quantifies the intensity of each dot (Fig. 2). The principal differences between normal mononuclear leukocytes and AML cells are clearly discernable, with AML showing expression of the myeloid antigens CD13, CD15 and CD33 (red), the stem cell (precursor) markers CD34 and CD117 (green) and a range of other antigens, including aberrant expression of the T-cell antigen CD4 (yellow). With lower grade leukaemias, the AML pattern can generally still be distinguished from a background binding pattern of normal leukocytes. However, when leukaemia cells comprise less than 10% of the total population, a secondary detection system such as fluorescent staining may be required to distinguish the leukaemia binding pattern.

DotScan™ has also been used to screen novel monoclonal antibodies of undefined CD antigen specificity submitted to the Human Leukocyte Differentiation Antigen workshop (HLDA8) for their reactivity with a panel of cell lines and patient samples. Antibody binding patterns for around 75 cell types could be subjected to hierarchical clustering to identify monoclonal antibody clones that bind to the same epitope [8]. DotScan™ is consequently able to provide a rapid and reliable method for the screening and characterization of the binding specificities of new monoclonal antibody clones.

3. Surface immuno-profiling of leukaemia samples from peripheral blood and bone marrow

The populations of mononuclear leukocytes isolated by Histopaque density gradient centrifugation from normal PB and BMA

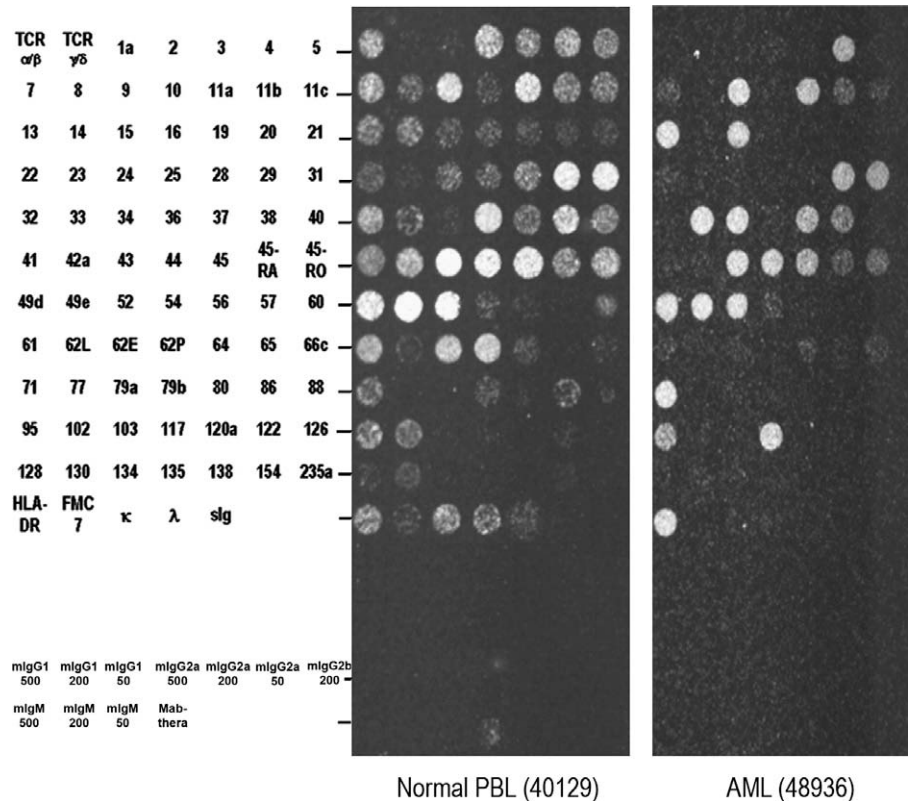


Fig. 1. Dot patterns obtained from leukocytes captured on DotScan™ microarrays for normal peripheral blood leukocytes (PBL) and a peripheral blood sample from a patient with acute myeloid leukaemia (AML). The numbers in the key refer to antibodies against the corresponding CD antigens; mIgG1, mIgG2a, mIgG2b and mIgM are murine isotype control antibodies tested at the concentrations shown in µg/mL; TCR α/β , TCR γ/δ , HLA-DR, FMC7, κ , λ and slg are antibodies against T cell receptors α/β and γ/δ , HLA-DR, FMC-7, kappa and lambda immunoglobulin light chains and surface immunoglobulin, respectively. Mabthera is a humanized monoclonal antibody against CD20.

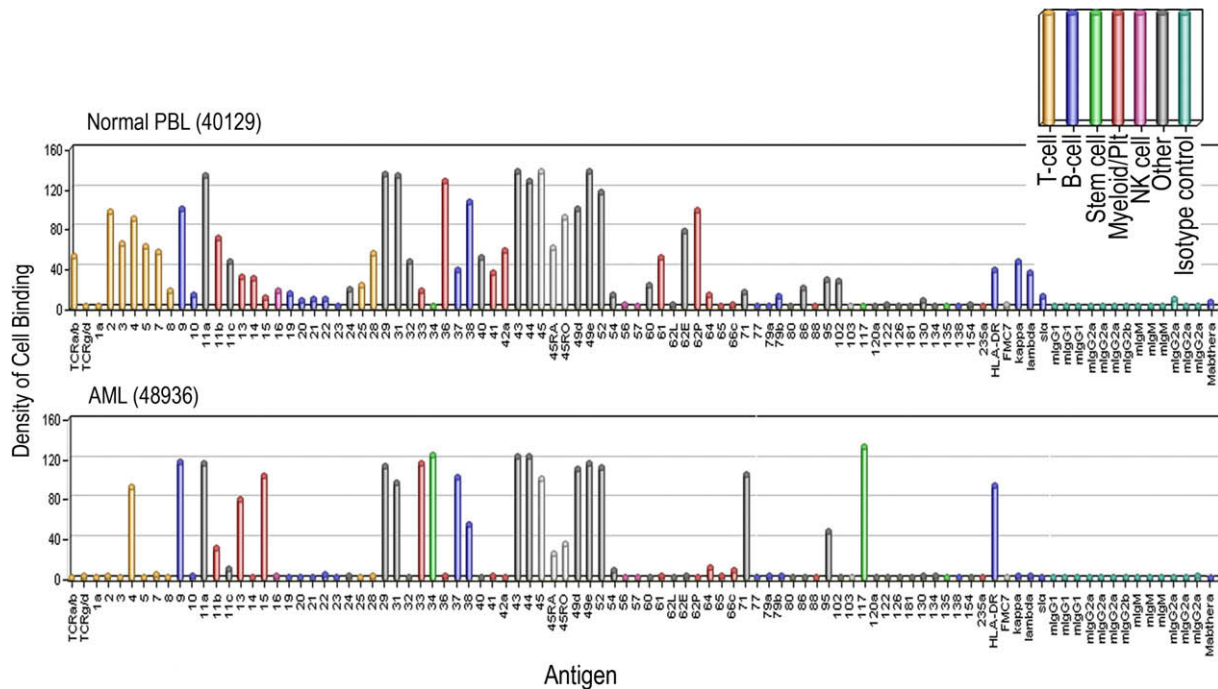


Fig. 2. Surface profiles obtained from the dot patterns in Fig. 1. The density of cell binding to each antibody above the background was calculated on an 8-bit pixel greyness scale from 1 to 256 using DotScan™ software. Abbreviations: as for Fig. 1; Plt, platelets.

have cellular and immunophenotypic differences. The PB fraction is principally mononuclear T- and B-lymphocytes and monocytes, while the BMA fraction contains cells at various stages of differentiation, mainly from the myeloid and B-lymphoid lineages [9]. Thus, the background of normal leukocytes found in the mononuclear PB and BMA fractions are distinct and yield different patterns when analyzed using DotScan™. The PB mononuclear fraction is comprised largely of T-lymphocytes (CD2, CD3, CD4, CD5, CD7, CD8, TCR α/β), with lower levels of myeloid cells (mainly monocytic CD13, CD14, CD64), B-lymphocytes (CD19, CD20, CD22, kappa and lambda immunoglobulin light chains of surface immunoglobulin) and contaminating platelets (CD41, CD42a, CD61, CD62P). Mononuclear BMA fractions contain high levels of myeloid (neutrophil) precursors (e.g., CD13, CD15, CD16, CD24, CD43, CD45RO and

CD66c [3]). This predominance of neutrophil precursors has been confirmed by flow cytometry and morphological analysis (N. Barber and W. Sewell, unpublished results). Consequently, samples of leukaemias in which the frequency of malignant clones is low (<10% leukaemia cells) will contain a significant proportion of non-malignant cells that contribute to the expression profile obtained from an optical scan of the microarray. Comparison of low level leukaemic surface profiles obtained with DotScan™ with equivalent normal samples enables identification of surface molecules that are differentially abundant as a consequence of the pathological leukaemic process. Fig. 3 shows the difference in surface profiles between normal samples from peripheral blood and bone marrow.

The binding patterns obtained for 'high grade' acute leukaemia samples from PB and BMA (>10% leukaemia cells) are usually

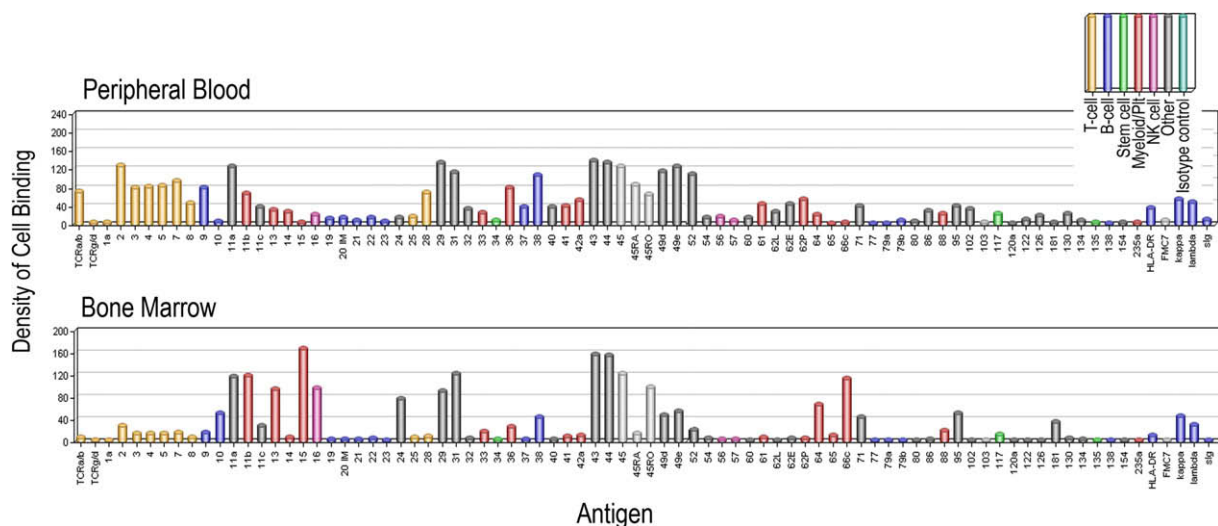


Fig. 3. CD antigen expression on control bone marrow and peripheral blood samples as determined by DotScan™. The density of cell binding to each antibody above the background was calculated on an 8-bit pixel greyness scale from 1 to 256 using DotScan™ software. Abbreviations: as for Fig. 1; Plt, platelets.

similar. Cell surface profiles for AML showed higher levels of surface CD117 (c-kit) from BMA compared with PB. For ALL, CD34 levels were higher for precursor B-ALL from BMA compared with PB [3]. These differences indicate that BMA samples contained a greater proportion of immature leukaemias; CD117 and CD34 expression is lost during maturation of myeloid (CD117 and CD34) and lymphoid (CD34) cells. The surface profiles obtained for B-lymphoproliferative disorders from PB and BMA samples were also similar. For 'high grade' leukaemias ($>10^7$ leukaemia cells per mL), the immunophenotype for a particular leukaemia will usually be similar between PB and BMA. Weinkauff et al. [10] found that immunophenotypes of PB and BMA blasts from the same patient analyzed by flow cytometry were similar; this observation should be confirmed using DotScan™ to determine whether there are site-specific homing, adhesion or other micro-environmental effects that may be of biological importance. The extensive CD antigen surface profiles obtained for leukaemias using DotScan™ are currently being compiled in a database to enable more detailed subtyping, to facilitate the prediction of patient responses to drugs and to define prognosis at the individual patient level.

4. Profiling of leukaemias during differentiation therapy

4.1. All-trans retinoic acid

Drugs that induce differentiation of acute leukaemias may have therapeutic benefit by slowing growth. All-trans retinoic acid (ATRA) is a successful anticancer drug used to treat acute promyelocytic leukaemia (APL) [11] by inducing cells to differentiate into

'abnormal' granulocytes [12]. Initial studies demonstrated that 23/24, 6/6 and 19/20 APL patients treated with ATRA achieved complete remission [13–15]. The human cell line HL-60, is derived from a case of the M2 subtype of AML and is used as a model for drug-induced differentiation of promyelocytic leukaemia [16]. Drugs such as ATRA, 12-O-tetradecanoyl phorbol-13-acetate (TPA) or 1- α ,25-dihydroxyvitamin D₃ (1,25D₃) induce HL-60 cells to differentiate into mature myeloid cells with loss of proliferative capacity [17]. ATRA induces major changes in membrane proteins, some of which are adhesion molecules that interact with neighbouring cells. The clinically significant and potentially fatal 'retinoic acid syndrome' may be due in part to interactions with neighbouring cells resulting from the induction of adhesion molecules and cytokines by ATRA [18]. We have used DotScan™ to analyze changes in CD antigen expression on HL-60 cells during differentiation induced by ATRA [19].

ATRA induces differentiation of HL-60 into cells that resemble neutrophils [20]. DotScan™ has been used to show that treatment of HL-60 cells with ATRA induces CD11b, CD11c, CD36, CD38, CD43, CD45RO, CD53, CD54, CD64 and CD66c and represses CD29, CD44, CD95 and CD117 [19]. The Venn diagram of Fig. 4A summarizes the expression of CD antigens on HL-60 cells treated with ATRA as compared to the surface profile obtained from normal neutrophils taken from the NCBI and PROW databases [21,22]. The surface profile of the neutrophil-like cells induced by ATRA is not identical to that obtained from normal neutrophils. HL-60 cells have activating mutations from their selection for cell culture not present in healthy cells. However, ATRA-differentiated HL-60 cells and normal neutrophils express a number of CD anti-

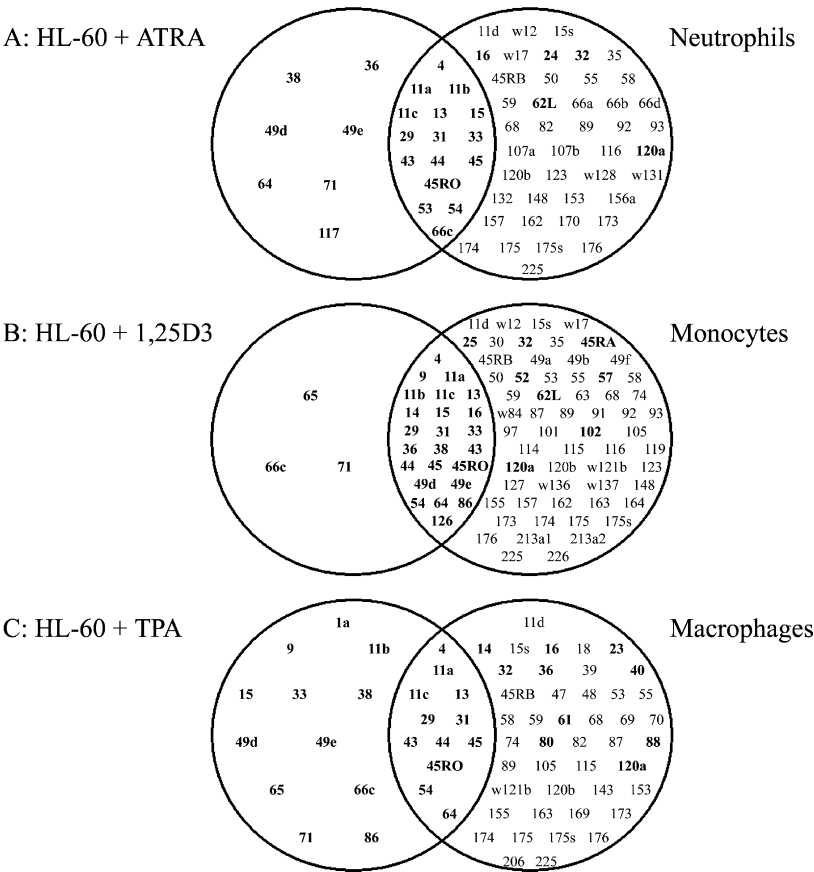


Fig. 4. Venn diagram comparing expression of CD antigens on differentiated HL-60 cells with normal leukocyte subtypes. (A) HL-60 cells treated with ATRA compared with neutrophils, (B) HL-60 cells treated with 1,25D₃ compared with monocytes and (C) HL-60 cells treated with TPA compared with macrophages. Numbers indicate CD antigens. Antigens in bold were tested on the DotScan™ microarray.

Table 1

Functions of differentially expressed CD antigens on HL-60 cells induced by ATRA, 1,25D₃ or TPA (Fig. 4). Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ma., macrophages; mo., monocytes; n., neutrophils; MC, mast cell, HPC, haematopoietic progenitor cell.

Antigen	Expression	Function	Ref.
CD4	n., mo.,ma.	Co-receptor for T-cell activation	[23]
CD9	mo.	Cell adhesion and migration	[24]
CD11a	n., mo., ma.	Leukocyte adhesion and co-stimulation	[25]
CD11b	n., mo.	Leukocyte adhesion	[26]
CD11c	n., mo., ma.	Leukocyte adhesion	[26]
CD13	n., mo., ma.	Signalling	[27]
CD14	mo., ma.	Receptor for the Gram-negative bacterial endotoxin, lipopolysaccharide (LPS) and some Gram-positive bacterial cell wall components	[28]
CD15	n., mo.	Crucial for capture of the neutrophil by the endothelium before subsequent adhesive interactions	[29]
CD16	n., mo., ma.	Binds monomeric IgG and immune complexes and is involved in ADCC	[30]
CD29	n., mo., ma.	Adhesion and migration across extracellular matrix proteins	[31]
CD31	n., mo., ma.	Transendothelial migration and adhesion of neutrophils to macrophages	[32]
CD36	mo., ma.	Signalling	[33]
CD38	mo.	Acts with MHC Class II and FcγRII to activate T-cells, cell adhesion	[34]
CD43	n., mo., ma.	Neutrophil movement	[35]
CD44	n., mo., ma.	Directed migration and polarisation of neutrophils and macrophages	[36]
CD45	n., mo., ma.	TCR and BCR mediated activation, neutrophil Fc receptor II activation, Fc receptor-mediated ADCC	[26,37]
CD45RO	mo., ma.	TCR and BCR mediated activation	
CD49d	mo.	Cell adhesion	[26]
CD49e	mo.	Cell adhesion	[26]
CD53	n., mo., ma.	Cell adhesion and migration	[24]
CD54	n., mo., ma.	Cell adhesion	[38]
CD64	mo., ma.	Facilitates MHC class I and class II-restricted antigen presentation to T-cells and mediates phagocytosis and ADCC	[39]
CD66c	n.	Neutrophil adhesion, phagocytosis, and activation of superoxide production	[40]
CD71	ma.	Transferrin receptor	[26]
CD86	mo.	Co-regulator of T-cell activation with CD80	[41]
CD117	MC, HPC	Growth factor receptor	[26]
CD126	mo.	Receptor for IL-6 involved in the immune response	[42]

gens in common (Fig. 4A): CD4, CD11a, CD11b, CD11c, CD13, CD15, CD29, CD31, CD33, CD43, CD44, CD45, CD45RO, CD53, CD54 and CD66c. The functions of differentially expressed antigens on HL-60 cells treated with ATRA are summarized in Table 1.

4.2. 1- α ,25-dihydroxyvitamin D₃

Although 1,25D₃ induces differentiation of leukaemia cells, therapeutic development has been limited due to hypercalcaemic side-effects in vivo. A number of analogues of 1,25D₃: EB1089, MC903, RO24-5531, 1- α -hydroxyvitamin D₂, 25-hydroxyvitamin D₃, 19-nor-1- α ,25-dihydroxyvitamin D₂ and 1- α -hydroxyvitamin D₅, induce differentiation without hypercalcaemia. These analogues have passed stringent pre-clinical toxicity trials in animals [43]. 1,25D₃ induces differentiation of HL-60 cells to monocyte-like cells with increased expression of CD14, CD11c, CD11b, CD54, CD86, CD38 and CD66c, while CD117, CD71, CD95, CD45, CD64 and CD13 are repressed. Low levels of CD16, CD36 and CD126 were also induced (Fig. 4B) [17]. The functions of these antigens are summarized in Table 1.

4.3. 12-O-Tetradecanoyl phorbol-13-acetate

TPA was introduced into clinical trials in China [44] achieving a reduction in bone marrow myeloblasts in 10 of 11 subjects with AML. This success led to a Phase I clinical trial of TPA in the USA with 14 patients [45]. Results of these trials indicate that TPA has significant clinical activity and that its toxicity profile and efficacy warrant further development in a Phase 2 study. TPA induces differentiation of HL-60 cells to macrophage-like cells with higher expression levels of: CD11c, CD9, CD11b, CD54, CD38, CD45RO and CD66c, while CD4 and CD117, CD95, CD71 and CD64 decrease (Fig. 4C) [17].

The surface profiles of the monocyte- and macrophage-like cells induced by 1,25D₃ and TPA, respectively, are not identical to those obtained from normal monocytes and macrophages [17]. However, Fig. 4B and C shows that normal monocytes and macrophages share many CD antigens with HL-60 cells treated with 1,25D₃ or TPA. HL-60 cells treated with 1,25D₃ express in common with monocytes CD4, CD9, CD11a, CD11b, CD11c, CD13, CD14, CD15, CD16, CD29, CD31, CD33, CD36, CD38, CD43, CD44, CD45, CD45RO, CD49d, CD49e, CD54, CD64, CD86 and CD126. HL-60 cells treated with TPA express in common with macrophages CD4, CD11a, CD11c, CD13, CD29, CD31, CD43, CD44, CD45, CD45RO, CD54 and CD64. The functions of differentially expressed antigens on HL-60 cells treated with 1,25D₃ or TPA are summarized in Table 1.

5. Cytotoxic therapy with purine analogues

Purine nucleoside analogues are effective anticancer drugs that are used in the treatment of B-lymphoproliferative disorders. Cladribine (2-chloro-2'-deoxyadenosine) is effective against some cases of chronic lymphocytic leukaemia (CLL), low-grade non-Hodgkin's lymphoma and hairy cell leukaemia [46]. Cladribine as the triphosphate derivative, inhibits ribonucleotide reductase and DNA synthesis [47] resulting in apoptosis [48]. Fludarabine (2-fluoroarabinosyladenosine) is also active as the triphosphate derivative with similar but not identical mechanisms of action to cladribine, resulting in apoptosis [49] with activity against CLL and indolent non-Hodgkin's lymphoma [50].

Using DotScan™, we have demonstrated that these purine analogues induce changes in the levels of some CD antigens on human MEC2 CLL and Raji Burkitt's lymphoma cell lines (Gez, Christopherson et al., manuscript in preparation). The effects of fludarabine nucleoside and cladribine (1 μ M, 24 h) on cell surface antigens on MEC2 and Raji cells have been determined using

DotScan™. Raji cells treated with cladribine up-regulated by more than 1.3-fold CD54, CD80, CD86 and CD95, with repression of CD22, while fludarabine nucleoside up-regulated CD20, CD54, CD80, CD86 and CD95. MEC2 cells treated with cladribine up-regulated CD20, CD45, CD54, CD62L CD80, CD86 and CD95, but fludarabine nucleoside had no effect.

6. Profiling of solid tumour phenotypes using fluorescence multiplexing

The cell surface CD antigen immuno-profiles presented here have been derived from patient samples where the leukaemia was predominant, or from monoclonal cell lines grown in culture. Under these circumstances, an optical scan of the pattern of captured cells on DotScan™ provides an accurate surface profile for those cells. However, many (pathology) samples are heterogeneous, with the cancer cells in low proportion. To obtain a surface profile of only a subset of captured cells of interest, a procedure of fluorescence multiplexing has been developed for the analysis of cell suspensions produced from surgical resections of colorectal cancer (CRC) [51]. Cells were captured on the microarray, fixed with formalin and then stained with a mixture of fluorescently-labelled soluble antibodies against chorio-embryonic antigen (CEA), epithelial cell adhesion molecule (EpCAM) and CD3. The optical scan of such a microarray had limited value because of the mixed population of cells with the CRC cells in the minority. Fluorescent scans for the soluble antibodies against CEA and EpCAM produced dot patterns specific to CRC cells, while the scan for CD3 showed the surface profile for tumour-infiltrating lymphocytes (TILs). The profiles obtained for the CRC cells dissected from the tumour were compared with those derived from normal mucosa from the same surgical sample to identify surface molecules differentially abundant on CRC. The profiles obtained for T-lymphocytes (CD3) in the CRC and normal mucosa fractions showed the presence of T-cell activation markers in the cancer characteristic of TILs. These profiles are being used to develop a new classification system for CRC, and some of the surface molecules identified may be potential targets for development of therapeutic monoclonal antibodies.

7. Conclusions

The DotScan™ antibody microarray enables the screening of live cell suspensions for the expression of large numbers of cell surface CD antigens in a single assay. The data obtained may be used to classify leukaemias and other diseases based upon the patterns of expression of cell surface CD antigens. Such profiles provide more robust results than classifications based upon the presence of one or several disease markers. In a plenary lecture at the first international workshop on Human Leukocyte Differentiation Antigens (HLDA1), Milstein (1984) speculated that the “complete definition of such mosaics” on cell surfaces would be “invaluable in defining cells of varying differentiation and pathological states”. While DotScan™ microarrays currently contain antibodies against up to 147 cell surface CD antigens, a ‘full house’ microarray containing antibodies against all of the currently defined CD antigens (around 360) could easily be constructed. However, the precise size of the complete repertoire of cell surface CD antigens expressed across the surface of cells from more than 200 different human tissues remains to be defined. While HLDA9, to be held in Barcelona in 2010, will certainly assign CD numbers to more cell surface proteins, the numbers involved will remain well below the total human complement. One of the criteria for designation as a CD antigen is the availability of several antibodies specific for extra-cellular epitopes of the protein. These monoclonal antibody reagents are in the public domain and could be readily

incorporated into extended DotScan™ antibody microarrays as required. The application of CD antigen signatures is far broader than just an application in human leukaemias and has the potential to ultimately fulfil predictions made by Milstein (1984), namely the extensive sub-phenotyping of normal and diseased cells using patterns of cell surface CD antigen expression.

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