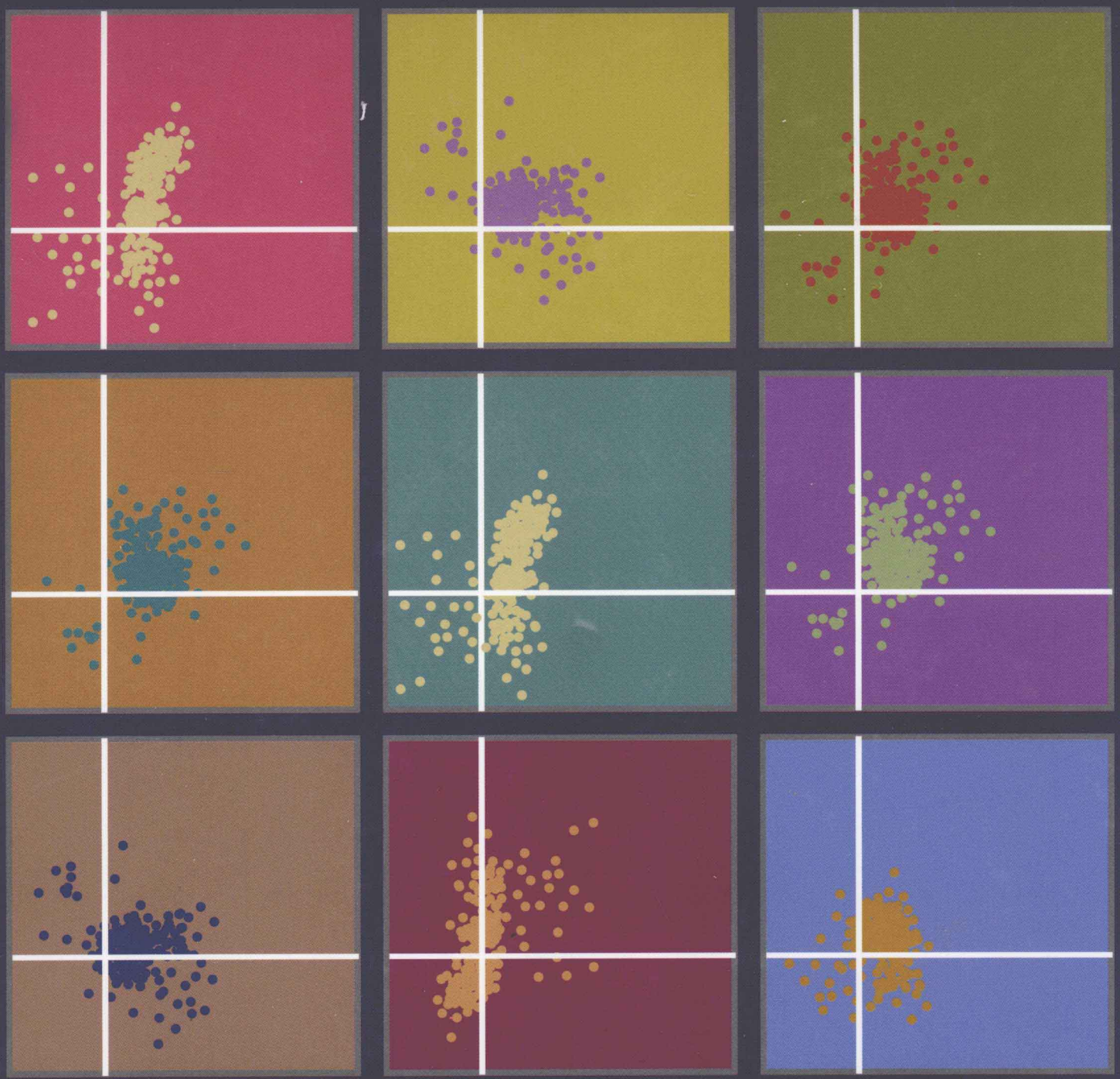
Flow Cytometry of Hematological Malignancies

Claudio Ortolani



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Flow Cytometry of Hematological Malignancies

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Foreword by Maryalice Stetler-Stevenson

Flow cytometry is a crucial tool in the diagnosis of hematolymphoid neoplasms, determining prognosis and monitoring response to therapy. Clinical flow cytometric immunophenotyping, however, is a complex field requiring extensive expertise in normal and abnormal patterns before clinical tests can be appropriately interpreted. Those new to the field are left with the conundrum of how best to achieve this expertise. At particular disadvantage is the resident or clinical fellow seeking to interpret flow cytometric data on a specific patient. The typical flow cytometry reference text is written in an encyclopedic format with extensive narrative that is not conducive to looking up the meaning of unusual test results. Furthermore, the general flow cytometry textbook, although a useful reference, cannot completely cover all the aspects needed to interpret clinical flow cytometry data. Therefore, *Flow Cytometry of Hematological Malignancies* fills a much needed role in hematopathology and hematology/oncology. The presentation is oriented toward the diagnostic laboratory in the academic center as well as in the general hospital.

Flow Cytometry of Hematological Malignancies is organized in a novel manner that makes it especially useful for the medical student and residents/fellows still in training, while still providing a valuable resource for hematopathologists, hematologists/oncologists and experts in the field of clinical flow cytometry. It lists antigens typically studied in clinical flow cytometry laboratories, from CD1 to CD138, followed by a discussion of general as well as flow cytometric features and hematolymphoid neoplasms expressing each antigen. Thus, when interpreting a clinical flow cytometry report, one can easily research an unusual antigen expressed by a leukemia or lymphoma. This pattern of organization makes more sense than only presenting lists of neoplastic processes and the expected flow cytometric findings. One has to first know the diagnosis on a particular patient before such a reference can be useful. *Flow Cytometry of Hematological Malignancies* also provides the usual description of typical flow cytometric immunophenotypical findings in the various hematolymphoid neoplasms. This is useful as a reference for panel design as well as diagnosis.

Flow Cytometry of Hematological Malignancies is being published at a time when the field is expanding rapidly and flow cytometry is assuming an even greater role in management of patients with hematolymphoid neoplasia. Dr Ortolani, an outstanding flow cytometrist, possesses extensive expertise in the clinical arena. For over 30 years he was employed in the Clinical Pathology Department of the Venice General Hospital, running one of the first diagnostic flow cytometry units in Italy. His main clinical activity was the diagnosis of hematological neoplasms, with a particular interest in lymphoproliferative diseases. Dr Ortolani has also been very active in teaching flow cytometry in many national and international courses. He has written what I believe to be an outstanding textbook covering the essential aspects of clinical flow cytometry. Dr Ortolani is to be commended for this brilliant contribution that is sure to become a well-used textbook in clinical centers around the world.

Maryalice Stetler-Stevenson, PhD, MD
Chief, Flow Cytometry Laboratory
National Cancer Institute, National Institutes of Health
Bethesda, MD, USA

Foreword by Bruno Brando

The European Society for Clinical Cell Analysis (ESCCA) is proud to present this volume by Dr Claudio Ortolani. *Flow Cytometry of Hematological Malignancies* is a benchtop companion for all who are involved in the complex process of characterization and diagnosis of leukemias and lymphomas by immunophenotypical techniques and flow cytometry.

This volume is a useful quick reference text for the matching of CD antigens with malignant hematological diseases, as defined by the WHO 2008 classification, taking into account antibody clones, features and behavior, with particular emphasis on variant forms and unexpected presentations.

After several decades of clinical and laboratory practice in this field, Dr Claudio Ortolani has meticulously prepared this book under the auspices of the ESCCA. It represents a major achievement for the dissemination of knowledge in one of the most important specialties within clinical cell analysis, as the book aims to improve and standardize the diagnostic process of malignant blood diseases. As a result, communication between clinicians and laboratory operators should benefit!

Bruno Brando
ESCCA President
Director, Hematology Laboratory and Transfusion Center,
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Preface and acknowledgments

The cytometric analysis of hematological malignancies is one of the most difficult applications of flow cytometry, requiring both a good knowledge of hematopathology and good control of the technique. Moreover, the effort of operators is made harder by the continuous evolution of the technology and by the continuous progress in the comprehension of the nature of the diseases.

This book was compiled from a series of notes originally intended for people practically involved in the field of diagnostic flow cytometry, and it is an example of what the author would have liked to consult at the beginning of his own career. The goal of this book is to offer the reader a quick and updated source of information on the phenotype of the hematological malignancies recognized by the last WHO classification, with the major exception of Hodgkin lymphoma which because of its peculiar nature is still beyond the limits of flow cytometry, even if things promise to change in the next few years.

The author may have unwittingly sown a number of mistakes and imprecisions, and he will be grateful to all colleagues who report these to him. He also realizes that this book could not have been written without the help of many friends and colleagues. Being unable to cite all of them, the author wants to particularly thank his friend Bruno Brando, current President of the European Society for Clinical Cell Analysis, for the continuous moral and practical support he has given over the years.

Claudio Ortolani MD
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Abbreviations

ABC	antibody-binding capacity
ABL	acute basophilic leukemia
a-CML	atypical chronic myeloid leukemia
ADP	adenosine diphosphate
AITL	angio-immunoblastic T cell lymphoma
ALCL	anaplastic large cell lymphoma
ALCL ALK+	anaplastic large cell lymphoma, ALK+
ALK	anaplastic lymphoma kinase
AML	acute myeloid leukemia
AMLL	acute mixed lineage leukemia
AMM	agnogenic myeloid metaplasia
ANKL	aggressive NK cell leukemia
APC	antigen-presenting cell
ASM	aggressive systemic mastocytosis
ATLL	adult T cell leukemia/lymphoma
ATRA	all-trans retinoic acid
AUL	acute undifferentiated leukemia
AZT	azidothymidine (zidovudine)
BAL	bronchoalveolar lavage
B-ALL	B cell acute lymphocytic leukemia
B-CLL	B cell chronic lymphocytic leukemia
B-CLL/PL	B-CLL in prolymphocytoid transformation
BCR	B cell receptor
BFU-E	burst-forming units/erythroid
BL	Burkitt lymphoma
B-LBL	B lymphoblastic lymphoma
B-NHL	B cell non-Hodgkin lymphoma
BPDC	blastic plasmacytoid dendritic cell
B-PLL	B cell prolymphocytic leukemia
B-SLL	B cell small lymphocytic lymphoma
c-ALCL	cutaneous anaplastic large T cell lymphoma
CEA	carcinoembryonic antigen
CEL	chronic eosinophilic leukemia
CFU-GM	colony-forming unit granulocyte-macrophage
CGH	comparative genomic hybridization
CINCA	chronic infantile neurological cutaneous articular syndrome
CLA	cutaneous lymphocyte antigen
CM	cutaneous mastocytosis
CML	chronic myeloid leukemia
CML-BC	CML in blastic crisis
CMML	chronic myelomonocytic leukemia
CMPN	chronic myeloproliferative neoplasm
CMV	cytomegalovirus
CNKL	chronic natural killer cell lymphocytosis
CNL	chronic neutrophilic leukemia
CLPD	chronic lymphoproliferative disease
CTCL	cutaneous T cell lymphoma
DFS	disease-free survival
DLBCL	diffuse large B cell lymphoma
EATCL	enteropathy-associated T cell lymphoma
EBV	Epstein–Barr virus
EMA	epithelial membrane antigen
ENKL	extranodal NK/T lymphoma
ENMZL	extranodal marginal zone lymphoma
EPC	endothelial progenitor cells
ET	essential thrombocythemia
FAB	French-American-British
FCCL	follicular cell cutaneous lymphoma
FDC	follicular dendritic cell
FDCS	follicular dendritic cell sarcoma
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
FL	follicular lymphoma
FSC	forward scatter
GM-CSF	granulocyte macrophage-colony stimulating factor
HAL	hybrid acute leukemia
HBLD	hairy B cell lymphoproliferative disorder
HCL	hairy cell leukemia
HCL-J	hairy cell leukemia, Japanese variant

HCL-v	hairy cell leukemia, variant
HES	hypereosinophilic syndrome
HPC	hemopoietic precursor cell
HSTCL	hepatosplenic T cell lymphoma
ICT	indeterminate dendritic cell tumor
IDCS	interdigitating dendritic cell sarcoma
Ig	immunoglobulin
IL	interleukin
ISM	indolent systemic mastocytosis
IVL	intravascular lymphoma
IVLBCL	intravascular large B cell lymphoma
JMML	juvenile myelomonocytic leukemia
KIR	killer cell immunoglobulin-like receptor
LBCL	large B cell lymphoma
LBP	lipopolysaccharide-binding protein
LCA	leukocyte common antigen
LCH	Langerhans histiocytosis
LDH	lactate dehydrogenase
LHL	lymphoepithelioid lymphoma
LIR	leukocyte Ig-like receptors
LPL	lymphoplasmacytic lymphoma
LPS	lipopolysaccharide
LRP	lung resistance protein
LyP	lymphomatoid papulosis
MBL	monoclonal B cell lymphocytosis
MCL	mantle cell lymphoma/mast cell leukemia
MDCL	myeloid dendritic cell leukemia
MDS	myelodysplastic syndrome
MDS-U	myelodysplastic syndrome, unclassified
MESF	molecules of equivalent soluble fluorochrome
MF	mycosis fungoides
MFI	mean fluorescence intensity
MGUS	monoclonal gammopathy of undefined significance
MHC	major histocompatibility complex
MM	multiple myeloma
M/NK-AL	acute leukemia of myeloid/NK precursors
MoAb	monoclonal antibody
MPAL	mixed phenotype acute leukemia
MPO	myeloperoxidase
MS	myeloid sarcoma
MVL	microvillous lymphoma
MZL	marginal zone lymphoma
NBS	Nijmegen breakage syndrome
NCA	non-specific cross-reacting antigen
NCAM	neural cell adhesion molecule
NK	natural killer
NMZL	nodal marginal zone lymphoma
PAL	pyothorax-associated lymphoma
PBL	plasmablastic lymphoma
PCH	pseudo Chediak–Higashi
PCL	plasma cell leukemia
PCMZL	primary cutaneous marginal zone lymphoma
PE	phycoerythrin
PEL	primary effusion lymphoma
PHA	phytohemagglutinin
PMA	phorbol myristate acetate
PMF	primary myelofibrosis
PPBL	persistent polyclonal B cell lymphocytosis
PRCA	pure red cell aplasia
PTCL	peripheral T cell lymphoma
PTCLnos	peripheral T lymphoma not otherwise specified
PTLD	post-transplant B lymphoproliferative disease
PV	polycythemia vera
RA	refractory anemia
RAEB	refractory anemia with excess of blasts
RARS	refractory anemia with ringed sideroblasts
RCMD	refractory cytopenia with multilineage dysplasia
RCUD	refractory cytopenia with unilineage dysplasia
RN	refractory neutropenia
RT	refractory thrombocytopenia
s-ALCL	systemic anaplastic large cell lymphoma
SCF	stem cell factor
SLL	small lymphocytic lymphoma
SLVL	splenic lymphoma with villous lymphocytes
SM	systemic mastocytosis
SM-AHNMD	systemic mastocytosis with an associated clonal hematological non-mast cell disorder
SMZL	splenic marginal zone lymphoma
SPTCL	subcutaneous panniculitic T cell lymphoma
SRCT	small round cell tumor
SRPL	splenic diffuse red pulp small B cell lymphoma
SS	Sézary syndrome
SSC	side scatter
T-ALL	T cell acute lymphocytic leukemia
TAM	transient abnormal myelopoiesis
T-CLL	T cell chronic lymphocytic leukemia
TCR	T cell receptor
TCRBCL	T cell-rich large B cell lymphoma
THL	true histiocytic lymphoma
T-LBL	T cell lymphoblastic lymphoma
T-LGL	T cell large granular lymphocytic leukemia
TMPD	transient myeloproliferative disorder
T-PLL	T cell prolymphocytic leukemia
TPO	thrombopoietin
TZL	T zone lymphoma
WD-EMT	well differentiated extramedullary myeloid tumor
WHO	World Health Organization

Contents

1 Antigens

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CD1 Antigens

General features

CD1 antigens are a group of at least four different glycoproteins, named CD1a, CD1b, CD1c and CD1d, which weigh 43–49 kD. They are encoded by a group of genes situated on the long arm of chromosome 1 [1] and play a role in the presentation of lipidic and glycolipidic antigens to NKT cells [1,2].

CD1 antigens are mainly expressed on cells belonging to T and B lineages and on antigen presenting cells (APC).

As for the T lineage, CD1 antigens have been demonstrated on the membrane of the cortical or "common" thymocytes [3] and on the membrane of some T lymphocyte subsets in cord and neonatal peripheral blood [4]; a low expression of CD1 antigens can be demonstrated in the cytoplasm of T lymphocytes activated by phytohemagglutinin (PHA) *in vitro* [5].

As for the B lineage, both CD1c and CD1d have been demonstrated on the precursors and on some subsets of mature B lymphocytes. More precisely:

- CD1c has been demonstrated on some subsets of B lymphocytes in the peripheral blood [6] [7], in the spleen [6,7] and in the mantle of the germinal center [6]
- CD1c+ B lymphocytes account for the majority of B cells in tonsils [8], in cord blood [4], in the peripheral blood of newborns [4], and in the peripheral blood of subjects submitted to autologous or allogeneic bone marrow transplantation during the first year following transplant [7]
- CD1d has been demonstrated on the membrane of bone marrow B precursors [9] and of B lymphocytes in peripheral blood [10], in the mantle of the germinal center [10], and in the spleen [11].

Finally, as for the antigen-presenting cells, CD1 antigens have been demonstrated on many cellular types. More precisely:

- CD1a has been demonstrated on Langerhans cells [12], where it is expressed at an intensity of 1600 molecules per cell [13], on some CD11b+ CD14+ mononuclear cells reported in the peripheral blood of burnt subjects and interpreted as Langerhans cell precursors migrating from bone marrow to epidermis [14], on monocytes activated with GM-CSF *in vitro* [15], and on *in vitro* monocyte-derived dendritic cells [16]
- CD1b has been demonstrated on monocytes activated with granulocyte macrophage-colony stimulating factor (GM-CSF) *in vitro* [15] and on a subset of Langerhans cells [17]
- CD1c has been demonstrated on monocytes activated with GM-CSF *in vitro* [15], on Langerhans cells [17], and on a minor subset of myeloid dendritic cells characterized by CD11c++ CD123± phenotype [18]
- CD1d has been demonstrated on "resting" monocytes [10], on dendritic cells of the dermis [19], and on *in vitro* monocyte-derived dendritic cells [19]
- CD1a, CD1b, CD1c and CD1d have been demonstrated in the "foam cells" of the atherosclerotic plaque [20].

Cytometric features

The cytometric demonstration of molecules belonging to the CD1 family should be performed while taking the following points into account:

- cytometric studies have demonstrated that activated T lymphocytes express CD1c on the membrane only when kept at room temperature, and fail to mount the molecule on the surface when kept at +4 or to +37°C [21]
- the expression of CD1a on the surface of the leukemic blasts can fluctuate spontaneously after a short period of incubation *in vitro* [22].

The antibodies specific for CD1 antigens do not behave in the same way. It should be kept in mind that CD1a features four different epitopes, the first of which is recognized by clones D47, Na1/34 and L119, the second by clone L404, and the third by clone L504 [23]; it should be noted that CD1a on the cells of B cell chronic lymphocytic leukemia (B-CLL) can be demostrated only with clones other than OKT6 or Na1/34 [24].

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The clones 7C4 and IOT6b recognize CD1b, and show different cellular reactivities as well [25].

Diagnostic features

CD1 antigens in neoplastic diseases of B cell precursors

CD1 antigens have been demonstrated in some cases of common acute lymphocytic leukemia [22]. In a group of 80 patients affected by childhood B lymphoblastic leukemia (B-ALL), the expression of CD1d has been demonstrated in 15% of the cases [9]. CD1d expression is significantly associated with pre-B phenotype, rearrangement of the gene MLL, and shorter global survival [9].

CD1 antigens in neoplastic diseases of T cell precursors

CD1 antigens are generally expressed on the cells of T lymphoblastic leukemia/lymphoma related to the stage of cortical or "common" thymocyte [3,22] (Fig. 1.1). According to the EGIL classification of T lymphoblastic leukemias, CD1 antigens are typically present in the T III form but missing in the T I, T II and T IV forms [26]. If CD13 is negative, the expression of CD1a is related to good survival [27], while the expression of CD1a together with CD10 is associated with the presence of the t(5;14) translocation [28].

CD1 antigens in acute myeloid leukemias

The expression of CD1a on the surface of the blasts of the acute myeloid leukemias has repeatedly been reported [22,29,30]. According to some authors, the expression of CD1a and CD1d is restricted to FAB subtypes characterized by a monocytic component [30].

CD1 antigens in neoplastic diseases of mature B cells

The presence of CD1 antigens on the surface of the elements of the neoplastic diseases of mature B cells has been demonstrated by different authors. More specifically, the cells of B cell chronic lymphocytic leukemia (B-CLL) have been reported to express CD1a [8,24], CD1c [6], and CD1d, whose intensity is more elevated in the cases without somatic hypermutations [31]; it is noteworthy that CD1a on B-CLL cells can be demostrated only with clones other than OKT6 or Na1/34 [24].

As for other neoplastic diseases of mature B cells, it has been reported that B cell prolymphocytic leukemia (B-PLL) cells express CD1c [8] [32], that Burkitt lymphoma (BL) cells do not express CD1c [6], that hairy cell leukemia (HCL) cells express CD1a [33] and CD1c [8], and that multiple myeloma (MM) cells express CD1d in the early stages, but tend to reduce its expression with disease progression [34].

CD1 antigens in neoplastic diseases of mature T and NK cells

The expression of CD1 antigens has sporadically been reported in rare cases of peripheral T cell lymphoma (PTCL) [35].

CD1 antigens in myelodysplastic and chronic myeloproliferative diseases

The expression of CD1d has been reported on the cells of the juvenile myelomonocytic leukemia (JMML) [30]. CD1a, CD1b and CD1c are expressed on the membrane of the blast cells in the 20% of cases of chronic myeloid leukemia (CML) in blastic crisis [22].

CD1 antigens in other pathological conditions

CD1a, CD1b and CD1c have been demonstrated with immunohistochemical techniques in the Langerhans cell histiocytosis (LCH) [36,37]. One of the most typical features of the pulmonary location of Langerhans cell histiocytosis (LCH) is the occurrence of more than 5% of CD1+ cells in the bronchoalveolar lavage (BAL) liquid [38].

CD1a expression has been reported in an anecdotal case interpreted as acute leukemia of Langerhans cell precursors on the basis of the presence of Birbeck granules and of the ability of blasts to develop dendritic processes when cultured *in vitro* [39].

CD1a has been demonstrated with immunohistochemical techniques in the indeterminate dendritic cell tumor (ICT) [40], but not in the follicular dendritic cell sarcoma (FDCS) nor in the interdigitating dendritic cell sarcoma (IDCS) [37].

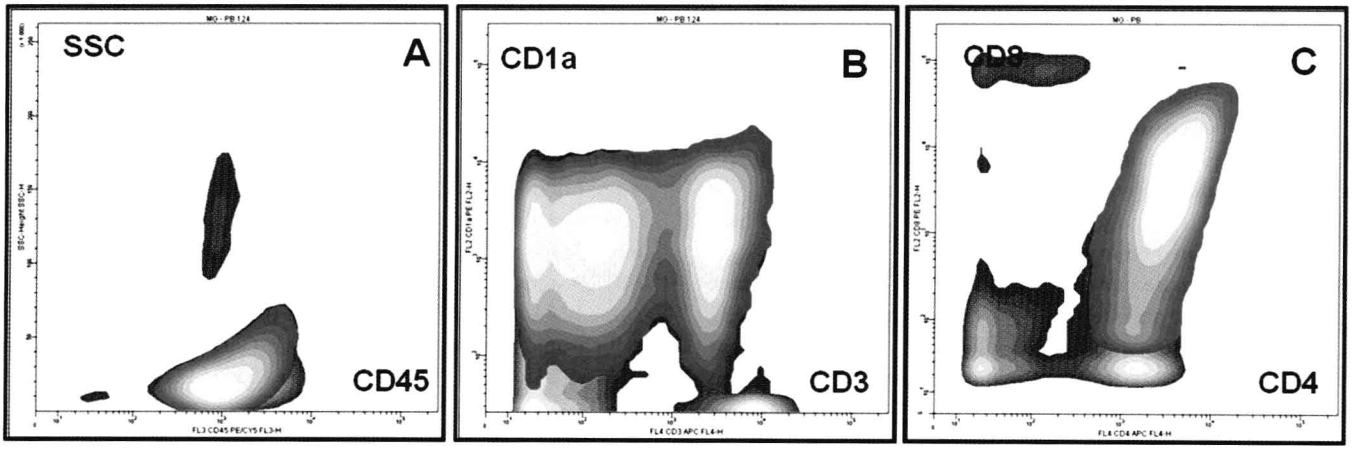

Figure 1.1 Peripheral blood from a subject affected by T lymphoblastic leukemia (T-ALL). The blasts (*red*) express the phenotype CD45 dim+, CD1a+, CD3+ (heterogeneous), CD4+, CD8+.

CD2 Antigen

General features

CD2 is a 45–58 kD glycoprotein belonging to the superfamily of the immunoglobulins, which is encoded by a gene situated on the short arm of chromosome 1 [41]. CD2 is an adhesion molecule, constitutes the ligand of the CD58 molecule [41], and interacts with CD48 and CD59 molecules as well [42].

CD2 is normally expressed on thymocytes, on whose membrane it begins to appear at the prothymocyte level [43], and on mature T lymphocytes [44].

Not all mature T lymphocytes co-express CD2. Indeed, it is well known that in the peripheral blood, small subsets of T lymphocytes exist that show CD3+ CD2– phenotype, and are characterized by the expression of T cell receptor (TCR) either with alpha/beta [45] or gamma/delta chains [46].

The expression of CD2 is not restricted to the T lineage. Indeed, it is well known that CD2 is expressed:

- on 70–90% of the NK cells negative for CD3 [47,48], where it is upregulated by activation [49]
- on a minority of follicular dendritic cells (FDC) [50]
- on a subset of mononuclear peripheral cells interpreted as precursors of myeloid dendritic cells [51]
- on a subset of peripheral monocytes characterized by the co-expression of Fc epsilon receptor (FcepsilonRI) [52]
- on a small subset of B cells in fetal liver [53], in fetal bone marrow [53], in thymus [54], in peripheral blood [55], and in the bone marrow of normal subjects [55].

Cytometric features

The staining of peripheral normal lymphocytes with an anti-CD2 monoclonal antibody generates a positive histogram with a narrow gaussian-like peak, clearly separated from the negative component, with a channel peak representing the presence of 24±7 E03 ABC (antibody binding capacity) [56].

Bimodal histograms can often be seen, especially when immune system activation is ongoing, because a higher number of CD2 molecules is expressed on activated cells [57] (Fig. 1.2).

In these cases the CD2 bright+ population tends to show higher values of forward and side scatter than the CD2 dim+ population.

CD2 bright+ cells nearly exclusively express CD45RO, while the CD2 dim+ population display either CD45RO or CD45RA [58]; the staining of CD2+ CD45RA+ cells with an anti-CD2 monoclonal antibody generates a histogram with a channel peak representing the presence of 21±4 E03 ABC while the staining of CD2+ CD45R0+ lymphocytes with the same MoAb generates a histogram with a channel peak representing the presence of 55±9 E03 ABC [56].

In accordance with the state of chronic activation caused by HIV infection, the lymphocytes of HIV-infected subjects seem to express a higher amount of CD2 molecules [59], while a reduced expression has been documented on the lymphocytes of elderly subjects [60]. According to some authors, the expression of CD2 on NK cells is dishomogeneous, being more intense in the CD16 dim+ CD56 bright+ subset than in the CD16 bright+ CD56 dim+ subset [61].

Not all the anti-CD2 MoAbs behave in the same way; some clones are able to inhibit the E-rosette formation [62], while others are able to activate T lymphocytes *in vitro* [63].

Diagnostic features

CD2 in neoplastic diseases of B cell precursors

Depending on the survey, the expression of CD2 has been reported in 1–4% of the observed cases [68,69,1065].

CD2 in neoplastic diseases of T cell precursors

CD2 is generally expressed on the blasts of the neoplastic diseases of T cell precursors, but it may be missing in the most immature forms [64]. According to the EGIL classification of T lymphoblastic leukemias, CD2 is typically present in the T II, T III and T IV forms, but is missing in the most immature form, T I [26]. CD2 is generally expressed by the cases with TCR alpha/beta, but only by some cases with TCR gamma/delta [65,66]; its presence in childhood cases is correlated with an increased probability of maintaining complete remission [67].

CD2 in acute myeloid leukemias (AML)

Depending on the survey, the expression of CD2 has been reported in 3–34% of the observed cases [70–77]. CD2 seems to be frequently expressed:

- on the blasts of pediatric AML-M2 negative for translocation t(8;21) [78]
- on the promyelocytes of AML-M3, with particular predilection for the microgranular variant (AML-M3v) [76,79,80], and for the presence of the "short" type of the PML-RARA fusion gene [77,80]

• on both the monocytic and non-monocytic neoplastic cells of AML-M4 [76,81,82]
• on the blasts of AML-M5 [82].

The presence of CD2 (and also of CD4, CD7 and CD56) on the blasts of AML is correlated with an increased risk of extramedullary disease (granulocytic sarcoma, and cutaneous, gingival and meningeal involvement) [83], and with a lower incidence of complete remission [84]. The CD2 expression has been reported in cases of AML with morphological anomalies mimicking the picture of Chediak–Higashi disease (pseudo Chediak–Higashi, PCH) [85], and in some cases of blastic plasmacytoid dendritic cell (BPDC) neoplasm [86]. The presence of CD2 on AML-M3 promyelocytes correlates with the occurrence of thrombotic events [87].

In AML-M4 with inv(16)/t(16;16), the expression of CD2 is variable, and has been reported as weaker in cases with fusion transcript CBFbeta-MYH11 other than type A [1736].

CD2 in neoplastic diseases of mature B cells

Sporadic reports exist signaling the presence of CD2 in isolated cases of B lineage non-Hodgkin lymphoma [88,89]. Since CD2 has been demonstrated on the surface of normal B lymphocytes [55], it is theoretically possible that these cases constitute a clonal expansion of very infrequent normal B cells rather than an expansion of B cell with an aberrant phenotype.

The expression of CD2 has occasionally been demonstrated in the sporadic B cell chronic lymphocytic leukemia (B-CLL) [55], but it seems particularly frequent in familial B-CLL, where it appears in 13% of the cases [90]; the demonstration of CD2 on the cells of a patient affected by B-CLL suggests that clinical investigations should be extended to the relatives as well [90].

Furthermore, CD2 has been demonstrated in some cases of follicular lymphoma (FL) [55], in some cases of diffuse large B cell lymphoma (DLBCL) [55,91], in some cases of diffuse large B cell lymphoma associated with pyothorax (PAL) [92], in some cases of hairy cell leukemia (HCL) [55], and in a case of multiple myeloma (MM) [93].

CD2 in neoplastic diseases of mature T and NK cells

CD2 is generally expressed on the cells of the neoplastic diseases of mature T and NK cells, but it may also be missing or expressed in an aberrant way. In the peripheral T lymphoma not otherwise specified (PTCLnos), about a third of the cases has been reported to show an aberrant antigen expression [94,95]. An aberrant CD2 expression has been reported with immunohistochemical methods in atypical cutaneous T cell infiltrates of subjects affected by mycosis fungoides [96], and with flow cytometric methods on neoplastic lymphocytes of subjects affected by Sézary syndrome [97], by T cell chronic lymphocytic leukemia (T-CLL) and by adult T cell leukemia/lymphoma (ATLL) [98].

The CD2 expression is more constant in the cases of angioimmunoblastic T cell lymphoma (AITL) [95], while in T cell large granular lymphocytic leukemia (T-LGL) it has been reported either as constant [99] or as variable [100]. The cases of CD8+ cutaneous T cell lymphoma (CD8+ CTCL) with CD2+ CD7– phenotype show a better prognosis than those with phenotype CD2– CD7+ [101].

In the neoplastic diseases of mature NK cells, CD2 may be missing [102] but it has been reported in most cases of chronic NK cell lymphocytosis (CNKL) [102–104], of aggressive NK cell leukemia (ANKL) [105,106], and of NK lymphoma [107].

CD2 in myelodysplastic and chronic myeloproliferative diseases

CD2, which is usually missing on normal mast cells [108], has been reported together with CD22 and CD25 on the neoplastic mast cells in systemic mastocytosis and mast cell leukemia [109–111]. Moreover, CD2 has been reported in a third of cases of chronic myelomonocytic leukemia (CMML) [82].

CD2 in other pathological conditions

CD2 has been demonstrated by immunohistochemical methods on the membrane of the cells of Langerhans cell histiocytosis (LCH)[36].

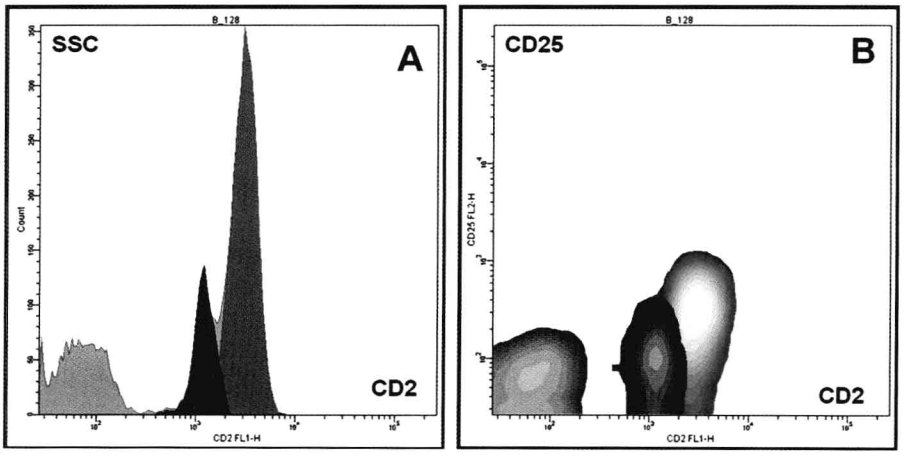

Figure 1.2 The histogram produced by the cytometric analysis of CD2 is bimodal (A), because the activated CD25+ lymphocytes (*red*) express more CD2 molecules than CD25– lymphocytes (*blue*) (B).

CD3 Antigen

General features

CD3 is made up of five different chains, i.e. gamma, delta, epsilon, zeta and eta. Chains gamma, delta, epsilon, and eta are encoded by a gene on the long arm of chromosome 11 [112], while chain zeta, separately clustered as CD247 [113], is encoded by another gene on the long arm of chromosome 1 [114]. In T cells, CD3 transmits the activation signal produced by the engagement of TCR [115,116].

Stechiometrical ratios between CD3/TCR components have not yet been completely understood. There is evidence that the CD3/TCR complex forms a multimeric array together with the tyrosine-phosphatase CD45, with a tyrosine-kinase, and with the CD7, which takes part in signal transmission [117].

The expression of the delta and epsilon chains is restricted to T lymphocytes, with two important exceptions:

- the fetal and adult activated NK cells, which can contain delta and epsilon chains in the cytoplasm [118,119]
- the plasmacytoid dendritic cells, in the cytoplasm of which epsilon chains have been demonstrated [120].

An isolated report exists, according to which eosinophils express low levels of CD3 together with a functional gamma/delta TCR [121].

As a rule, gamma and zeta chains are present in NK cells as well [122], as either homodimers or heterodimers [123]. In NK cells, both chains are not covalently linked with the transmembrane tail of the CD16, and transmit the signal produced by the linkage between CD16 and the IgG crystalizable fragment [124–126]. Signal transduction by zeta chain is carried out by the intracytoplasmic protein ZAP-70 [127,128].

During normal T cell maturation, the CD3 appears in the cytoplasm at the prothymocyte level, but is only expressed on the membrane from the common thymocyte stage on [3,129–134].

During normal T cell maturation, the TCR and the CD3 complex are assembled together before they are expressed on the surface [135]; consequently, the TCR is not normally expressed on the membrane in the absence of CD3, and vice versa [136,137].

CD3 has been demonstrated with immunohistochemical methods in the cytoplasm of Warthin–Finkeldey polykaryocytes, which can be seen in tonsils during the measles prodromic period, and are probably derived from T lymphocytes [138].

Cytometric features

Almost all anti-CD3 monoclonal antibodies are specific for an epsilon chain epitope [139,140]. The staining of peripheral normal lymphocytes with an anti-CD3 epsilon monoclonal antibody generates a positive histogram with a narrow gaussian-like peak, clearly separated from the negative component, with a channel peak representing the presence of 57 ± 7 E03 ABC [56].

Evidence does exist that the number of CD3 epsilon chains is not the same for every T lymphocyte, but is particularly high on gamma/delta T cells [141], which express roughly 116 ± 15 E3 ABC per cell [142].

Among peripheral T lymphocytes, CD3 expression tends to vary depending on the T lymphocyte subset. Evidence does exist that, in comparison to CD8 bright+ T lymphocytes, the CD3 mean fluorescence intensity (MFI) of positive cells is almost twice as intense in CD4+ T cells, while T CD8 dim+ lymphocytes behave similarly to CD4+ lymphocytes. This behavior does not depend on cellular dimensions, inasmuch as in CD4+ lymphocytes scatter values are even lower than in CD8 bright+ ones [58].

A reduced expression of CD3 has been reported in other cases:

- in alveolar T cells, with a negative modulation greater for CD4+ cells [143]
- in activated T cells that infiltrate nasal polyps [144]
- in intrathyroidal T lymphocyte subsets in autoimmune thyroid disease [145]
- in intestinal intraepithelial T lymphocytes [146]
- in T cells of patients given OKT3 rescue treatment for kidney rejection [147]
- in T cells of patients with HIV infection [59,148]
- in T cells of aged subjects [60]
- in a minor subset of peripheral T lymphocytes characterized by low CD4 expression, and positivity for CD25 and HLA-DR [149].

This CD3 downmodulation might be due to the activation state common to the great majority of the cases reported; it is important to bear in mind that a CD3 downmodulation can be caused by apoptosis as well [150].

In some cases, the positive histogram can appear with a bimodal shape, mostly due to the presence of a consistent subset of gamma/delta T cells, which actually bear more TCR/CD3 epsilon complexes than alpha/beta T cells on the membrane [142] (Fig. 1.3).

In our experience, this behavior does not occur with the gamma/delta T lymphocytes mounting Vdelta1/Jdelta1 sequences stained by deltaTCS1 MoAb (Fig. 1.4).

Sometimes it is possible that the bimodality of the CD3+ peak is due to the presence of a clonal T cell population, homogeneously expressing the molecule at an intensity that differs from other normal residual T cells. This behavior is frequently reported in patients affected by mature T cell malignancies [94,151].

In comparison to mature T cells, thymocytes express CD3 with a different intensity; as a rule, most common or cortical CD1+ CD4+ CD8+ thymocytes express low amounts of CD3, while mature or medullar CD1– and CD4+ or CD8+ thymocytes express the molecule in the same way as mature T lymphocytes [130,152], with a differential higher expression on CD4+ CD8– T cells [58].

A thymocyte CD4+ CD8+ subset has been reported expressing high levels of CD3; it is hypothesized that this subset is a late differentiation stage between cortical and medullar thymocytes [153].

As mentioned previously, the CD3 can be looked for both on the membrane and in the cytoplasm of the cell. The demonstration of the intracytoplasmic molecule requires the use of permeabilization techniques which allow intracellular entry of the antibody. Although they could be improved by some optimization procedures [154], such techniques can rely on the use of standardized commercial permeabilizing solutions [155–158].

MoAb OKT3, SK7/Leu4 and UCHT-1

The three monoclonal antibodies OKT-3, SK7/Leu4 and UCHT-1 recognize CD3 epsilon chains in cells transfected with genes coding for epsilon and delta chains or for epsilon and gamma chains, but do not recognize CD3 epsilon chains in cells transfected with genes coding for epsilon chains only [159]. This behavior suggests that the three antibodies recognize a conformational epsilon chain epitope, depending on the association of epsilon chain with delta or gamma chain, and are not able to detect isolated intracytoplasmic epsilon chains [159].

Consequently, a negativity for intracytoplasmic epsilon chains accomplished with one of the aforementioned antibodies is not sufficient proof of epsilon chain absence, and should be validated using an antibody specific for isolated epsilon chains, such as SP34 and APA 1/1, or a polyclonal rabbit antiserum raised against a synthetic polypeptide mimicking a sequence on the intracytoplasmic tail of the epsilon chain [160].

This point is of some practical importance. Given that in thymocyte cytoplasm delta and epsilon chains are simultaneously expressed from the prothymocyte level onwards [161], these three antibodies are perfectly suitable for demonstrating the intracytoplasmic CD3 antigen in T cell malignancies, but could miss it in some cases of NK neoplasms. It has been reported that MoAb UCHT-1 is able to stain the cerebellar Purkinje cells [162].

MoAb WT31

In the same way as OKT-3, SK7/Leu4 and UCHT-1, the monoclonal antibody WT31 recognizes CD3 epsilon chains in cells transfected with genes coding for epsilon and delta chains or for epsilon and gamma chains, but do not recognize CD3 epsilon chains in cells transfected with genes coding for epsilon chains only [159]. This behavior confirms that, contrary to the original hypothesis [163] and in keeping with successive remarks [139], MoAb WT31 is not specific for a TCR alpha/beta determinant, but binds a conformational epitope on CD3 epsilon chains, and should be considered a *bona fide* anti-CD3 antibody.

Nevertheless, it should be stressed that the epitope stained by MoAb WT31 is particularly accessible to this MoAb in the case of TCR alpha/beta co-expression; this condition makes MoAb WT31 fit for the presumptive identification of TCR alpha/beta T cells, expecially if used in combination with a second antibody specific for the same chain. In this case, the sterical hindrance between the two antibodies blocks the binding between WT31 and the epsilon chain of T cells bearing gamma/delta TCR, and WT31 behaves like a MoAb specific for alpha/beta TCR only.

In these conditions, the staining of peripheral normal T lymphocytes with the WT31 monoclonal antibody generates a histogram with a negative peak encompassing T cells bearing gamma/delta TCR (Fig. 1.5).

The removal of the sterical hindrance allows the WT31 monoclonal antibody to bind the epsilon chain of T cells with gamma/delta TCR, although in a weaker way than alpha/beta T cells. Indeed, if we stain a sample containing a high number of gamma/delta T cells using both the WT31 monoclonal antibody and a second monoclonal antibody specific for TCR gamma/delta, the WT31 monoclonal antibody will generate a histogram with a first positive peak which encompasses gamma/delta negative T cells, and a second positive but intermediate peak which encompasses gamma/delta positive ones (Fig. 1.6).

From a practical point of view, the possibility of sterical hindrance between the WT31 MoAb and another anti-CD3 epsilon monoclonal antibody suggests that a sequential staining procedure should be performed, in which the sample is incubated first with WT31 alone and then with the other anti-CD3 epsilon antibody.

MoAb T3

The FITC-conjugated form of T3 displays unexpected behavior [164]. In a multicolor analysis which combines a MoAb specific for TCR gamma/delta (clone 11F2) and a second anti-CD3 epsilon MoAb (clone SK7), the FITC-conjugated form of T3 does not recognize gamma/delta T cells (Fig. 1.7).

It is interesting to notice that in this model, T3-FITC behaves very similarly to WT31, which is shown for comparison (Fig. 1.8).

The anomalous behavior of T3-FITC is difficult to explain. The small molecular volume of FITC rules out a sterical hindrance effect, and the independence of the phenomenon from the length of incubation does not suggest affinity variations induced by the conjugation procedures.

It has been observed that, owing to a different glycosylation pattern, CD3 delta chains in gamma/delta T cells display a more acidic isoionic point than CD3 delta chains in alpha/beta T cells [165]. It could be hypothesized perhaps that FITC increases the total negative charge of the FITC-conjugated antibody, allowing

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