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Assessment of CD96 expressionin Acute Myeloid Leukemia Patients

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Abstract

Background:Acute myeloid leukemia (AML) is a clonal malignant disease of hematopoietic tissues that is defined by the accumulation of leukemic blast cells in the marrow resulting in hematopoietic failure.Among human peripheral blood cells,Cluster of Differentiation 96 (CD96) expression was observed on T and Natural Killer (NK) cells but not on the majority of B cells, monocytes, and granulocytes. In contrast to the role of CD96 participating in immune surveillance of tumors, CD96 itself was identified as a tumor marker. Indeed, well before first studies deciphered its functions, CD96 was reported to be upregulated in subpopulations of T-Acute lymphoblastic leukemia (ALL) and AML. Increased expression of CD96 was shown in several subsequent studies to correlate with poor prognosis and enhanced resistance to chemotherapy. A promising treatment strategywould therefore be to sort out CD96-expressing stem cells before autologous transplantation of AML patients.

Keywords:Acute myeloid leukemia (AML).Cluster of Differentiation 96 (CD96).

Acute Myeloid Leukemia:

Definitions

Leukemia is a cancer of the marrow and blood and it represents a group of hematological malignancies characterized by clonal expansion of hematopoietic cells with uncontrolled proliferation, decreased apoptosis, and blocked differentiation **(1).**

AML is a clonal hematopoietic disorder that may be derived from either an HSC or a lineage-specific progenitor cell. AML is characterized both by a predominance of immature forms (with variable, but incomplete, maturation) and loss of normal hematopoiesis. Single or multiple hematopoietic lineages may comprise the leukemic clone. The requisite blast percentage is 20% in the peripheral blood and bone marrow; a lower percentage is acceptable in cases with AML-defining translocations and in acute erythroid leukemia **(2).**

Epidemiology

AML is the most common acute leukemia in adults**(3)**. During the past 10 years, the frequency of hematological malignancies (HMs) has increased with a complete difference between the developing and developed countries. **(4).** In Egypt, leukemia is the most common presented hematological malignancy (75%), nearly half of leukemic cases were acute myeloid leukemia **(5).**

The prevalence of AML cases is slightly more common in men compared to women. According to the American Cancer Society's estimations for AML cases in the USA for 2019, there will be 21,450 new cases and 10,920 deaths from AM. Among all the leukemia cases, 32% of cases in adults are due to AML **(6)**.

The incidence of AML increases with age and is highest in adults aged 65 years and older. The median age at diagnosis is 68 years. It is estimated that over half of new cases of AML are diagnosed in individuals 65 years and older, with approximately one-third of patients diagnosed at the age of 75 years or older. Surprisingly, the incidence of AML increased in most countries, but the rise was partly due to an increasing the prevalence of therapy for AML as more patients treated with cytotoxic chemotherapy are cured of their primary malignancy (**7).**

Etiology

Although there are several well-recognized risk factors for the development of AML, little is known about the etiology of most cases. Like most of the malignancies, there is no recognized factor common to most cases of AML. Proven or possible risk factors for AML include genetic, environmental, therapy-related, and pre-existing hematological disorders **(8).**

Leukemogenesis is a multistep process that requires the susceptibility of a hematopoietic progenitor cell to inductive agents at multiple stages. The different subtypes of AML may have distinct causal mechanisms, suggesting a functional link between a particular molecular abnormality or mutation and the causal agent. Generally, known risk factors account for only a small number of observed cases. Most cases of AML arise de novo without recognized leukemogenic exposure **(6).**

Immunophenotyping (IPT) classification:

Immunophenotyping using multiparameter (commonly at least 3- to 4-color) flow cytometry is used to determine the lineage involvement of a newly diagnosed acute leukemia **(9)**. Flow cytometry determination of blast count should not be used as a substitute for morphologic evaluation **(2).** To identify AML (Table 2), the percentage of positive reacting blasts should be greater than 20% with one or more myeloid-associated antigens CD33, CD13. Other myeloid markers as CD11b, CD14, CD15, CD65, CD86, cytoplasmic MPO, CD34, and HLA-DR might be present. The use of panels of monoclonal antibodies has identified certain phenotypic groups that might be clinically important such as the association between M2 subtype with AML t(8,21) cytogenetic abnormality and the expression of CD34 & the B-cell associated cell surface antigen CD19 **(10).**

Table 1: Immunophenoytping markers in AML**(10).**

Markers	*M0*	*M1*	*M2*	*M3*	*M4*	*M5*	*M6*	*M7*
HLA-DR	++	++	++	-	++	++	+	+
CD11b	+	+	+	-	++	++	-	-
CD13	+	++	++	++	++	++	+	+/-
CD14	-	+	+	-	++	++	-	-
CD15	-	-	+	+	+	+	-	-
CD33	+	++	+++	+++	+++	+++	+	+/-

CD41,CD61	-	-	-	-	-	-	-	+++
Glycophorin A	-	-	-	-	-	-	++	-
TdT	++	+	-	-	-	-	-	-
CD34	++	+	-	-	-	-	-	+

Cluster of Differentiation 96 (CD96)

The cluster of differentiation 96 (CD96) was discovered in 1992 and named originally TACTILE, (T cell activation increased late expression). Although identified as a marker distinguishing a subset of acute leukemias, CD96 did not receive further attention for more than a decade. This changed when CD155 (CD155) formerly addressed as a receptor for poliovirus (PVR), was detected as an interaction partner mediating cell adhesion.

Furthermore, these findings suggested a role of the CD155/CD96 axis in target cell elimination by NK cells **(11).**Ironically, **(12)** already mentioned PVR in their publication because it showed up among other polypeptides in a similarity search. Indeed, CD96 and CD155 are membrane-bound receptors of the immunoglobulin superfamily (IgSF) and are distantly related to each other **(13).** However, in contrast to CD155 which is expressed by a huge variety of cell types, available data indicated that CD96 expression is largely restricted to cells of hematopoietic origin, in particular to T and NK cells **(14).** This was confirmed by **(15).**

Yet attempts to demonstrate a direct role of CD96 in NK-mediated killing in vitro failed, a flaw that was resolved later on when it was shown that CD96 can suppress NK cells in vivo **(16)**.

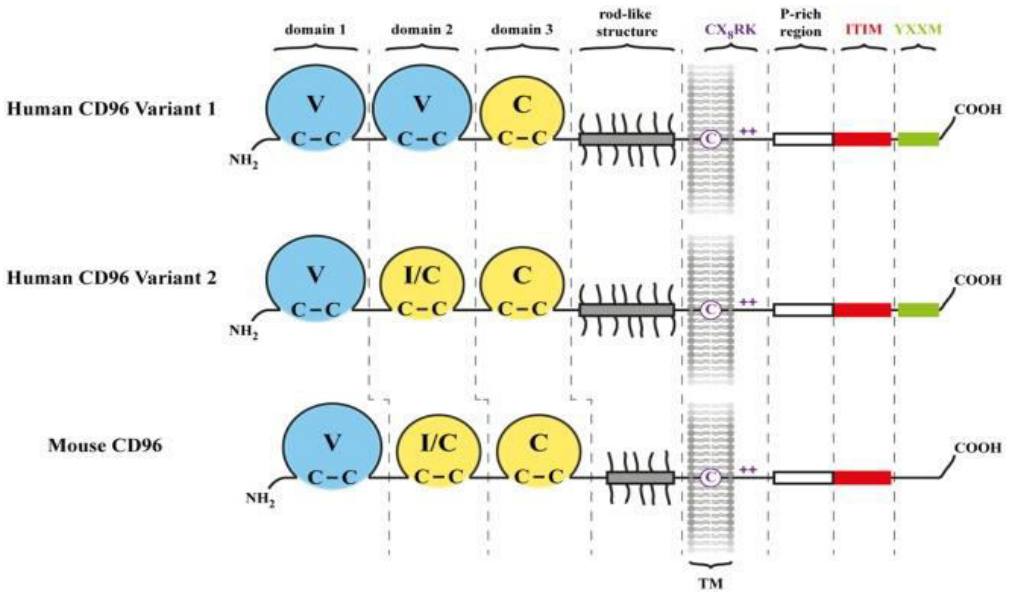

Figure 1: Architecture of CD96. Shown are the two human CD96 (hCD96) isoforms (variant 1 and variant 2) along with mouse CD96 (mCD96). Three Ig-like domains comprise the N-terminal (NH2) part of CD96 in mouse and hCD96 where V indicates a V-like domain and C indicates a C-like domain. The second domain is predicted to fold as an I-like or C-like domain in hCD96 variant 2 and mCD96. The proline/serine/threonine-rich region (gray bar) contains many potential O-linked sugar modification sites (short protrusions) and may adopt a rod-like shape. The transmembrane (TM) and cytoplasmic domain harbor motifs of potential importance for signaling

triggered by CD96. The C denotes a cysteine residing in the TM region, and the + indicates positively charged amino acid residues **(11).**

Structure of CD96:-

i-The IgSF-Part of the Ectodomain:-

CD96 represents a single-pass transmembrane receptor that is heavily *N*-glycosylated **(15).**

The crystal structure of the CD96 ectodomain is not resolved wherefore its folding pattern was deduced from comparisons with other IgSF members. According to this, the outermost domain represents a V-like domain in h/mCD96 and mediates binding to h/mCD155 in trans (**17**).
An N-terminally located V-like domain is a common feature shared by all CD155 family members and as far as investigated, extracellular binding to themselves or other family members (but also to viruses) is invariantly restricted to this domain. Available data from crystal structures of human/mouse nectins, CD155, and TIGIT revealed a consensus binding interface that consists of amino acids **(18)**.

ii-The Stalk Region:-

The three Ig-like domains are separated from the transmembrane (TM)-domain by an unusually long region that is rich in proline, serine, and threonine 3**.** This allows for extensive O-linked glyco-modification that would confer to this domain a rod-like structure. As a consequence of this, the Ig-like domains should protrude from the glycocalyx layer markedly exposing them to contacting cells. Proline/serine/threonine-rich stalks are also present in other TM receptors like CD44 or CD8α/β. Interestingly, the degree of sialylation of the O-linked oligosaccharides on the CD8β chain impacts on co-receptor function during the development of T cells in the thymus (**19**).
Therefore, the stalk-like region of CD96 may play a role in the orientation/presentation of the Ig-like domains representing a tool for how cells could modulate the capacity of CD96 to interact with binding partners **(11).**

iii-The TM/Cytoplasmic Domain:-

The intracellular domain of h/mCD96 is rather short (45 amino acids) but possesses several interesting motifs of potential importance for CD96 function. By this, there is a high degree of conservation between man and mouse in this domain (80% as compared with 54% for the ectodomain). A split motif consisting of an intra-TM cysteine and charged residues at the TM/cytoplasmic border (CX_8RK) may serve for constitutive association with SRC-like kinases. Similarly, composed motifs are present in other immune-relevant receptors such as CD28, CD2, CD4, CD8α, FcεRIβ, TIGIT, and CD44. Another feature conserved between hCD96 and mCD96 is a proline-rich (P-rich) tandem (RPPPFKPPPPPIK) that is flanked by arginine and lysine residues. A similar but longer P-rich sequence was found in Fas ligand (FasL) **(20)**.
P-rich motifs represent binding sites for the SH3 domain-containing signaling components.
In FasL, binding of SRC-like kinases triggers tyrosine phosphorylation and along with mono-ubiquitination of the flanking lysine **(21)**residues this results of FasL sorting into secretory lysosomes **(22)**.

Expression of CD96:

Among human peripheral blood cells, CD96 expression was observed on T and NK cells but not on the majority of B cells, monocytes, and granulocytes **(14).** In non-hematopoietic tissue, CD96 is expressed in the convoluted tubular epithelium of the kidney, the mucosal epithelium of the small and large intestines, and the vascular endothelium. It was also reported that CD96 is expressed in nearly 30% of human AML samples, regardless of disease subtype **(21)**.

Moreover CD96 was described as a tumor marker for myelodysplastic syndromes, T-ALL, AML and for the AML stem cell in particularCD96 has additionally been proposed as a cancer stem cell marker in leukemia**.** CD96 expression is present on a proportion of hematopoietic stem cells, T cells, NK cells, and a subpopulation of B cells αβ and γδ **(23)**.

CD96 is not expressed on other immune cells, and expression is generally low or absent in organs without lymphocyte infiltrate **(17)**.

CD96 Functions

Initial investigations of CD96 biology suggested a role in mediating NK- cell adhesion to CD155-expressing target cells and was also proposed as a weak NK-cell–activating receptor **(14).** CD96 was also described as an adhesion molecule to CD155 **(15).**

i- Functions of CD96 in NK cells:-

NK cells are innate lymphocytes that play a critical role in the early defense against transformed cells, and they are particularly important in the control of cancer metastasis and hematological malignancies (**24**). NK cells can directly kill tumor cells, secrete various cytokines such as interferon (IFN) and tumor necrosis factor (TNF) to initiate antitumor responses, and recruit other immune cells into the antitumor defense **(25).**

Regulation of Natural Killer (NK) cell activity is achieved by the integration of both activating and inhibitory signals acquired at the immunological synapse with potential target cells. NK cells express paired receptors from the immunoglobulin family (e.g, CD96) which share common ligands with the nectin family (e.g, CD155) of adhesion molecules **(26)**. Alterations in NK cells, for example, excessive expression of inhibitory receptors or reduced expression of activating receptors, can result in impaired cytotoxicity against tumor cells and a decreased ability to recruit other immune cells **(27).** CD96 is expressed by all resting human and mouse NK cells **(16).**

In humans, CD96 promotes adhesion of NK cells to tumor cells expressing CD155, an interaction that may assist in NK-mediated destruction of the targets **(14).**CD96 competes with TIGIT and DNAM1 for CD155 binding **(28)**. Also, mouse CD96 has been shown to bind to CD111 (also known as nectin 1 or PVRL1) **(15).**

In 2019, human CD96 was also proposed to bind CD111, with overexpression of CD111 rendering K562 cells more sensitive to NK cell lysis in vitro. Paradoxically, mouse CD96 is reported by one group to inhibit NK cell function in vivo **(28).**

CD96 has an immunoreceptor tyrosine-based inhibitory motif in its cytoplasmic tail that is predestined to initiate inhibition, CD96 exists as splice variants in humans but not in mice; this affects the characteristics of interactions between cells**.** Little is known about the function of CD96 **(36)**, but it may besynergistically regulated by the CD226–TIGIT–CD96 family members, although these receptors share the same ligands, the inhibitory receptors (TIGIT and CD96) maintain binding priority over stimulatory receptor CD226, which is proposed to be the mechanism for preventing the CD226-mediated chronic activation of NK cells **(12).**

CD96 can also promote NK cell activation, although less efficiently than CD226 and other activating NK cell receptors. Notably, CD96 stimulated freshly activated NK cells, but not NK92, suggesting that the stimulatory function of CD96 may require expression and functional cooperation of other molecules that are absent in NK92 **(12)**

The role of TACTILE in controlling the activity of NK cells is unclear and additional studies are required, as this receptor has motifs of activation and inhibition that could mediate both positive and negative signals in these cells**(29).**

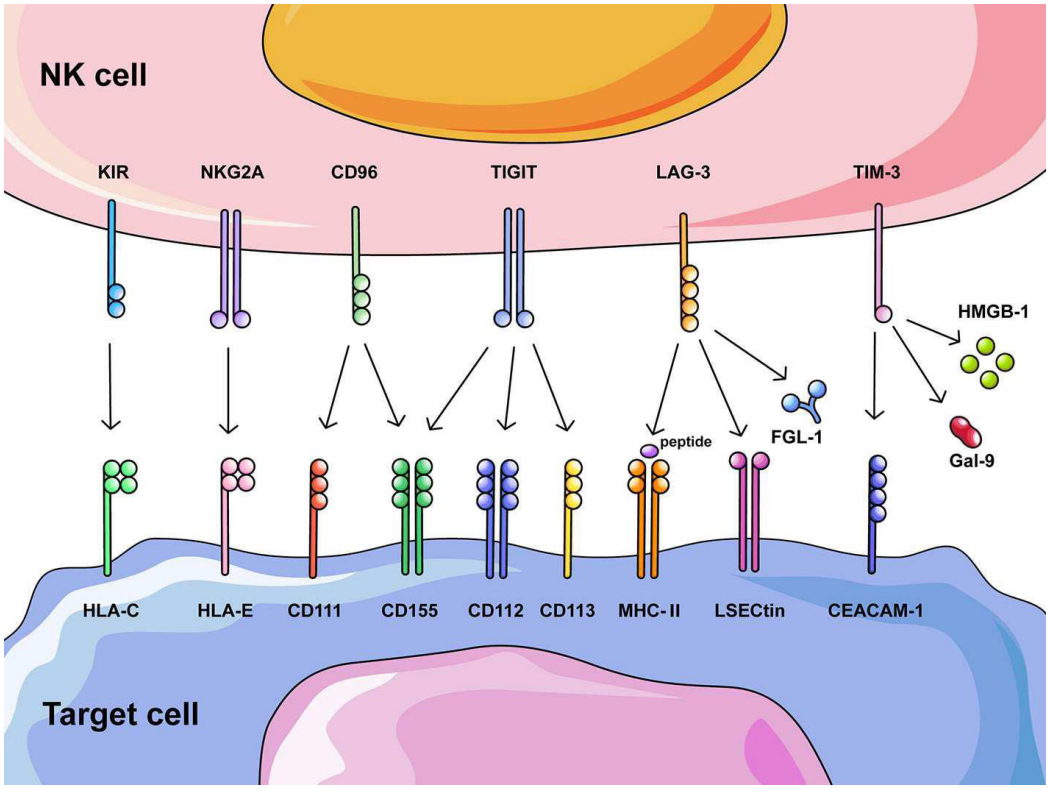

FIGURE 2: Overview of potential NK cell checkpoint molecules and their corresponding ligands. Recognition and clearance of tumor cells by NK cells are regulated through activating and inhibitory receptors on NK cells that bind their corresponding ligands on tumor cells. This figure summarizes inhibitory receptors on NK cells that could also act as checkpoints in cancer immunotherapy, including HLA class I-specific receptors and those recognizing ligands other than HLA class I molecules (CD96, TIGIT, LAG-3, and TIM-3) **(30).**

ii- Function of CD96 in T Cells

T cells, as the main component of cellular immunity, are highlighted for participating in defense against cancer and virally infected cells. After T cell activation, effector cells differentiate,

proliferate, and migrate to sites of inflammation to promote efficient immune responses through direct killing (e.g., CD8+ cytotoxic T cells) or cytokine production (e.g., CD4+ T helper cells) **(31).** Th9 cells are a subset of CD4+ T cells that secrete IL9 and IL10 **(32).**

CD96 is expressed on CD4+ T cells, CD8+ T cells, and NK cells and belongs to a family of molecules interacting with nectins and nectin-like proteins **(33)**. , the level of CD96 expression on CD8+T cells from HIV1-infected patients with high and low viral loads was analyzed (**34**).

Interestingly, a downregulation of CD96 on a fraction of CD8 T cells present in the patients with high viral loads was found. Functional characterization of the $CD96^{+}$ and $CD96^{-}$ CD8 T cells showed that both are potent producers of IFNγ but that the $CD96^{-}$ cells also produced perforin. This raises the possibility that in chronic infection CD96 negatively regulates perforin production in CD8+T cells. Dissimilar effector functions were also observed among CD96and CD96 TH9 cells generated in vitro **(35).**

The CD96 +ve subpopulation was found to be less pathogenic, produced less cytokines, and propagated less efficiently when compared with CD96−TH9 cells. These observations would be in line with the assumption that CD96 inhibits selective T cell effector functions. But again, more information is required to draw more general conclusions **(11).**

iii- Dissecting CD96 Functions in Comparison with CD226 and TIGIT:-

CD96 belongs to a network of interactions that manipulates in a multifaceted fashion adhesion, activation, and inhibition of participating cells. CD226 was reported to activate T and NK cells whereas TIGIT and CD96 act as inhibitors upon interaction with CD155-expressing cells (**16**).

Also, the functional activities triggered by its engagement appear identical to a large extent despite some black boxes. Most importantly, a direct inhibitory role of CD96 was proven only for murine NK cells and explored in vivo mainly in the context of tumor models. Conclusive evidence that this also applies to human NK cells is missing so far **(36)**.

Besides, there is currently a wealth of data documenting that CD226 activates T and NK cells, but concerning TIGIT, most publications demonstrate its role in inhibiting T cells, especially CD8 T and regulatory T cells **(37)**.

Fewer data were presented that documented inhibition of CD4 T or NK cells by TIGIT (**38**).

It remains to be seen whether this illustrates a functional bias of these two inhibitory receptors in that TIGIT predominantly suppresses CD8 T and regulatory T cells whereas CD96 mainly inhibits NK cells. Possibly, this view is misleading and just reflects the current lack of information especially regarding CD96 which was much less thoroughly investigated compared with CD226 or TIGIT **(11).**

Table 2. Biological roles of CD96 in lymphocyte function and outcomes of pathway inhibition relevant to immunotherapy

	CD96
T-cells	• Role of CD96 in T-cell function currently unknown **(12)** • Surface expression of CD96 upregulated on activated human T-cells **(12)** • CD96 mRNA expression increased and associated with a T-cell signature in nonsquamous non-small cell lung cancer cohort **(39)** • Reduced expression on CD8+ T-cells from chronic HIV infected patients compared to healthy controls (**34)**
NK	• Putative adhesion molecule of NK cells (**17**)

cells	• Putative activating receptor for NK cells **(14).** • Blockade with anti-CD96 mAb increases NK production of IFNγ (**23**) • Blockade with anti-CD96 mAb increases control of NK cell dependent tumors and metastases (**23**)

CD96 in Diagnosis and Potential Therapeutic Target in AML

In contrast to the role of CD96 participating in immune surveillance of tumors, CD96 itself was identified as a tumor marker. Indeed, well before first studies deciphered its functions, CD96 was reported to be upregulated in subpopulations of T- ALL and AML. Increased expression of CD96 was shown in several subsequent studies to correlate with poor prognosis and enhanced resistance to chemotherapy **(40).**

Firmly establishing CD96 as a diagnostic marker. Following the hierarchical theory of cancer development, it is assumed that in leukemia the disease-causing incident(s) occur among stem cells generating an LSC that shares self-renewal potency with the stem cells **(41)**.

In line with this, Hosen et al. identified CD96 as a potential target in an LSC-specific therapy to treat AML **(21)**. In approximately two-thirds of the AML cases analyzed, the majority of AML-LSC was found to be $CD96^+$ whereas only a small fraction of approximately 5% was $CD96^+$ among hematopoietic stem cells from healthy donors. A promising treatment strategy would therefore be to sort out CD96-expressing stem cells before autologous transplantation of AML patients **(21).**

A classical approach of a CD96-based therapy would engage mechanisms such as ADCC and complement dependent-cytotoxicity to eliminate AML cells but must take into account that this might affect other CD96-expressing cells as well **(42)**.
The functional role CD96 plays in AML-LSC biology remains elusive, and its expression may turn out irrelevant or of inferior importance for the neoplastic properties of these cells. **(11).**

NK Cell Expressed CD96 as Therapeutic Target in Cancer
Although there are increasing numbers of cases documenting CD96 involvement in controlling tumors and their metastases in mouse models, up to date there is no study translating a concept of a moAb-based neutralization of CD96 into human therapy. However, the design of such treatment strategies is impaired by the lack of conclusive evidence as to whether CD96 inhibits or activates human NK cells. Since investigations in vitro were not helpful in this regard the ex vivo analysis of NK cells obtained from tumor patients could provide at least indirect evidence. This is exemplified by CD226 that is frequently downregulated as part of an immune evasion mechanism in NK cells controlling tumors overexpressing CD155 **(43)**.

Unfortunately, analogous information for CD96 is very limited yet would suggest that in cases of pancreatic cancer CD96 rather activates human NK cells**.** However, more studies are required to corroborate this**(11).**

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