# TIM-3 Is a Novel Therapeutic Target for Eradicating Acute Myelogenous Leukemia Stem Cells

**Koichi Akashi**

**Abstract** Acute myelogenous leukemia (AML) is derived from self-renewing leukemic stem cells (LSCs). We found that T-cell immunoglobulin mucin-3 (TIM-3) is expressed on LSCs in most types of primary AML, except for acute promyelocytic leukemia (M3 by the FAB classification). TIM-3 is not expressed in normal hematopoietic stem cells (HSCs). In a xenogeneic transplantation system, we showed that targeting of TIM-3 by an anti-TIM-3 cytotoxic antibody is sufficient to eradicate human AML LSCs without affecting normal human hematopoiesis. These data strongly suggest that TIM-3 is a promising therapeutic target to cure AML patients.

**Keywords** Acute myelogenous leukemia • Leukemic stem cell • Cancer stem cell • TIM-3 • Hematopoietic stem cell • Xenotransplantation

## Introduction

In normal hematopoiesis, human hematopoietic stem cells (HSCs) reside within the $CD34^+CD38^-$ cell fraction of bone marrow cells. They self-renew and differentiate into mature cells to maintain normal hematopoiesis. Similarly, in acute myelogenous leukemia (AML), leukemic blast cells are derived from a small population called leukemic stem cells (LSCs) or leukemia-initiating cells, which also resides within the $CD34^+CD38^-$ cell fraction [1, 2]. LSCs self-renew and give rise to clonogenic leukemic cells, whereas non-LSCs lack the potential to self-renew or maintain leukemia [1, 3, 4] indicating that AML is hierarchically organized initiating from LSCs.

Conventional chemotherapy currently achieves complete remission in ~90 % of AML cases [5, 6]. However, a considerable proportion of AML patients (~60 %) eventually relapse after intensive chemotherapies. The recurrence of AML in these

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patients may be caused by regrowth of surviving LSCs. To selectively kill AML LSCs while sparing normal HSCs, one of the most practical approaches is to target AML LSC-specific surface molecules or molecules required for LSC function. To achieve such specificity, the target molecule should be expressed on LSCs at a high level but not on normal HSCs [7]. The molecule can be expressed in mature blood cells or progenitors, because these cells can anyway be replenished by normal HSCs.

## Search for Surface Antigens Specific to AML LSCs

A number of candidate surface molecules for eradicating AML LSCs have been reported mainly by utilizing cDNA microarray analysis of purified LSCs. Figure 1 shows the results of transcriptome profiling of purified LSCs from AML patients and normal adult HSCs [8]. The molecules strongly expressed in AML LSCs, including CLL-1 [9], CSF1R [10], CD96 [11], and CD99 [12], are specifically expressed in LSCs. CLL-1 is a transmembrane glycoprotein [13]. The proportion of CLL-1-expressing $CD34^+CD38^-$ AML cells, however, is highly diversified in cases [9]. CD96 is a member of the Ig gene superfamily. CD96 is expressed on activated T cells [14]. The expression level of CD96 protein is also high enough to clearly distinguish AML LSCs from normal HSCs. T-cell immunoglobulin mucin-3 (TIM-3) is expressed in LSCs of most AML types (except for M3) at high levels, but is not expressed in normal HSCs [8]. The expression level of CD25 [15], CD32 [15], CD44 [16], and CD47 [17] in LSCs was only two- to threefold higher at the mRNA level as compared with normal HSCs, and in some AML cases, LSCs did not express these molecules. CD33 and CD123 [18] proteins are expressed at a high level in normal HSCs and myeloid progenitors, including CMPs and GMPs [19], suggesting that targeting these molecules should harm normal hematopoiesis.

It might also be important to understand the function of these therapeutic target molecules in the development of AML. A previous study has shown that anti-CD44 monoclonal antibodies reduced the leukemic burden and blocked secondary engraftment in a NOD-SCID model [16]. This effect on LSCs was mediated in part by the disruption of LSC-niche interactions [16]. Anti-CD47 antibodies can block LSC reconstitution in a NOD-SCID model [17], and this might be due to the activation of phagocytosis by macrophages through inhibition of interaction of CD47 with SIRPA [20].

Recently, we have reported that TIM-3 is expressed on the cell surface of LSCs in most AML types [8, 21]. TIM-3 is not expressed in normal human HSCs [8] (Fig. 1). Furthermore, a recent study has succeeded in prospectively isolating LSCs from residual HSCs within the $CD34^+CD38^-$ fraction in de novo AML patients by using TIM-3 as a positive LSC marker [12]. Here, we summarize recent progress in studies of TIM-3 and discuss the potential usefulness of TIM-3 for eradicating AML LSCs. TIM-3 has several advantages over other candidate markers. First, TIM-3 protein is not detectable in normal HSCs or in other myelo-erythroid or lymphoid progenitors, although TIM-3 is upregulated in monocyte-lineage committed progenitors.

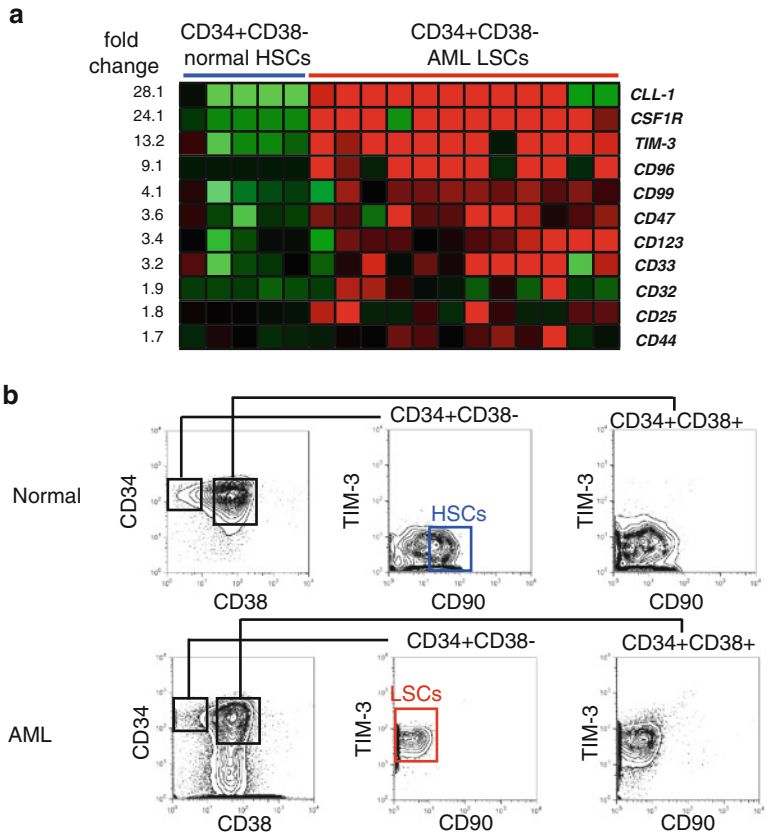


**Fig. 1** TIM-3 expression in normal HSCs and AML LSCs. (**a**) Results of gene expression analysis comparing $CD34^+CD38^-$ normal HSCs and AML LSCs. Surface molecules highly expressed in LSCs are shown. (**b**) FACS analysis of TIM-3 protein expression in normal HSCs and AML LSCs. Both $CD34^+CD38^-CD90^-$ LSCs and $CD34^+CD38^+$AML cells express TIM-3, whereas $CD34^+CD38^-CD90^-$ HSCs completely lack TIM-3 expression. TIM-3 expression originates within the $CD34^+CD38^+$ progenitor fraction in normal human hematopoiesis. A representative FACS analysis is shown here

Second, TIM-3 marks all functional LSCs that can reconstitute human AML in immunodeficient mice in the majority of M0, M1, M2, and M4 AML cases, and its expression level is sufficient to eradicate LSCs by antibody-based treatment.

## TIM-3 Expression and Functions in Normal Hematopoiesis

TIM-3 was originally identified as a surface molecule expressed in interferon (IFN)- γ-producing $CD4^+$ Th1 cells and $CD8^+$ T cytotoxic type 1 (Tc1) cells [22] in murine hematopoiesis. TIM-3, a type 1 cell-surface glycoprotein, has a structure

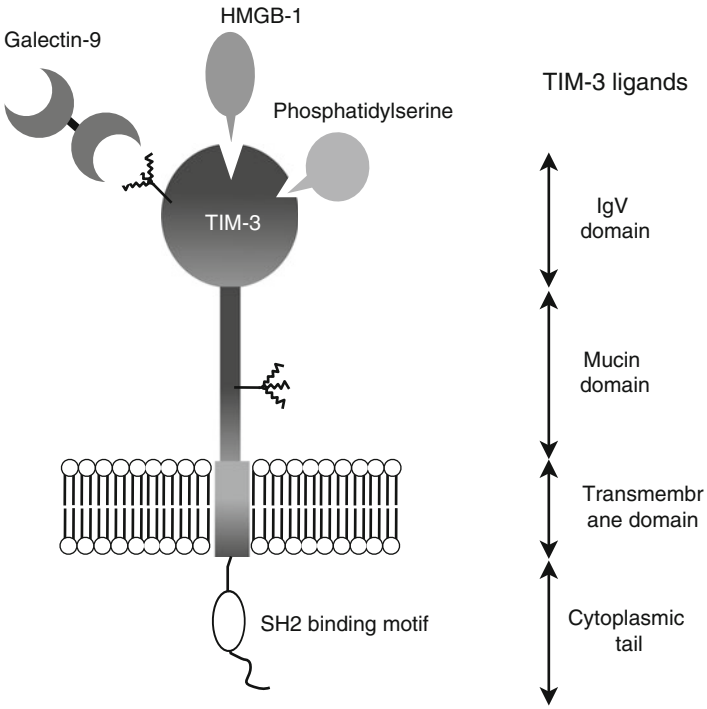


**Fig. 2** Structure of TIM-3 molecule and its ligands. TIM-3 is a type 1 cell-surface glycoprotein and has a structure that includes an N-terminal immunoglobulin variable domain followed by a mucin domain, a transmembrane domain, and a cytoplasmic tail with highly conserved six tyrosine residues and a SH2 binding motif. Galectin-9, HMGB1, and PS have been identified as ligands of TIM-3

that includes an N-terminal immunoglobulin variable domain followed by a mucin domain, a transmembrane domain, and a cytoplasmic tail (Fig. 2). In steady-state human hematopoiesis, TIM-3 is expressed in monocytes and in a fraction of NK cells, but not in granulocytes, B cells, or T cells [8]. However, TIM-3 is upregulated in T cells in response to immune reactions. TIM-3 plays an important role in regulation of Th1-dependent immune responses and immune tolerance [22–24]. Galectin-9, an S-type lectin, has been reported as a TIM-3 ligand in lymphocytes. Galectin-9 has two distinct carbohydrate recognition domains and binds to carbohydrate chains on the IgV domain of TIM-3. TIM-3 has highly conserved six tyrosine residues and a Src homology 2 (SH2) binding motif in its cytoplasmic tail, and stimulation of TIM-3 by galectin-9 results in increased phosphorylation of tyrosine residues in T cells [25]. Engagement of TIM-3 by galectin-9 induces apoptosis of Th1 cells and inhibits their IFN-γ production [26]. These data collectively suggest that TIM-3 is a negative regulator of Th1- and Tc1-driven immune responses.

TIM-3 is also known as a marker of "exhausted" $CD8^+$ T cells. Exhausted T cells show impaired proliferation and effector function under antigen stimulation.

One of the major markers for exhausted T cells is the inhibitory molecule programmed cell death 1 (PD-1), and T cell function is partially restored by blocking the interaction between PD-1 and PD-1 ligand in mice [27]. TIM-3 is also expressed on exhausted $CD8^+$ T cells in patients with chronic viral infections, including human immunodeficiency virus (HIV) [28], hepatitis B virus [29], and hepatitis C virus (HCV) [30]. Blockade of both TIM-3 and PD-1 ligation can significantly restore T cell proliferation and effector potential, suggesting that both TIM-3 and PD-1 pathways play a major role in $CD8^+$ T cell exhaustion [31].

TIM-3 can also modulate the immune reaction pathway to regulate innate immunity. NK cells and some myeloid cells, including monocytes/macrophages, dendritic cells, and mast cells, express TIM-3 in both human and mouse hematopoiesis. In NK cells, TIM-3 is induced on their surface on activation [32, 33], but the function of TIM-3 in NK cells remains controversial. It has been reported that TIM-3 is a human NK cell co-receptor to enhance IFN-γ production [32], but another report showed that NK cell-mediated cytotoxicity was reduced by cross-linking of TIM-3 [33].

In terms of the myeloid lineage, TIM-3 is expressed in monocytes/macrophages, dendritic cells (DCs), and mast cells [34–37]. In human bone marrow, $CD34^+CD38^-CD90^+$ normal HSCs and the majority of $CD34^+CD38^+$ progenitor cells do not express TIM-3. Within the $CD34^+CD38^+$ progenitor fraction, human myeloid progenitors can be divided into three subpopulations, such as common myeloid progenitors (CMPs), granulocyte/macrophage progenitors (GMPs), and megakaryocyte/erythrocyte progenitors (MEPs) [38]. TIM-3 is expressed only in a fraction GMPs, but not in CMPs and MEPs. Purified $TIM\text{-}3^+$ GMPs give rise mainly to colony-forming unit-macrophage (CFU-M), whereas $TIM\text{-}3^-$ GMPs can generate various types of myeloid colonies, suggesting that upregulation of TIM-3 occurs in concert with monocyte lineage commitment at the GMP stage in humans [8].

In mature monocytes or dendritic cells, TIM-3 signaling synergizes with that of Toll-like receptors to promote secretion of tumor necrosis factor-α (TNF-α) inflammatory responses [34]. In addition, TIM-3 on macrophages and DCs recognizes phosphatidylserine (PS) in apoptotic cells through its IgV domain. Binding of PS to TIM-3 does not interfere with that of galectin-9 to TIM-3, as the binding sites of these molecules are located on opposite sides of the IgV domain. In TIM-3-expressing DCs, recognition of PS by TIM-3 induced enhancement of phagocytosis of apoptotic cells and cross-presentation of apoptotic cell-associated antigen to $CD8^+$ T cells [35]. TIM-3 expression and functions in hematopoietic cells are summarized in Fig. 3.

## TIM-3 Is a Marker of Malignant Stem Cells in Human AML

We have identified TIM-3 as an AML LSC-specific surface molecule. We first compared the gene expression profiles of $CD34^+CD38^-$ AML cells and normal HSCs by using cDNA microarray analysis (Fig. 1a). As shown in Fig. 1b, TIM-3 protein is not expressed in $CD34^+CD38^-CD90^+$ normal HSCs, but the vast majority

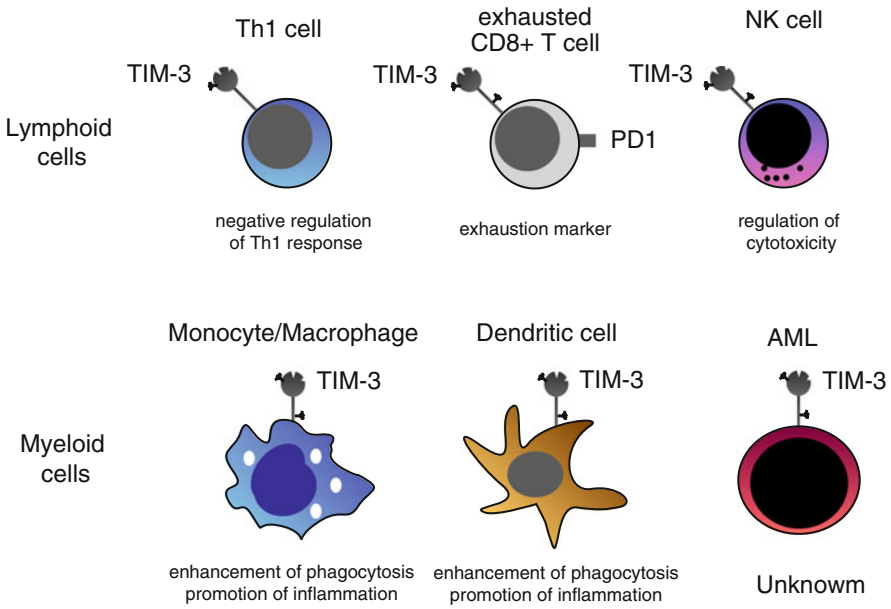


**Fig. 3** TIM-3 expression and functions in normal hematopoietic cells. TIM-3 is expressed in Th1 cells, exhausted $CD8^+$ T cells, NK cells, monocytes, and dendritic cells in normal hematopoiesis. The functions of TIM-3 differ by cell type and context

of the $CD34^+CD38^-$ LSCs and the $CD34^+CD38^+$ cells expressed TIM-3 at a high level in patients with most types of AML except for acute promyelocytic leukemia (M3) [8, 21]. Another group has also reported that the expression level of TIM-3 is especially high in immature AML cells with core-binding factor translocations or mutations in *CEBPA* [21].

It is important to note that the TIM-$3^+$ fraction in AML patients contained all functional LSCs. We separated AML cells into the TIM-$3^+$ and TIM-$3^-$ populations and transplanted each population into sublethally irradiated immunodeficient mice, and found that only TIM-$3^+$ AML cells, but not TIM-$3^-$ cells, reconstituted human AML in these mice [8]. These data suggest that targeting TIM-$3^+$ cells is sufficient for eradication of LSCs in AML patients.

## Targeting AML-LSCs by Monoclonal Anti-TIM-3 Killing Antibodies in a Xenograft Model

To utilize TIM-3 to target AML LSCs, it is critical to establish anti-human TIM-3 antibodies that can kill TIM-3-expressing cells in vivo. To achieve successful antibody-based treatment, antibody-dependent cellular cytotoxicity (ADCC) and

complement-dependent cytotoxicity (CDC) activities are critical to eliminate target cells [39]. Additionally, recent studies have suggested that antibody-dependent cellular phagocytosis (ADCP) could play an important role in killing target cells in vitro [40] and in vivo [41].

An anti-TIM-3 monoclonal antibody (IgG2b) was obtained by immunizing Balb/c mice with L929 cells stably expressing human TIM-3 and soluble TIM-3 protein [8]. In this antibody, the variable portions of the VH regions of the cloned hybridoma that recognize TIM-3 were grafted onto IgG2a Fc regions, because the IgG2a subclass is most efficient to induce ADCC activity in mice [42, 43]. The clone called ATIK2a was established, and it was effective in killing TIM-3-expressing cell lines by its CDC and ADCC activities [8].

We then tested the effect of ATIK2a on the growth of AML LSCs or normal HSCs in xenograft models. NOD-SCID mice transplanted with $10^5$ $CD34^+$ cord blood cells were treated with ATIK2a. These mice were reconstituted with normal hematopoiesis with nearly equal percentages of human cell chimerisms, although human mature monocytes were depleted. In contrast, in mice reconstituted with human AML, ATIK2a exerted profound effects on leukemia development. The mice were transplanted with human AML of M0, M1, and M4 types, and after confirmation of AML development in these mice, ATIK2a was injected six times over 2 weeks. Strikingly, human AML cells disappeared in mice treated with ATIK2a but not in those with control IgG treatment. These data strongly suggest that targeting of AML LSCs by utilizing anti-TIM-3 killing antibodies is a practical approach to cure human AML.

## TIM-3 Is a Functional Molecule for AML LSC Maintenance

Since TIM-3 has a tyrosine residue and SH2 domain that can activate Src family proteins, we hypothesized that TIM-3 signaling has some function to maintain AML-LSCs. We found that the serum levels of galectin-9, a TIM-3 ligand, were significantly (>10-fold) elevated in AML patients but not in normal individuals on an ELISA assay. Furthermore, $TIM\text{-}3^+$ AML cells had abundant galectin-9 protein in their cytoplasm, and they secreted galectin-9 in the sera of mice transplanted with human AML. Mice reconstituted with normal human HSCs or B cell acute lymphoblastic leukemia did not have detectable levels of serum galectin-9. These results collectively suggest that AML cells secreted galectin-9 in an autocrine manner. Furthermore, TIM-3 stimulation by galectin-9 in AML cells in vitro induced significant gene expression changes including NF-κB target genes (unpublished data). Collectively, it is suggested that AML LSCs had growth and survival advantages through an autocrine stimulation loop of the TIM-3/galectin-9 system.

## Conclusion

TIM-3 has been shown to play pivotal roles in modulating immune reactions. By transcriptome analysis, we newly identified TIM-3 as a surface molecule specific to AML LSCs but not expressed in normal HSCs. Our in vivo xenogeneic transplantation analysis directly showed that targeting TIM-3 could be an efficient, useful therapeutic approach to eradicate AML LSCs.



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