TIM-3 as a novel therapeutic target for eradicating acute myelogenous leukemia stem cells

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Abstract Acute myelogenous leukemia (AML) originates from self-renewing leukemic stem cells (LSCs), which represent the ultimate therapeutic target for AML. Recent studies have identified several AML LSC-specific surface antigens as candidate targets of therapeutic molecules. T cell immunoglobulin mucin-3 (TIM-3) is expressed on LSCs in most types of AML, with the exception of acute promyelocytic leukemia, but not on normal hematopoietic stem cells (HSCs). In xenograft models reconstituted with human AML LSCs or HSCs, an anti-human TIM-3 mouse IgG2a antibody with cytotoxic activities eradicates AML LSCs in vivo, but does not affect normal human hematopoiesis. Thus, TIM-3 is a promising therapeutic target for the eradication of AML LSCs.

Keywords Acute myelogeneous leukemia · Leukemic stem cell · TIM-3

Introduction

Human hematopoietic stem cells (HSCs) reside within the $CD34^+CD38^-$ cell fraction of bone marrow. HSCs have the potential to self-renew and differentiate into multilineage mature hematopoietic cells and thereby maintain normal hematopoiesis. By virtue of their organization into such a hierarchical system, HSCs are able to regulate dynamic homeostasis of hematopoiesis. Similarly, in acute myelogenous leukemia (AML), a small number of leukemic cells, called leukemic stem cells (LSCs) or leukemia-initiating cells, are found within the same $CD34^+CD38^-$ cell fraction. LSCs self-renew and give rise to clonogenic leukemic cells, whereas non-LSCs lack the potential to self-renew or maintain leukemia, thus AML is also organized as a hierarchy, which originates from LSCs [1–3]. The concept of LSCs has been proposed based on the finding in the 1980s that only a limited fraction of AML cells can give rise to blast colonies in vitro. In 1994, Dick et al. showed that the $CD34^+CD38^-$ AML leukemic cells, but not other AML cells, including $CD34^+CD38^+$ and $CD34^-$ leukemic cells, can reconstitute human AML in immunodeficient mice, providing direct evidence for the existence of LSCs [1]. Although recent studies have suggested that LSCs in some types of AML may be present within the $CD34^+CD38^+$ fraction [4] or the $CD34^-$ leukemic cell fraction as well [5, 6], the $CD34^+CD38^-$ population concentrates the LSCs in a vast majority of cases [7]. The $CD34^+CD38^-$ phenotype is identical to that of very primitive human HSCs with long-term reconstitution activity [8, 9] and multipotent progenitor cells, suggesting the possibility that LSCs originate from these very primitive HSCs or progenitor cells [10].

In the clinic, conventional chemotherapy currently achieves complete remission in ~ 90 % of AML cases [11, 12]. However, a considerable fraction (~ 60 %) of AML patients eventually relapse after intensive chemotherapies. The recurrence of AML in these patients may be caused by re-growth of surviving LSCs. Thus, LSCs should be the ultimate therapeutic target if we are to achieve a cure for AML. 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

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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 [13]. It does not matter whether the molecule is expressed in normal blood cells or normal progenitor cells, because if physiologic HSCs are spared, they are able to replenish all mature blood cells after the conclusion of treatment.

Recently, two groups have identified T cell immunoglobulin mucin-3 (TIM-3) as a surface molecule expressed in LSCs of most AML types [14, 15]. TIM-3 is a promising therapeutic molecule for the targeting of LSCs while sparing normal residual HSCs [14]. Furthermore, a recent study has succeeded in the prospective separation of LSCs from residual HSCs in de novo AML patients by TIM-3 expression, allowing us to clarify the clonal relationship between LSCs and HSCs in the same patient [16]. In this review, we summarize recent progress in studies of TIM-3 and discuss the potential utility of TIM-3 for eradicating AML LSCs while leaving normal HSCs unaffected.

TIM-3 in normal hematopoiesis

TIM-3 was first identified as a surface molecule expressed in interferon (IFN)-γ-producing CD4$^+$ Th1 cells and in CD8$^+$ T cytotoxic type 1 (Tc1) cells [17] in murine hematopoiesis. TIM-3, a type 1 cell-surface glycoprotein, has a structure that includes an N-terminal immunoglobulin variable domain followed by a mucin domain, a transmembrane domain and a cytoplasmic tail. Figure 1 shows the structure of TIM-3 and its ligands. TIM-3 plays an
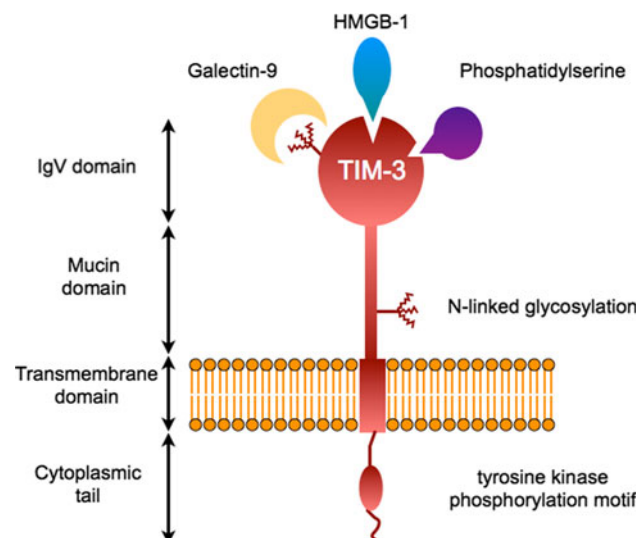
important role in regulating Th1-dependent immune responses and in inducing immune tolerance [17–19]. In lymphocytes, galectin-9, an S-type lectin, has been reported as a TIM-3 ligand. Galectin-9 has two distinct carbohydrate recognition domains and can bind to carbohydrate chains on the TIM-3 IgV domain. TIM-3 has highly conserved six tyrosine residues and an 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 [20]. Engagement of TIM-3 by galectin-9 induces apoptosis of Th1 cells and inhibits their IFN-γ production [21]. Thus, TIM-3 is considered to be a negative regulator of Th1- and Tc1-driven immune responses.

Another aspect of TIM-3 expression in the T cell lineage is as a marker of "exhausted" CD8$^+$ T cells. Exhausted T cells show impaired proliferation potential and effector function in response to antigen stimulation. Such exhausted T cells express the inhibitory molecule programmed cell death 1 (PD-1) on their surface, and T cell function can be partially restored by blocking the interaction between PD-1 and PD-1 ligand in mice [22]. In addition to PD-1 expression, recent studies have shown that TIM-3 expression also marks exhausted CD8$^+$ T cells in patients with chronic viral infections, including human immunodeficiency virus (HIV) [23], hepatitis B virus [24] and hepatitis C virus (HCV) [25]. Blockade of both TIM-3 and PD-1 pathways can 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 [26].

TIM-3 also plays a major role in innate immunity, by modulating immune reaction pathways. Among innate immune cells, NK cells and some kinds of myeloid cells, including monocytes/macrophages, dendritic cells and mast cells, express TIM-3 in both humans and mouse. In NK cells, TIM-3 is shown to be an inducible surface receptor on activation [27, 28], but its function remains controversial. Gleason et al. [27] reported TIM-3 as a human NK cell co-receptor to enhance IFN-γ production, whereas Ndhlovu et.al reported the inhibition of NK cell-mediated cytotoxicity by TIM-3 cross-linking [28]. Further studies are necessary to clarify the precise function of TIM-3 in NK cells. In the myeloid lineage, TIM-3 is expressed in monocytes/macrophages, dendritic cells (DCs) and mast cells [29–32]. TIM-3 expressed on monocytes or DCs synergizes with Toll-like receptors to promote tumor necrosis factor-α (TNF-α) secretion and enhances inflammatory responses [29]. 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,

Fig. 1 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 an SH2 binding motif. Galectin-9, HMGB1 and PS have been identified as ligands of TIM-3

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 [30]. In addition to the previously identified TIM-3 ligands, galectin-9 and PS, a recent study revealed that high mobility group box 1 (HMGB1) serves as a ligand for TIM-3 in tumor-associated DCs (TADCs). TIM-3 is highly expressed in TADCs and suppresses nucleic acid-mediated innate immune response independently of galectin-9 or PS. HMGB1 has the potential to trigger the transportation of nucleic acids into endosomes in DCs. TIM-3 inhibits the recruitment of nucleic acids to endosomes by trapping HMGB1, and attenuates the innate immune response against tumor [33]. As described above, TIM-3 is expressed in various types of hematopoietic cells and seems to have lineage- or cellular context-dependent signal transduction pathways or functions [29]. Further studies will help us to understand the detailed function of TIM-3.

In steady-state human hematopoiesis, TIM-3 is expressed mainly in monocytes and a fraction of NK cells, but not in granulocytes, B cells or the vast majority of T cells [14]. In human bone marrow, $CD34^+CD38^-CD90^+Lin^-$ normal HSCs and the vast majority of the $CD34^+CD38^+$ progenitor cells lack TIM-3 expression. Within the $CD34^+CD38^+$ progenitor fraction, human myeloid progenitors can be divided into three progenitors, including common myeloid progenitors (CMPs), granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs), by the expression of CD123 and CD45RA [34]. Of these myeloid progenitors, TIM-3 is expressed only in a fraction GMPs, but not in CMPs and MEPs. The vast majority of purified $TIM-3^+$ GMPs mainly give rise to colony forming unit-macrophage (CFU-M), whereas $TIM-3^-$ GMPs give rise to various types of myeloid colonies, including colony forming unit-granulocyte/macrophage (CFU-GM), colony forming unit-granulocyte (CFU-G) and CFU-M, suggesting that upregulation of TIM-3 occurs in concert with monocyte lineage commitment at the GMP stage in human [14].

TIM-3 in AML

Many studies of TIM-3 have focused on its role as an immune modulating molecule and intensively investigated in the field of immunology; however, the studies on its role or expression in hematological malignancies have been limited so far. We and another group identified TIM-3 as an AML LSC-specific surface molecule based on the transcriptome analysis between gene expression profiles of $CD34^+CD38^-$ AML cells and normal HSCs (Fig. 2a). As shown in Fig. 2b, TIM-3 protein is not expressed in $CD34^+CD38^-CD90^+Lin^-$ normal HSCs, but the vast majority of the $CD34^+CD38^-$ LSCs as well as the

$CD34^+CD38^+$ cells in most types of AML except for acute promyelocytic leukemia express TIM-3 protein at a high level [14, 15]. Notably, it has been reported that the expression level of TIM-3 is high in AML with core-binding factor translocations or mutations in *CEBPA* [15].

Strikingly, the $TIM-3^+$ population in the bone marrow contains all AML LSCs, and residual HSCs are always included in patients' $TIM-3^-$ population. To test the significance of this, $TIM-3^+$ and $TIM-3^-$ AML populations were transplanted into sublethally irradiated immunodeficient mice, and it was found that only $TIM-3^+$ AML cells reconstituted human AML in mice [14]. Jan et al. [15] reported that normal human hematopoiesis is always reconstituted in the mice transplanted with $CD34^+CD38^-TIM-3^-$ cells from AML patients, suggesting that TIM-3 may be a useful marker to separate residual HSCs from AML LSCs. Jan et al. also compared the gene mutation status of the prospectively isolated residual $CD34^+CD38^-CD99^-$ $TIM-3^-$HSCs and $TIM-3^+$AML cells in the same AML patients by exome sequencing, and found that the presence of pre-leukemic HSCs harboring identical gene mutations to those of paired AML cells, revealing the multi-step leukemogenesis from self-renewing HSCs [16]. These studies reveal that TIM-3 expression is very specific to AML and is useful in clearly distinguishing HSCs from LSCs.

Targeting AML LSCs by anti-TIM-3 killing antibodies

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. In terms of the antibody-based treatment, the antibody-dependent cellular cytotoxicity (ADCC), as well as the complement-dependent cytotoxicity (CDC) activities is critical to eliminate target cells [35]. Additionally, recent studies have suggested that antibody-dependent cellular phagocytosis (ADCP) could play an important role in killing target cells in vitro [36] and vivo [37].

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. 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 IgG2a subclass is most efficient to induce ADCC activity in mice [38, 39]. The established clone, ATIK2a, was effective in killing TIM-3-expressing cell lines via both CDC and ADCC effects [14].

The effect of ATIK2a on normal and malignant AML hematopoiesis was tested in xenograft models. NOD-SCID mice transplanted with 10^5 $CD34^+$ cord blood cells with or without ATIK2a treatment developed nearly equal percentages of human hematopoietic cells. In mice injected

Fig. 2 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. Representative FACS analysis results of TIM-3 expression are shown here
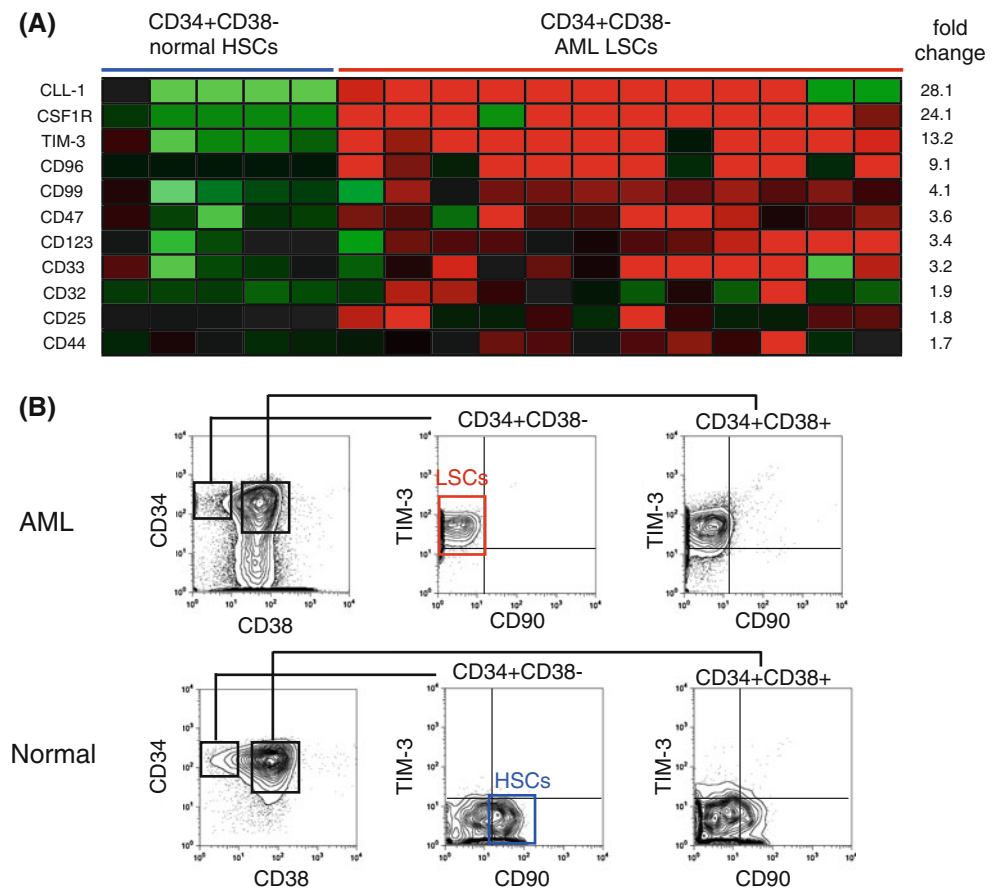
with ATIK2a, however, human CD33$^+$TIM-3$^+$ mature monocytes were depleted, suggesting that targeting TIM-3 does not affect development of normal hematopoiesis, killing only the normal TIM-3$^+$ cells.

In contrast, against AML cells, ATIK2a exerted profound effects on leukemia development. In mice transplanted with human AML of M0, M1 and M4 types, ATIK2a treatment significantly reduced the human CD45$^+$ AML burden as well as the CD34$^+$CD38$^-$ LSC cell numbers in vivo. Re-transplantation of the remaining AML cells in primary recipients treated with ATIK2a into the secondary recipients failed to reconstitute human AML, indicating that ATIK2a treatment successfully eradicated functional LSCs in primary recipients. These data suggest that eliminating AML LSCs by using anti-TIM-3 killing antibodies is a practical approach to cure human AML.

Perspective

To target surface antigens on AML LSCs, it is critical to specifically kill LSCs sparing normal HSCs with high sensitivity. 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. 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. The expression level of other candidate molecules, including CD25 [40], CD32 [40], CD44 [41] and CD47 [42] in LSCs, was only two- to threefold higher at the mRNA level as compared to normal HSCs in our own transcriptome analysis, and in some AML cases, LSCs did not express these molecules. CD33 and CD123 [43] proteins are expressed at a considerably high level in normal HSCs and myeloid progenitors including CMPs and GMPs [44], suggesting that targeting these molecules should harm normal hematopoiesis. In fact, prolonged cytopenia has been observed in AML patients treated with gemtuzumab [45], a recombinant humanized anti-CD33 monoclonal antibody conjugated with the cytotoxic antibiotic calicheamicin. In contrast, CLL-1 [46], CSF1R [47], CD96 [48] and CD99 [16] are all specifically expressed in LSCs. CLL-1 is a transmembrane glycoprotein [49]. The proportion of CLL-1-expressing CD34$^+$CD38$^-$ AML cells is highly diversified in cases [46]. CD96 is a member of the Ig gene superfamily. CD96 is expressed on activated T cells [50]. Of note, like in the case of TIM-3, CD96$^+$ but not CD96$^-$

AML cells efficiently reconstituted AML in the immuno-deficient mice [48], suggesting that CD96 can mark all functional AML LSCs. The expression level of CD96 protein is also high enough to clearly distinguish AML LSCs from normal HSCs. The sensitivity of TIM-3 is likely to be the highest among these molecules, at least for AML M0, M1, M2 and M4.

It is important to understand the function of these therapeutic target molecules on the development of AML. For example, it has been shown that anti-CD44 monoclonal antibodies reduce the leukemic burden and block secondary engraftment in an NOD-SCID model [41]. This effect on LSCs was mediated in part by the disruption of LSC-niche interactions [41]. Anti-CD47 antibodies can block LSC reconstitution in an NOD-SCID model [42], and this might be due to the activation of phagocytosis by macrophages through inhibition of interaction of CD47 with SIRPA [51]. Since the pathway for eradication of LSCs by anti-CD44 or anti-CD47 treatment is different from that by anti-TIM3 antibodies, the combination of these antibodies might be critical for future treatments targeting AML LSCs. Unlike the cases in CD44 and CD47, the function of TIM-3 molecule in AML is still largely unknown. TIM-3 is expressed in the various types of hematopoietic cells and seems to have lineage- or cellular context-dependent signal transduction pathways or functions. TIM-3 expression and functions in hematopoietic cells are summarized in Fig. 3.

Recent studies reported the ectopic expression of TIM-3 in lineages other than hematopoietic cells, particularly in primary cancer cells, including melanoma [52], non-small cell lung cancer [53], cervical cancer [54] and liposarcoma [55]. Importantly, patients with TIM-3 positive cancer cells had a significantly shorter survival time than those with TIM-3 negative cancer cells in non-small cell lung cancer [53] and cervical cancer [54]. TIM-3 expression in these various types of malignant cells raises the possibility of tumorigenic function of TIM-3. Further studies will help us to find out the TIM-3 function in malignant cells.

Conclusion

TIM-3 plays pivotal and diverse roles in modulating immune reaction. We identified TIM-3 as a specific surface molecule expressed in AML LSCs through transcriptome analysis of normal HSCs and AML LSCs. TIM-3 expression can distinguish AML LSCs from normal HSCs, indicating that targeting TIM-3 may be a useful therapeutic approach. We have developed antibody-based therapeutic xenograft model of primary AML cells, and shown that targeting TIM-3 by cytotoxic monoclonal antibodies kills functional LSCs in vivo. Thus, TIM-3 is a promising therapeutic target for the eradication of AML LSCs.

Conflict of interest None.

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

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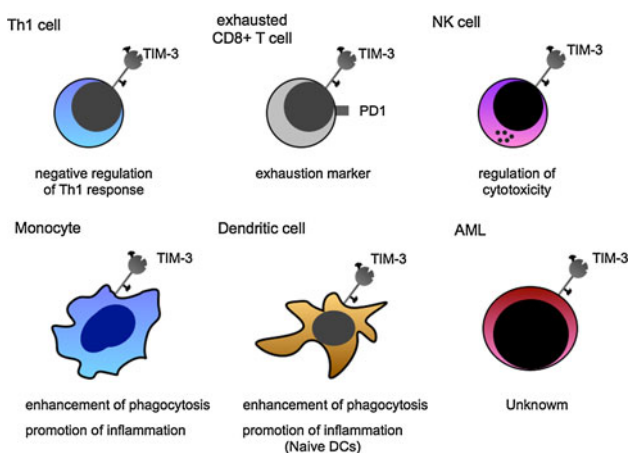
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