

CXCL12-Abundant Reticular (CAR) Cells: A Review of the Literature with Relevance to Cancer Stem Cell Survival

Hussam S Eltoukhy, Garima Sinha, Caitlyn Moore, Khadidiatou Guiro, and Pranela Rameshwar*

From the Department of Medicine, Division of Hematology-Oncology, Rutgers, New Jersey Medical School, Newark, NJ.

Abstract: The bone marrow niche is an intricate microenvironment with multiple cell types, gradient of cytokines and oxygen concentration. The niche has a substantial and intricate role in both health and disease, including the accommodation of cancer cells in the dormant phase. Since the cancer stem cells can survive as dormant cells in the bone marrow, an understanding of the niche would be important for hematological as well as an understanding of cancer stem cell in a physiological situation. One of the cell types in the bone marrow is CXCL12-abundant reticular cells, commonly referred to as CAR cells. Discovered relatively recently, some progress has been made in understanding their origin and function. The function and location of the CAR cells indicate that these cells may be important therapeutic target for both hematological and oncological diseases. Since the CAR cells have been shown to promote and maintain hematopoietic stem cells, further research may demonstrate the same effect with cancer stem cells. We review here the current literature regarding CAR cells and their potential for future research as well as the roles in bone marrow diseases, such as their role in sustaining the cancer stem cells within bone marrow.

Keywords: Cancer stem cells, CAR cells, VSEL, CXCL12, Hematopoietic stem cells, CXCR4.

INTRODUCTION

The bone marrow is a complex organ, comprising of several cell types such as mesenchymal stem cells (MSCs), osteoprogenitor cells, chondrocytes, adipocytes, neuronal cells, glial cells, and a primitive type cell with primordial marker, referred to as VSEL or very small embryonic-like cells [1, 2]. In addition, the bone marrow is innervated and the neurotransmitters linked to the nerve fibers can modulate hematopoiesis [3–5]. Although hematopoietic functions have been well studied, there are additional cells being identified, including very small embryonic cells (VSEL) and CXCL12-abundant reticular cells, also known as CAR cells [2, 6–9]. The CAR cells are located adjacent to sinusoids in the bone marrow (Figure 1). The relatively recent discovery of CAR cells leaves us with many unanswered questions and many open doors to understand the functions not only in hematopoiesis but also in hematological malignancies.

CAR cells were first identified in a study exploring cellular niches and developmental processes within the bone marrow [10]. CXCL12 was studied due to its involvement in several bone marrow functions including

hematopoietic regulation [11]. CXCL12 expression in CAR cells was identified in experimental studies with mice in which the green fluorescence protein (GFP) gene was knocked into the CXCL12 locus [10]. The investigators noted cells with high CXCL12 expression and morphologically showed cellular processes [10]. Originally denoted as CXCL12-expressing cells, they were found to be located distant to the surface of the bone and to osteoblasts (Figure 1). They were distinguished from endothelial cells and osteoblasts as they did not express their markers.

Secondary to their relative recent discovery, the data regarding CAR cells is limited. We are interested in elucidating their role, function, and interaction in the bone marrow niche because their increased expression of CXCR12 leads us to believe that they may be a substantial therapeutic target due to CXCL12's role in tumor growth and metastasis [12]. We discuss also CAR cells' proposed interaction with cancer stem cells (CSCs) and methods that can be used to isolate and study them.

CAR IN HEMATOPOIETIC STEM CELL (HSC) FUNCTION

The general hypothesis is that CAR cells are involved in B-cell development. This was deduced from the analyses of the various stages of B-cells and their location with respect to the CAR cells. It is thought that multipotent cells come in contact with the processes located on the CAR cells,

*Correspondence: Pranela Rameshwar, Ph.D., Department of Medicine – Division of Hematology/Oncology, New Jersey Medical School, Rutgers School of Biomedical Health Science, Newark, NJ 07103 USA; E-mail: rameshwa@njms.rutgers.edu
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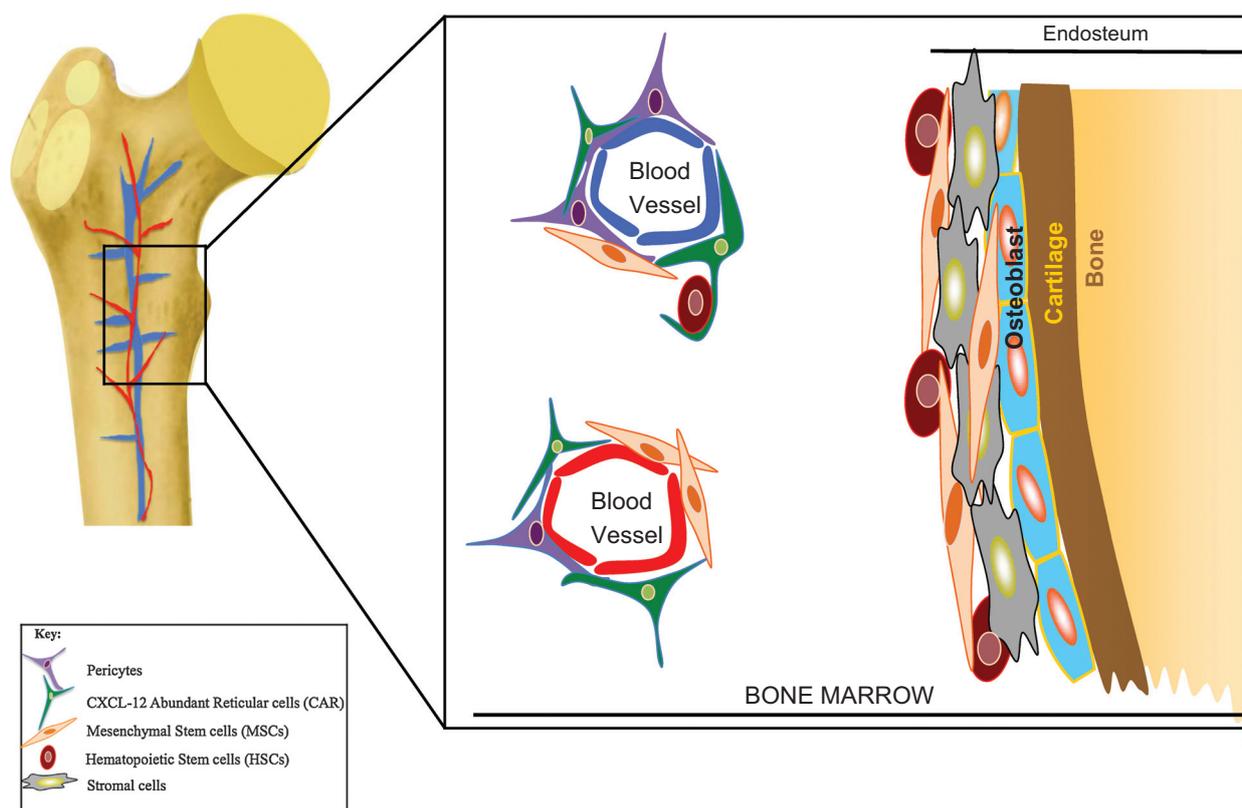


Figure 1. Schematic representation the anatomy of the bone marrow niche with CAR cells located adjacent to the sinusoids and appearing as reticular cells.

develop into pre-pro-B cells and then migrate to other cells within the bone marrow in order to complete the developmental processes [10]. Later studies using immunohistochemistry identified CAR cells as the major producer of CXCL12 in the bone marrow and showed their localization as mainly near the endosteum and adjacent to the sinusoids, usually co-localized with Hematopoietic Stem Cells (HSCs) in the bone marrow [8]. To further elucidate the relationship between HSCs and CAR cells, a later study was performed in which CAR cells were conditionally ablated with transgenic mice expressing the diphtheria toxin receptor-green fluorescent protein fusion protein knocked into the CXCL12 locus [13]. The results further demonstrated the importance of CAR cells in the maintenance of both HSC and B-cells by showing a decrease in quantity and an increase in quiescent activity of the HSCs. Their HSC maintenance function was again supported when it was shown that by deletion of *Foxc1*, a transcription factor found to be highly expressed in CAR cells, the quantity of HSCs was significantly reduced in the marrow [14].

DISTINCT CELL LINE VERSUS MSC LINEAGE

The origin and destiny of CAR cells have yet to be clearly elucidated. Flow cytometry of cells with high GFP expression in mice with GFP knocked into the CXCL12 locus showed expression of VCAM-1, CD44, CD51, PDGFR α , and PDGFR β [13]. Transcript analyses showed increases

in lineage-specific genes, *C/EBP α* , *PPAR γ* , *RUNX2*, and *Osterix* in CAR cells relative to other cell populations [13]. These findings suggested a possible adipogenic or osteogenic potential of CAR cells. If so, this would argue against these cells being MSCs or multipotent cells.

It has been suggested that CAR cells may be a sub-population of MSCs [15]. The literature reported on nestin-positive cells that were assigned as MSCs, based on the ability to differentiate into mesenchymal lineages [16]. However, these nestin positive cells were found to have many characteristics that resemble what are now known as CAR cells. The similarities included their perivascular location usually in the center area of the marrow, their morphologic similarity to vascular pericytes that are found close to the region of the HSCs, and the expression of a high level of CXCL12 [15, 16]. Although the findings do not suggest that the nestin-expressing MSCs and CAR cells are the same cell type, the information suggests that both cell populations may be overlapping. It may very well be that CAR cells are MSC progenitors given their many similarities with MSCs and their adipogenic and osteogenic potential described above.

CANCER STEM CELLS (CSCs), DRUG RESISTANCE, AND THE BONE MARROW

The hypothesis of CSCs as the driving force behind the indefinite proliferation and metastasis of tumors has been a prominent player in cancer research in the more recent

literature [17]. This model proposes that oncogenesis incorporates a multi-step hierarchy similar to other normal stem cells such as the hematopoietic stem cells. In a hierarchical model, the CSCs are the core stem cell giving rise to more differentiated tumor cells, but never fully mature to undergo senescence [18]. This model proposes that CSCs share the features of normally occurring stem cells such as that they exist in low numbers, are in a state of quiescence, have the ability of self-renewal, and that they differentiate into a progeny of cells.

Drug resistance

Clinical evidence supports dormant CSC as one of the main reasons for cancer relapse [19, 20]. CSCs have the ability to escape any conventional therapeutic agents, partly because these agents rely on the increased proliferation for uptake into the cell whereas the CSCs are normally found in the quiescent G_0 state [21]. Thus, despite several rounds of chemotherapy, CSCs continue to reside in the niche unharmed. CSCs, apart from being dormant, are chemoresistant. There are several factors that play a key role in their chemoresistance including the expression of ATP-binding cassette (ABC) transporters and aldehyde dehydrogenase (ALDH) [22].

ABC transporters belong to the family of ATP dependent pumps that use energy to efflux small molecules/compounds across the membrane, thus responsible for multiple drug resistance [22]. The ABC transporter has a nucleotide-binding domain that hydrolyzes energy in the form of ATP and a transmembrane domain that keeps check on the small molecules/compounds [23, 24]. The human ABC family is divided into seven subfamilies denoted by the letters A through G, thus performing a wide variety of functions [23, 24]. ABCG2, which has been found to be expressed in both the normal and CSC population of cells in the liver and breast, is responsible for drug efflux thus providing chemoresistance [25, 26]. ABCB1 (multiple drug resistance protein 1 (MDR1) or p-glycoprotein (p-gp)), under normal conditions, is either not expressed or expressed at a very low level [23]. In a study done in the CSC population of thyroid and osteosarcoma, ABCB1 was upregulated along with ABCG2 suggesting it provides the CSCs with a strong defense against chemotherapy [27, 28]. In other studies, the CSC population has been shown to be chemo- and radio-resistant [29, 30].

ALDH is a cytoplasmic enzyme responsible for oxidation of aldehyde to carboxylic acid. Within the ALDH family, chemoresistance by ALDH1 against cyclophosphamide was first reported in L1210 leukemia [31]. In later studies, ALDH1 expression was reported in hematopoietic stem and progenitor cells and its inhibition caused these cells to become chemosensitized [32–34]. The expression of ALDH1 has now been widely shown on both normal cells and CSCs and is also used as one of the diagnostic markers to identify CSCs [35]. In a study performed in

2009, it was shown that there was an increase in ALDH1 positive breast tumor after neoadjuvant treatment without a change in the $CD44^+CD24^-$ population (then used as a breast CSC marker), suggesting ALDH1's expression in a non-CSC population can induce chemoresistance [36]. The retinoic acid signaling pathway is also important in both normal cells and CSCs [37, 38]. Retinol is oxidized to retinoic acid (RA) after a series of enzymatic reactions that include ALDH1. During differentiation of stem cells, RA binds to the RA receptor (RAR) giving negative feedback to ALDH1 [39]. When the level of RA is downregulated, it binds to the RA response element and its enhancer element to transcriptionally activate ALDH1 to increase RA formation and chemoresistance [39, 40].

Bone marrow- 'the niche' for CSCs

CSCs mostly micro-metastasize to different organs and remain undetected in a dormant state for a long period of time [17, 41, 42]. In breast cancer, one of the favorable organs of metastasis for these CSCs is the bone marrow (BM) [18, 43, 44]. Clinical evidence shows that most breast cancer relapse has its origin from the BM [20, 45–47]. The BM microenvironment includes the stromal cells, MSCs, endothelial cells and HSCs, and it is likely the pathways and environment that support these cells are also involved in the maintenance of CSCs. Similar to HSCs, the hypoxic microenvironment in the BM is critical for the CSCs to induce and maintain dormancy [48, 49].

CSCs enter the BM via central vasculature [50]. The cells surrounding the vasculature maintain high CXCL12 expression and its expression seems to be downregulated in cells close to the endosteum [43, 51]. This CXCL12 gradient may be important for the CSC to migrate and stabilize in the BM. The current literature suggests that the area of the highest level of CXCL12 may facilitate migration whereas the region with the lower level of CXCL12 may support dormancy. The latter could occur by the low level of CXCL12 inducing gap junctional formation between the CSC and the BM microenvironment [52, 53]. In colorectal cancer, the associated fibroblasts have been shown to be involved in the expansion of the CSC population [54]. This expansion has been attributed to the release of exosomes from the fibroblasts to favor the CSCs, resulting in cycling quiescence and chemoresistance [54]. The interaction between the CSC and the BM cells is not limited to gap junction, but also via exosomes released by the niche cells that signal the CSCs to undergo dormancy [55].

Despite extensive understanding of the interaction between the CSC and BM microenvironment, CSC similarity to the resident normal stem cells limits any chemotherapy to be approved for targeting CSCs. The reversal of dormancy does not happen until the microenvironment is favorable. Therefore, further dissection of these interactions is important in identification of survival pathways, which is exclusive to the CSC population.

CSCs and CAR CELLS

As stated previously, CAR cells have been shown to be involved in HSC maintenance [14]. This function is relevant to the discussion on CSCs because both types of malignant and normal stem cells are located in the bone marrow. Since both are surrounded by the same niche, it is likely that the information gained from studies of HSCs could be used to understand how the CSCs can be supported by the signaling cascades identified for HSCs. When CAR cells were depleted in mice, this resulted in a decrease in the amount of HSCs with a concomitant decrease in the otherwise cycling erythroid and lymphoid progenitors [13]. More importantly, the depletion of CAR cells led to reduced number of HSCs with the expression of myeloid genes in these cells. This observation was similar to what would occur if HSCs were cultured with a niche.

One of the possible explanations for the support of HSCs by CAR cells might be due to the fact that the stromal cells are the main producers of stem cell factor (SCF) and CXCL12, both of which can contribute to the maintenance of HSCs [8, 10]. It is possible that this same effect can be extended to CSCs and by inhibiting CAR cells, the depletion of SCF and CXCLs may decrease the population of CSCs and hence preventing the regrowth of tumor following chemotherapy. Other factors such as hepatocyte growth factor, extracellular nucleotides and two bioactive phosphorylated sphingolipids that are derivatives of sphingolipid metabolism, sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P) can be chemoattractants for HSCs [56]. Thus, it will be of interest to determine if, in addition to CXCL12, S1P and C1P among the other chemoattractants can also be produced by CAR cells. If so, this will expand the support of CAR cells on CSCs.

It is possible that CAR cells may play a role in other mechanisms that allow CSCs to survive. Although not studied with regard to CAR cells, one such mechanism that CSCs use to escape normal scavenging by the body is by the upregulation of CD47, a cell surface receptor which mediates the removal of old and damaged cells by macrophages [57]. Under normal physiology, this surface receptor is upregulated by HSCs when they enter the bloodstream to prevent phagocytosis. A previous study has shown that CAR cell depletion induces HSC mobilization and it would be interesting to note if this has the same effect on CSCs [58]. It has yet to be seen what, if any, effect CAR cells have on the expression of CD47 and the regulation of other surface receptors involved in the lack of immune response by the body to CSCs. This and other such characteristics are future avenues of research with CAR cells.

3D IN VITRO MODELING SECTION FOR CAR CELL REVIEW

In order to best study CAR cell behavior and interactions *in vitro*, the model utilized should replicate the structure

and function of the BM as closely as possible. Two-dimensional (2D) systems provide a simple, powerful *in vitro* method for investigating cell behavior [59]. However, 2D systems inherently lack the appropriate dimensionality to allow for proper cell-cell and cell-matrix interactions, rendering them less capable of accurately mimicking *in vivo* physiological conditions [59–63].

On the other hand, three-dimensional (3D) systems have been found to support cell morphology and behaviors, migration, gene and protein expression, signal transduction, and drug tolerance in a way that is comparable the endogenous environment [64–67]. In addition, topographical, mechanical, and physiochemical properties of the 3D matrix can be tuned to support these physiological parameters [68–70]. Critically, oxygen and chemokine gradients that are responsible for many cellular interactions and activities can be better established in 3D models [71, 72].

3D systems developed with the aim of reconstructing the BM microenvironment have been applied to investigation of cancer metastasis, cancer dormancy, drug resistance, resident stem cell behaviors, and hematopoietic niche physiology [71, 73–78]. There are a wide variety of 3D systems available for the recapitulation of BM in 3D. Scaffold-based models can be composed of natural or synthetic biomaterials. Collagen I [73, 79], silk [76, 77], fibrin [71], Matrigel [71, 79], fibronectin [79], poly-caprolactone (PCL) [80], acrylamide polymers [81], GELFOAM [82], and polyethylene glycol (PEG) [74] are some biomaterials that have been utilized in 3-D models. Also, microfabricated devices that model the BM in 3D have recently become a critical investigational tool that incorporate patient-specificity and the high-throughput nature of microfluidic strategies [75, 83–85].

We propose that 3D *in vitro* models of the BM can be extended and adapted to the study of CAR cells. 3D models will provide the benefit of better recapitulating the parameters of the BM microenvironment, helping to reveal more about the behavior and function of CAR cells *in vivo*.

FUTURE DIRECTION

The importance of CXCR4 in cancer research is well known. CXCR4 has been known to be expressed highly in numerous cancers and is involved in facilitating the migration, invasion, and proliferation essentially contributing to tumorigenesis and metastasis [12]. This leads us to believe that CAR cells have an important role in cancer treatment. CAR cells may theoretically be an important target in preventing the metastasis of cancer to the bone marrow. One such application may be in breast cancer therapy, where CXCR4 is proposed to be implicated in metastasis through binding of its ligand CXCL12 and by promoting cancer growth through promotion of angiogenesis, cell proliferation, and recruitment of dendritic cells which suppress antitumor activity [86]. However, before

any treatment can be proposed, the function of CAR cells need to be more thoroughly investigated and its lineage fully elucidated in order to understand the potential consequences of impairing CAR cell function *in vivo*. As discussed, this may be facilitated with 3D modeling of the bone marrow environment and further research into the relationship between CAR cells, CSCs and the body's normal resident stem cells. The literature on CAR cells on immune therapy during cancer treatment and the development of immune cells are areas to look for in the forthcoming literature. Currently, the area is new but would evolve with the prediction exponentially.

CONFLICT OF INTEREST

None of the authors has any conflict of interest.

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