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Review Article

The role of surface molecule CD229 in Multiple Myeloma

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ABSTRACT

The outcome of Multiple Myeloma (MM) patients has dramatically improved, however, most patients will still succumb to their disease. Additional therapeutic options are urgently needed and novel immunotherapies are enormously promising in the therapeutic armamentarium against MM. The first step in the development of any immunotherapy needs to be the identification of an appropriate target structure. In this review we present the current knowledge on surface molecule CD229, a member of the Signaling Lymphocyte Activation (SLAM) family of immune receptors. We believe that based on its characteristics, including (1) strong and homogenous expression on all myeloma cells, (2) expression on myeloma precursors, (3) absence from most normal tissues, (4) a central function in the biology of MM, CD229 (SLAMF3) represents a promising target for anti-MM immunotherapies. The introduction of novel anti-CD229 approaches into the clinic will hopefully lead to more durable responses, or maybe even cures, in MM.

1. Introduction

Multiple Myeloma (MM) is a hematologic malignancy which develops from the malignant transformation of a plasma cell clone in the patient's bone marrow (BM). MM can cause renal failure, immunosuppression with infections, bone marrow failure with anemia, hypercalcemia, and lytic bone lesions [1]. In the past 15 years we have seen impressive advances in the patients' outcome, however, almost all patients will still eventually develop refractory disease and suffer a fatal relapse.

It has been proposed that most relapses are due to the persistence of chemotherapy-resistant precursor cells [2–4] in the BM even after destruction of the bulk of tumor cells [5–8]. Accordingly, it has repeatedly been shown that the persistence of minimal residual disease (MRD) after conventional anti-myeloma therapies results in a reduced survival in MM patients [9]. The identification of appropriate immunotherapeutic targets expressed by chemotherapy-resistant myeloma precursors could potentially result in the eradication of the disease eventually resulting in cures.

A crucial step in the design in design of any novel immunotherapeutic approach is the identification of a promising target antigen. We have previously defined a number of characteristics, which can help to rank potential candidates. We think that an ideal myeloma-associated antigen for antibody-based approaches…

1) …must be expressed on the surface of myeloma cells
2) …should be expressed by as few normal tissues as possible
3) …should be expressed by a sufficiently large proportion of myeloma patients
4) …should homogeneously be expressed by the tumor cells of a given patient
5) …should have a central function in the biology and/or pathophysiology of myeloma in order to prevent its downregulation under the selection pressure of an effective immunotherapy

In this review we will determine whether the SLAM family member CD229 fulfills these criteria and whether this receptor could be developed into a target for antibody-mediated immunotherapies for MM.

2. SLAM family member CD229

CD229 (Ly9, SLAMF3) belongs to the Signaling Lymphocyte Activation Molecule (SLAM) family of immune receptors. Like other members of the SLAM family, CD229 has been implicated in lymphocyte development and function and is highly expressed on a variety of lymphocyte subsets [10–15]. Interestingly, the extracellular domain (ECD) of CD229 is composed of 4 immunoglobulin-like domains unlike the typical 2 domains found in other SLAM family receptors [10]. The first and third domains are V-like domains while the second and fourth

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domains are similar to IgSF-truncated C2 set domains [10]. The ECD of CD229 interacts homophillicaly through its N-terminal V-like domain; this interaction appears to occur regardless of species as human CD229-Ig has been shown to bind cells expressing mouse CD229 and vice versa [16]. The intracellular domain of CD229 includes two immunoreceptor tyrosine-based signaling motifs (ITSMs) as well as additional tyrosine residues that function as SH2 domain binding sites following phosphorylation - these domains are critical for CD229 signaling and endocytosis [17,18].

The heart of CD229 signaling lies in its two intracellular ITSMs (T-V/I-Y-xx-V/I). The SH2 domain of SLAM associated protein (SAP) in T-cells and NK cells competes for binding of pTyr residues Y558 and Y581 in the ITSMs of CD229 with the inhibitory protein-tyrosine phosphatase SHP-2 [17,18]. Similarly, in B cells, the SH2 domain of Ewing sarcoma transcript 2 (EAT-2) competes for binding of pTyr residues in CD229's ITSMs with SHP-2 [17]. Due to the relatively low dissociation constant of the SAP–ITSM interaction, SAP tends to dominate binding of the intracellular domain of CD229 where it can then promote phosphorylation of additional Tyr residues via recruitment of src-like protein tyrosine kinases (PTKs) FynT and Lck [17,19].

CD229 signaling has been shown to decrease ERK phosphorylation downstream of TCR signaling, resulting in decreased IFNγ production [20]. The competition between SAP/EAT-2 and tyrosine phosphatases such as SHP-2 allows the cell to tightly regulate CD229 function. The importance of the fine tuning of immunity regulated by this mechanism is exemplified in the autoimmune disorder systemic lupus erythematosus. Here, a polymorphism in the first ITSM of CD229 results in increased SAP-CD229 binding affinity and a subsequent increased level of CD69 upregulation after CD3 stimulation [21]. Ultimately, the presence of this polymorphism has been shown to confer susceptibility to SLE in linkage studies [22].

CD229 signaling is further regulated by rapid internalization following receptor ligation. Del Valle et al. demonstrated that CD229 is the only member of the SLAM family to bind the u2 (AP50) domain of the clathrin-associated adaptor complex AP-2 in T and B lymphocytes [23]. Association of the adapter complex AP-2 with CD229 is crucial for receptor endocytosis [23]. Interestingly, it was later determined that growth factor receptor-bound protein 2 (Grb2) also plays an important role in CD229 endocytosis [20]. Although Grb2 is classically known as an adapter protein in Ras/MAPK signaling, it has also been shown to play a role in EGFR endocytosis [24,25]. In the case of CD229, it was shown that Grb2 binds the cytoplasmic domain of CD229 and promotes receptor internalization [20]. Specifically, site-directed mutagenesis of the critical Grb2 SH2 domain binding residue in CD229 greatly reduced CD229 internalization following receptor ligation [20]. Interestingly, TCR and BCR signaling promote phosphorylation, likely through downstream activation of Fyn kinase, and subsequent endocytosis of CD229 [19,23]. CD229 phosphorylation following TCR ligation, specifically, has been shown to be essential for Grb2 recruitment and endocytosis of CD229 [20]. The interplay between CD229 and TCR/BCR signaling is clearly a highly regulated and finely tuned process; the importance of CD229 in regulating TCR and BCR signaling can be seen in the autoimmune phenotype found in CD229−/− mice as will be discussed later in this review.

3. CD229 function in normal B cells

CD229 function in B cells remains poorly understood with only two studies seeking to address this question. The first study to address CD229 function in B cells established its importance in protection against an autoimmune B cell phenotype. In this study, de Salort et al. observed increased anti-nuclear, ds-DNA and nucleosome antibody production in CD229−/− mice vs WT BALB/c mice [14]. Additionally, a significant increase in numbers of T1 transitional, marginal zone (MZ) and germinal center (GC) B cells accompanied by splenomegaly was observed in CD229−/− mice [14]. Expansion of innate-like B cell subsets and splenomegaly are both implicated in autoimmunity [26–28]. Taken together, this data suggests that CD229 may play a role in restraining B cell immunity in order to prevent the formation of a B cell mediated autoimmune phenotype.

Three years later the same group published another paper further elucidating the function of CD229 in B cells. Once again, the group demonstrated increased levels of innate-like splenic B cells including T1, MZ and B1a B-cells [15]. The elevated levels of MZ and B1a cells correlated with an increase of humoral IgG3 antibody as MZ and B1a cells are a known primary source of IgG3 in the absence of antigen [15]. Medsker et al. also found increased antibody response to T-independent type II antigens, this response included increased production of 2,4,6-Trinitrophenyl (TNP)-specific IgG2a, IgG2b and IgG3 [15]. Since the group observed no change in marginal zone structure or signaling, they concluded that the elevated B cell response to antigen in CD229−/− mice is B cell intrinsic and not due to changes in spleen architecture. Treatment of BALB/c mice with a CD229 mAb (Ly9.7.144) completely depleted the MZ B cell population and significantly reduced the B1 cell population resulting in reduced antibody production against T-dependent and T-independent type I and type II antigens [15]. Interestingly, this effect was shown to be independent of NKT cells as treatment of NKT-deficient CD1d−/− mice with CD229 mAb had the same effect on the T cell-dependent response [15]. CD229 mAb treatment also unexpectedly decreased surface levels of the B cell co-receptor complex, composed of CD19, CD21 and CD81 [15]. The researchers hypothesized that the downregulation of CD19 may drive some effects not seen in CD229−/− mice, including reduced B cell proliferation and survival. Ultimately, it is difficult to determine whether the effects on B cell function seen with treatment of CD229 mAb are strictly due to CD229 activation, especially since CD19 levels have not been studied in CD229−/− mice.

4. Expression of CD229 in Multiple Myeloma

We first identified CD229 as a potential therapeutic target in MM when we applied a protein array to lysates of myeloma cell lines and described CD229 as a surface molecule overexpressed on MM cells [29]. We were also able to show that CD229 is the only SLAM family member that is strongly expressed both at the RNA level as well as on the surface protein of 100% of all established myeloma cell lines. Even more importantly, our analyses showed for the first time that CD229 is consistently and homogeneously found on the cell surface of the malignant plasma cells from all patients with MM. We also observed strong and homogeneous expression of CD229 independent of the patients' disease stage or current treatment [29], a finding which was later confirmed by Muccio and coauthors [30].

In a subsequent study we confirmed that CD229 is not only overexpressed on the malignant plasma cells from patients with MM but also on the tumor cells of patients with the myeloma precursor lesion Monoclonal Gammopathy of Undetermined Significance (MGUS) and those from patients with indolent Smoldering Multiple Myeloma [31]. The finding of equally strong and homogenous expression of CD229 on MGUS and MM plasma cells was confirmed by others [32] and Ishibashi et al. underlined the fact that CD229 is highly and constitutively expressed on plasma cells from patients with MGUS, SMM and MM regardless of disease stage or previous treatment [33]. Strong and homogenous expression of CD229 was even detected on extramedullary MM manifestations and plasmacytomas [34]. Accordingly, Pojero et al. concluded that out of a variety of myeloma-associated surface markers CD229 is the most reliable alternative to CD38 or CD138 for the identification of plasma cells, for example in patients undergoing anti-CD38 or anti-CD138 therapy [34].

Looking at the presence of soluble CD229 protein in the patients' serum instead of surface expression, Ishibashi et al. found that soluble CD229 levels were significantly higher in symptomatic MM than in asymptomatic MM and MGUS and markedly increased in advanced

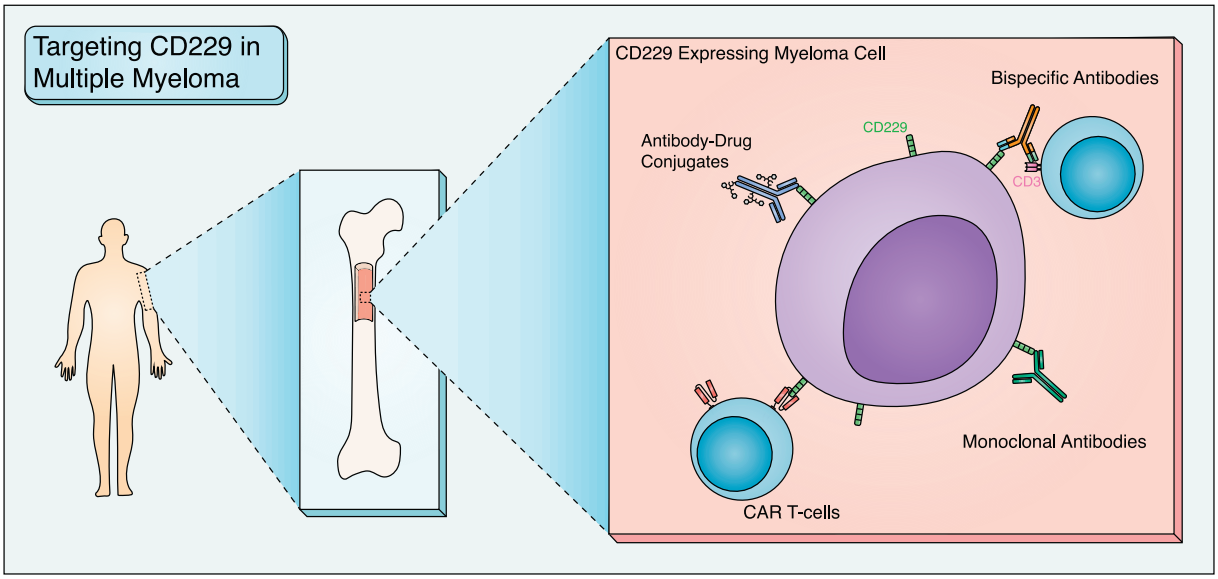

Fig. 1. Targeting CD229 in Multiple Myeloma.
The elevated expression of CD229 on abnormal plasma cells provides numerous avenues for tumor-directed treatment in multiple myeloma. Antibodies specific for CD229 can be used to promote complement or antibody cellular dependent cytotoxicity (CDC/ADCC), recruit cytotoxic effector cells to the tumor microenvironment (bispecific antibodies) or confer tumor specificity to chemotherapeutic drugs (antibody-drug conjugates). Additionally, CAR T-cells targeting CD229 provide direct and prolonged immune response to myeloma cells.

MM. Furthermore, they showed that MM patients with relatively high levels of soluble CD229 evidenced more aggressive clinical characteristics and shorter progression-free survival times than those with relatively lower levels [33].

The question whether CD229 is expressed on all conventional tumor cells from MM patients is obviously of major relevance, however, we believe that it is of equal importance to determine whether the antigen is present on the subpopulation of tumor cells promoting the development, survival, and progression of MM if the goal is to achieve cures. In order to destroy the tumor bulk as well as MM-promoting precursors and eventually eradicate the disease, it may be necessary to attack it from different biological angles using a variety of modalities, including immunotherapeutic approaches,

It has recently been shown that myeloma-propagating activity is the exclusive property of a cell subpopulation characterized by its ability for bidirectional transition between the dominant CD19-CD138+ plasma cell fraction and a small fraction of pre-PCs expressing a CD19-CD138- phenotype [3]. It has also been demonstrated that pre-PCs are more quiescent, are enriched in epigenetic regulators, and are up to 300-fold more drug-resistant than the common malignant PCs of myeloma patients [3]. Importantly, we have shown that in the BM of myeloma patients conventional PC as well as CD138-negative pre-PC myeloma-propagating cells expressed similarly high levels of surface molecule CD229 [31].

These combined data suggested to us that CD229 could potentially serve as a novel target for anti-myeloma immunotherapies such as monoclonal antibodies or chimeric antigen receptor (CAR) T cells if its expression is sufficiently specific for the malignant cells.

5. CD229 expression in normal tissues

Studying CD229 mRNA expression within a wide variety of healthy human tissues using real-time PCR we found relatively high levels of CD229 mRNA only in lymphatic tissues such as the thymus, spleen and tonsillar tissue as well as in the bone marrow and within PBMC. No expression or only trace levels of CD229 mRNA were found in the other human tissue tested [29]. Further dissecting CD229 expression within human lymphatic organs, flow cytometry was applied to leukocytes derived from normal human bone marrow, tonsils, and peripheral blood. We detected CD229 surface expression on T helper cells (CD3 + CD4+), cytotoxic T cells (CD3 + CD8+), natural killer cells (CD3-CD56+), and B cells (CD3-CD19+). In contrast, CD229 was not expressed on monocytes (CD14+) or neutrophils (CD15+). Importantly, CD229 was also absent from CD34+ hematopoietic progenitor cells isolated from donor-derived bone marrow [29].

When we compared CD229 expression levels on myeloma cells versus normal blood and bone marrow cells, we found that the intensity of CD229 staining was always higher on myeloma cells compared to other lymphocyte cell types, such as normal T cells and B cells, found within the bone marrow of the myeloma patients [29]. Furthermore, compared to healthy controls, MM patients generally showed higher CD229 expression on their BM-residing plasma cells [29].

6. Function of CD229 in Multiple Myeloma

As explained above, we believe that it is of an advantage if a therapeutic target plays an important role in the biology of a given tumor type. We examined the function of CD229 in MM by downregulating its expression in MM and found that CD229 protects the malignant cells from spontaneous apoptosis [29]. In accordance with our findings, Ishibashi et al. have recently demonstrated that CD229 overexpression in KMS34 cells promoted the proliferative and antiapoptotic potential of the cells [33]. In addition, we found that co-treatment of myeloma cell lines with CD229 siRNA and conventional anti-myeloma drugs melphalan and bortezomib increased anti-myeloma cytotoxicity indicating a potential use for CD229 as an adjunct to conventional therapeutic approaches in MM [29].

Importantly, asking whether CD229 gene silencing would also have an effect on myeloma-propagating progenitors we used a standard colony-forming assay and we detected a significantly reduced number of cell colonies after transfection with CD229-specific siRNA [29].

7. Therapeutic use of CD229 in Multiple Myeloma

Different types of anti-CD229 approaches are currently under development in our lab. In a pilot experiment, using a murine CD229-

specific antibody, we observed induction of strong antibody-dependent cellular cytotoxicity (ADCC) as well as complement-dependent cytotoxicity (CDC) against MM cell lines. These findings indicated to us that a monoclonal antibody directed against surface molecule CD229 could in principle be developed into a promising instrument for the therapy of MM [29].

We propose that based on its characteristics, which include (1) strong and homogenous expression on all myeloma cells, (2) expression even on myeloma precursor cells, (3) absence from most normal tissues, and (4) a central function in the biology of MM, CD229 represents a preferable target for anti-MM immunotherapies. Since CD229 is expressed on the surface of the myeloma tumor cell, it can be used as target for monoclonal antibodies, immunoconjugates, bi-specific T cell engagers (BiTEs), and chimeric antigen receptor (CAR) T cells (Fig. 1). Our group has developed CAR T cells targeting CD229 and this approach is currently being translated into the clinic.

A potential disadvantage of CD229 as a therapeutic target is its expression on normal lymphocyte subsets such as B cells, NK cells, and T cells. Although the expression of CD229 seems to be lower on normal lymphocytes compared to malignant myeloma plasma cells, this could potentially result in on-target and off-tissue toxicity for example after the use of CD229-specific CAR T cells. However, the expression of CD229 on earlier-stage B cells may also be an advantage allowing for the elimination of myeloma cell precursors thereby helping to reduce relapse rates after a CD229-specific immunotherapy.

8. Conclusions

CD229 is an intriguing new therapeutic target for the treatment of MM, due to its strong and homogeneous expression on the malignant plasma cells of patients with newly diagnosed as well as relapsed MM and its absence from most healthy tissues. Importantly, CD229 is present not only on the bulk of MM cells but, in contrast to other MM-specific immunotherapy targets, also on chemotherapy-resistant clonogenic MM precursors. CD229 is centrally involved in healthy B cell function and essential for MM cell viability, indicating that its expression may be indispensable for the malignant cells and downregulation unlikely during selective immunotherapeutic pressure. Overall, we hypothesize that the development of novel anti-CD229 approaches has the potential to lead to more durable responses, or maybe even cures, in MM.

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