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CD38 and bone marrow microenvironment

Antonella Chillemi[1], Gianluca Zaccarello[1], Valeria Quarona[1], Mirca Lazzaretti[2], Eugenia Martella[3], Nicola Giuliani[4], Riccardo Ferracini[5], Vito Pistoia[6], Alberto L. Horenstein[1,7], Fabio Malavasi[1,7,8]

[1]Laboratory of Immunogenetics, Department of Medical Sciences, University of Torino Medical School, Torino, Italy, [2]Department of Bioscences, University of Parma, Parma, Italy, [3]Anatomia e Istologia patologica, Azienda Ospedaliero-Universitaria di Parma, Parma, Italy, [4]Hematology and Blood and Marrow Transplantation (BMT) Center, University of Parma, Parma, Italy, [5]Department of Orthopedics and Traumatology, Citta della Salute e della Scienza Hospital, Torino, Italy, [6]Laboratory of Oncology, Department of Experimental and Laboratory Medicine, Istituto Giannina Gaslini, Genova, Italy, [7]Centro di Ricerca in Medicina Sperimentale (CeRMS), University of Torino Medical School, Torino, Italy, [8]Transplantation Immunology Service, Citta della Salute e della Scienza Hospital, Torino, Italy

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1. ABSTRACT

This review summarizes the events ruled by CD38 in shaping the bone marrow environment, recapitulating old and new aspects derived from the body of knowledge on the molecule. The disease models considered were myeloma and chronic lymphocytic leukemia (CLL). CD38 has been analyzed considering its twin function as receptor and enzyme, roles usually not considered in clinics, where it is used as a routine marker. Another aspect pertaining basic science concerns the role of the molecule as a member of an ectoenzyme network, potentially metabolizing soluble factors not yet analyzed (*e.g.,* NAD^+, ATP, NAM) or influencing hormone secretion (*e.g.,* oxytocin). The last point is focused on the use of CD38 as a target of an antibody-mediated therapeutic approach in myeloma and CLL. A recent observation is that CD38 may run an escape circuit leading to the production of adenosine. The generation of local anergy may be blocked by using anti-CD38 antibodies. Consequently, not only might CD38 be a prime target for mAb-mediated therapy, but its functional block may contribute to general improvement in cancer immunotherapy and outcomes.

2. INTRODUCTION

The microenvironment has become the most recent experimental playground for researchers to test *in vivo* hypotheses derived from *in vitro* evidence. The advantage of using the microenvironment is that it allows the pooling together of apparently unrelated findings from physiology (and pathology) and anatomical structures. The most widely-investigated tissues are currently lymph node and bone marrow (BM), because they represent the convergence of different cells and soluble factors in a discrete architecture and allow a dissection and testing of the actions driven by the different components of the complex system. As the most accessible system in humans, the bloodstream was the first complete model to be studied, thus shaping most of the information over the last thirty years. A concerted international effort based on monoclonal antibodies (mAbs) defined new phenotypes (and consequently the ability to identify cells) and the possibility of monitoring what happens when cells communicate in the context of the family or outside. The majority of these effects are mediated by physical interactions between cells. The communication is enriched by using soluble factors,

like cytokines, chemokines and hormones along with the network of their specific receptors. This notion drew attention to the signals implemented in different cell populations, to the events involved and to the biological effects triggered.

Dissection of the different components is a difficult task when working in an open system like the bloodstream, so attention was shifted to closed systems, where discrete tissue architecture drives circuits and pathways. These systems may now be more precisely defined as microenvironments. The different working hypotheses tested in such systems were validated by using tumor models. The conclusions derived from pathology have taught us about the normal functioning of the system and how these mechanisms can be subverted for the needs of metabolic demands specific for the different tumors. These include metabolism, the escape from immune defenses and, at the same time, the necessity to migrate from the place of origin, to colonize target organs or the entire organism.

Interest in this field is dual: on the one hand, this information enriched our knowledge of the physiology of cell growth, differentiation, and apoptosis in different tissues. On the other, this information had a direct impact on experimental treatments by designing drugs able to interfere with all of these processes in the tumor microenvironment. This has led to the construction of several models of targeted therapies, some of which are already undergoing clinical trials.

This overview provides analysis and perspectives hinging on CD38, an old molecule described in the late 1970s. Originally considered a mere activation marker and, as such, used in clinical diagnosis, the same molecule was the focus of different groups who eventually attributed unexpected functions to it. CD38 was later demonstrated to be a member of a family of multifunctional ectoenzymes, initially considered a topological paradox. However, the number of ectoenzymes is now known to make up almost 5% of cell surface molecules, indicating and underlining a natural design still to be fully understood.

CD38 was shown to govern the synthesis of ADPR and cADPR, both signaling molecules. Besides this, it was demonstrated that CD38 has a non-substrate ligand, and thus an adhesion molecule. The latest findings seem to support the hypothesis that CD38 is also involved in negative regulation of the immune response, contributing to immunosuppression.

In synthesis, this is what is known in human cell biology. Furthermore, experience derived from using genetically modified animal models has highlighted that CD38 plays a key role in the release of oxytocin (OT), a phylogenetically extremely conserved hormone and which is now emerging as a controller of multiple functions, especially in behavior.

We now intend to analyze bone marrow from the perspective provided by selected ectoenzymes which govern the balance in production of compounds. The aim is to investigate the properties exerted in this system by CD38 (and its paralogue CD157) and their role in the production of OT. In addition, we will test the role played by the CD39/CD73 axis, which is the main pathway of adenosine (ADO) production. To frame the information coming from different fields into a unified model and to make it accessible to a wider audience, here we report the latest findings concerning all the components of the system, with special emphasis on the translational opportunities for clinical applications.

3. BIOLOGY OF CD38

Human CD38 is one of the first surface molecules identified by means of murine mAbs during the pioneering work of Reinherz and Schlossman (Dana-Farber Cancer Center, Boston, MA) (1). The work was focused on the identification of membrane molecules involved in antigen recognition, while CD38 appeared as linked to cell activation. CD38 attracted the attention of basic and clinical scientists because of distribution in normal tissues, as well as in selected leukemias. The molecule no longer appears as strictly dependent on cell lineage or activation. Human mature resting cells and lymphocytes, however, do express limited amounts of surface CD38.

3.1. CD38 gene

The human *CD38* gene maps to chromosome 4p15 and consists of 8 exons (2). The exon 1, the largest coding exon, determines the intracytoplasmic and transmembrane regions and the part of the extracellular region (3, 4). Intronic sequences represent over 98% of the *CD38* gene. Intron 1 is also the location of a single nucleotide polymorphism (SNP; rs6449182; C→G variation) (5), that binds the transcription factor E2A (6). This allelic variation may differentially influence CD38 expression and its ability to be shed in biological fluids.

The control of human *CD38* expression appears to be multitiered. The first layer of control lies in the 5'-flaking promoter region characterized by the lack of a TATA box and the presence of a CpG island, a methylation-controlled region more frequently associated with housekeeping than tissue-specific genes (3). A second level of control is likely to be upstream of the CpG island. Potential binding sites have been identified for transcription factors [*e.g.,* for T cell transcription factor-1α (TCF-1α), nuclear factor for interleukin-6 (NF-IL-6) and interferon-responsive element-1 (IRF-1)] and binding sites for glucocorticoid hormones (3). The last level of control is located at the 5'-end of intron 1 and is involved in the regulation of CD38 via a retinoic acid-responsive element (RARE) (7). The effects are mediated by binding to the retinoic acid receptors (RARs). RARs is also capable to interact with other nuclear receptors, thus expanding their spectrum of action on gene expression.

CD38 has a well-characterized single-nucleotide polymorphism (SNP) located at the 5'-end of this intron C→G), which leads to the presence (or absence) of a Pvu II restriction site. The frequency of the three genotypes has

been established in healthy Italian-born adults as 70% CC, 26% CG, and 4% GG. Similar frequencies have been reported in Spanish and Irish populations. The SNP is located in an intronic hot spot, containing part of the CpG island + RARE mentioned above. In addition, evidence based on a novel truncated CD38 mRNA transcript suggests that a further mechanism for controlling *CD38* gene expression involves transcriptional elongation, where a stop-or-go decision is taken within the 5'- end of intron 1 (8).

The most common retinoid also used for clinical applications is all-*trans* retinoic acid (ATRA), an active metabolite of vitamin A. The ATRA/RAR/RARE binding cascade is the early phase of a multistep process (phase 1), which includes a delayed phase mediated through response elements in the 5'-upstream region of CD38 (phase 2). This second step requires de novo synthesis of protein kinase C-δ (PKC-δ), which likely acts on a sequence of elements for c/Enhancer Binding Protein β (c/EBPβ), a non-canonical ATRA-responsive element (9).

ATRA activates both phase 1 and 2. Newly identified synthetic derivatives of retinoids (*e.g.*, tamibarotene, Am80) have been identified. These molecules may contribute to the regulation of CD38 expression, but their effects only involve the early phase response (10).

3.2. CD38 protein structure

Human CD38 is made up of a single chain of 300 amino acids (aa) with a corresponding molecular weight of ≈45 kDa. It is characterized by a short cytoplasmic tail (21 aa), a small transmembrane domain (23 aa) and a large extracellular domain (256 aa). CD38 is a glycoprotein comprising 2 to 4 N-linked oligosaccharide chains containing sialic acid residues. The overall structure of the CD38 molecule is stabilized by six pairs of disulphide bonds (8). In addition to the canonical 45 kD structure, experiments showed that the molecule may exist in a soluble form present in biological fluids in normal, para-physiological and pathological conditions (11, 12).

The monomeric membrane-bound forms of CD38 are flanked by its soluble form of approximately 78 kDa (p78) (13) and a high-molecular weight form of 190 kDa (p190) (14). The latter fits with a tetrameric conformation of the molecule, both displaying enzymatic activities (15).

The transition from monomers to dimers (or multimers) modulates the functions of the molecule (16). Additional control is rendered by the dynamic localization of CD38 in lipid microdomains of the plasma membrane, where the molecule has a tendency to associate with other proteins, forming large supramolecular complexes (8). CD38 is also found in exosomes, membrane vesicles secreted by B cells and likely a component of an intercellular communication network (17).

4. RECEPTORIAL FUNCTIONS OF CD38

One function initially attributed to CD38 was the regulation of activation and proliferation of human T lymphocytes (18, 19). After ligation by agonistic mAbs, CD38 induces rapid Ca^{2+} release and triggers the phosphorylation of intracellular substrates, leading to activation of the nuclear factor-κB (NF-κB) complex (20, 21). Protracted effects include initiation of genetic programs causing cytokine secretion and proliferation of T lymphocytes (22).

CD38 as receptor was initially assessed by the use of a panel of specific mAbs. First evidence have shown that CD38 engagement was followed by the activation of selected peripheral blood mononuclear cell populations (18). The identification of a first putative ligand was obtained by exploiting the observation that human T lymphocytes tended to adhere to endothelial cells (23). Experiments blocking this adhesion concluded that CD31 [also known as PECAM-1, a member of the immunoglobulin superfamily (24)] acted as a CD38 non-substrate ligand. The interaction of CD38 with CD31 expressed by cell surfaces may reproduce the results obtained with the agonistic mAbs triggering the same intracellular processes (25). The interplay between CD38 and CD31 is crucial for leukocyte migration through the endothelium (26). CD38-mediated signals are regulated at distinct levels: a flip-flop mechanism of membrane positioning has been recently proposed, with a type III form of CD38 displaying its catalytic site in the cytoplasm (27). The second level is based on the dynamic localization of CD38 in lipid microdomains within the plasma membrane. Lateral associations with other molecules determine a third level of control. Lipid raft localization and association with professional signaling complexes are pre-requisites for signals mediated through CD38 (25).

5. ENZYMATIC FUNCTIONS OF CD38

CD38 has a striking similarity (≈35% aa identity) to the enzyme adenosine diphosphate (ADP) ribosyl cyclase presents in a soluble form in the ovotestis of the mollusk *Aplysia californica* (28). In *Aplysia* this enzyme has the ability to convert NAD^+ to cADPR, a universal second messenger that controls Ca^{2+} levels in an IP_3-independent way (29). Under appropriate conditions, a limited amount of cADPR is converted to ADPR. In mammals, CD38 maintained these enzymatic activities and became localized on the cell surface, thereby developing into an ectoenzyme (30). As an ectoenzyme, CD38 metabolizes NAD^+ to cADPR and ADPR. A further enzymatic activity attributed to CD38 is the pH-dependent conversion of $NADP^+$ to $NAADP^+$. The binding of these products to different receptors and channels influences the regulation of Ca^{2+} and activates critical signaling pathways (31). Thus, CD38 regulates extracellular NAD^+ levels, thereby limiting the availability of this substrate to a larger family of enzymes, including ADP ribosyl transferases (ARTs), poly (ADP-ribose) polymerases (PARP) and sirtuins. The role of CD38 and of its products in regulating a wide range of physiological functions is demonstrated by the multiple impairments revealed in *CD38* knock-out mice. These include alterations of neutrophil chemotaxis, defective oxytocin (OT) release and aberrant social behavior (32).

Table 1. Distribution of human CD38

Tissue	Cell population
Lymphoid	
Blood	T cells (precursors, activated)
	B cells (precursors, activated)
	Myeloid cells (monocytes, macrophages, dendritic cells)
	NK cells
	Erythrocytes
	Platelets
Bone Marrow	Precursors (very early $CD34^+$cells are $CD38^-$)
	Plasma cells
Cord Blood	T and B lymphocytes, monocytes
Thymus	Cortical thymocytes
Lymph nodes	Germinal center B cells
Non-lymphoid	
Bone	Osteoclasts
Brain	Purkinje cells
	Neurofibrillary tangles
	Cultured astrocytes
	Cerebellum
Eye	Cornea
	Retinal gangliar cells
Gut	Intraepithelial lymphocytes
	Lamina propria lymphocytes
Pancreas	β cells
Muscle	Sarcolemma (smooth and striated muscle)
Prostate	Epithelial cells
Kidney	Glomeruli

6. TISSUE DISTRIBUTION OF CD38

Human CD38 is surface expressed by various cells of both hematopoietic and non-hematopoietic lineages. CD38 is expressed in the T cell compartment by a significant fraction of human thymocytes, mainly at the double-positive stage and progressively lost during maturation. Expression in B cells is tightly regulated during cell ontogenesis, being present at high levels in BM precursors and in terminally differentiated plasma cells. CD38 is expressed also in circulating monocytes (but not in resident macrophages), and in circulating and residential NK cells and granulocytes.

CD38 is also present in many tissues other than haematopoietic cells, including normal prostatic epithelial cells, pancreatic islet cells and the brain, where it is detected in perikarya and dendrites of many neurons, such as the cerebellar Purkinje cells, in rat astrocytes and in perivascular autonomic nerve terminals. Other $CD38^+$ cells include smooth and striated muscle cells, renal tubules, retinal gangliar cells and cornea (8).

7. CD38 IN TUMOR MICROENVIRONMENT

7.1. Bone marrow niche

Hematopoietic progenitor cells (HPCs) home to and engraft in highly specific BM microenvironments, or niches, that regulate their survival, proliferation, and differentiation (33, 34). These niches have been defined by the association of particular stromal cell types and by their elaboration or secretion of specific signaling molecules, growth factors and cytokines (35). At least two distinct HPC-supportive niches have been identified in the BM. The osteoblastic niche, in which molecules including bone morphogenetic protein, osteopontin, angiopoietin-1 and Notch appear to play important regulatory roles. The vascular niche remains to be fully molecularly defined (36, 37).

Although defects in hematopoiesis are frequently observed in patients with malignant involvement of the BM, the molecular bases of these phenomena, and whether they might reflect perturbations in HPC-supportive niches, are unknown (38). Suppression of normal hematopoiesis can occur in the setting of relatively low tumor burden and thus does not necessarily reflect anatomic crowding out of benign cells (39, 40). For example, leukemic proliferation in the BM alters the stromal microenvironment and creates malignant niches that outcompete native HPC niches for $CD34^+$ cell engraftment. Normal $CD34^+$ cells engaged by the malignant niche exhibit abnormal behavior. Conceivably, derangements in hematopoiesis and HPC mobilization could impair anti-tumor immune responses (41).

7.2. Bone marrow and chronic lymphocytic leukemia (CLL)

CLL is a common adult leukemia which results from the accumulation of small B ($CD19^+/CD5^+/CD23^+$) lymphocytes in blood, BM, lymph nodes (LN) and other lymphoid tissues (42). The latter districts represent permissive niches, where lymphocytes can proliferate in response to microenvironmental signals (43). The percentage of cells within a CLL clone that express surface CD38 is an indicator of the potential and actual degree of cellular activation of the clone. High levels of CD38 in CLL cells are generally associated with advanced disease stage, higher incidence of lymphadenopathy, high-risk cytogenetics, shorter lymphocyte doubling time, shorter time to first treatment and poorer response to therapy. Besides being a prognostic marker, CD38 is a component of a molecular network which delivers growth and survival signals to CLL cells (44). The aggressiveness of $CD38^+$ cells appears to rely upon their ability to migrate and take advantage of interactions with the microenvironment. ZAP-70 also appears to be involved in this action. (44) CD38 can work in association with chemokines and their receptors, mainly CXCL12/CXCR4, influencing the migratory responses and contributing to the recirculation of neoplastic cells from blood to lymphoid organs (45) and with specific adhesion molecules belonging to the integrin family (46, 47).

Investigation into the origin and development of this form of leukemia has provided solid evidence in favor of the current view that survival and proliferation of CLL cells depends on the microenvironment (48, 49). The host microenvironment and the resulting interplay between the genetic background and environmental influences thus play a crucial role in disease progression, as well as in resistance to treatment. Several findings presented support the view that a favorable microenvironment can provide the optimal combination of antigen and co-signals for expansion of the

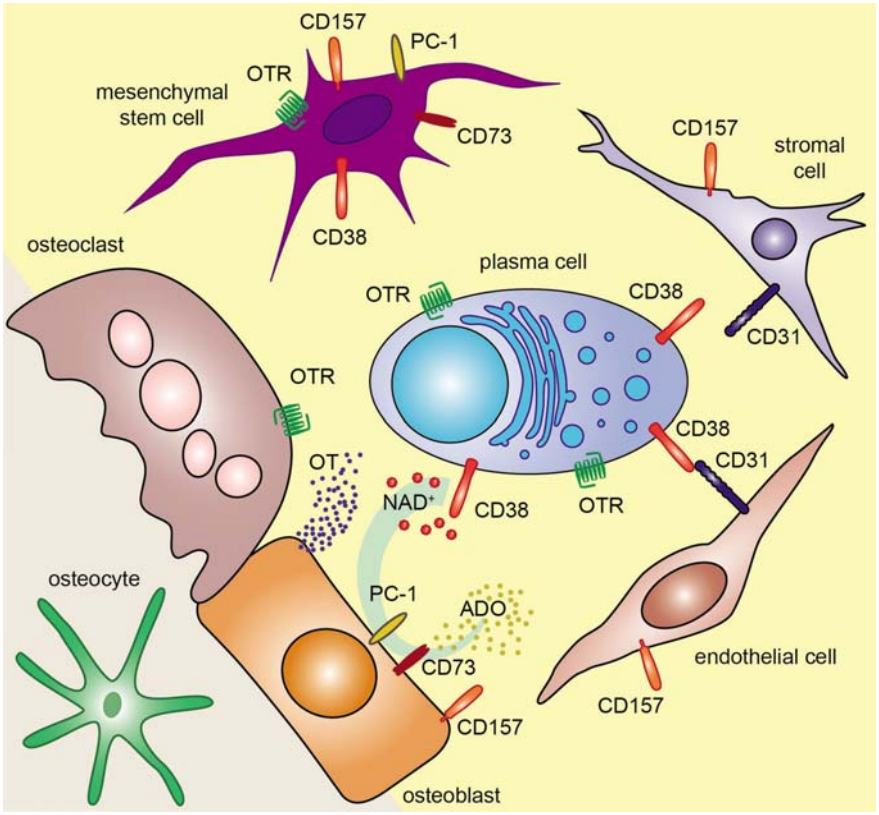

Figure 1. Schematic model of bone marrow niche.

CLL-initiating cells, leading to disease progression (50). Instead, under environmental conditions which do not favor growth, proliferation of the same CLL cells is impaired, and the molecular mechanisms preventing apoptosis prevail. The consequence is that the independent expression of CD38 by the leukemic clone provoked and increased migration to LN, a microenvironment protecting from drug and simultaneusly feeding the metabolic need of the cells. Under analysis is the hypothesis that the enzymatic functions of the molecule may be subverted to the emergencies of the leukemic cells, in energy and in escaping immune responses. An instance is represented by the process of generation of ADO (51).

7.3. Multiple myeloma (MM)

MM is characterized by the accumulation of clonal plasma cells in the bone marrow, the presence of monoclonal immunoglobulin (Ig) in the serum or urine, osteolytic bone lesions, renal disease and immunodeficiency (52, 53). Progression to MM is associated with a series of complex genetic events in myeloma cells, as well as changes in the BM microenvironment, including increased angiogenesis, suppression of the immune response, increased bone resorption and the establishment of aberrant signaling loops involving cytokines and growth factors associated with the clinical features of MM and its resistance to treatment (54).

There are several issues suggesting that CD38 plays significant roles in MM. First, CD38 is expressed by normal and tumoral plasma cells at high levels in cells which tend to eliminate the majority of surface molecules. Second, myeloma express CD31 molecule, the CD38 ligand, in a significant portions of cases, as it is for monoclonal gammopathies of undetermined significance (MGUS) (55). Third, experiments in murine models showed that CD38 is a key regulator of OT release (32) in biological fluids and the specific receptor (OTR) is detectable on myeloma cells (56). Fourth, the BM environment (osteoclasts, osteoblasts, stromal cells, endothelial cells, among the others) has the ability to concentrate soluble factors (*e.g.*, NAD^+, ATP and ADO) not yet considered in a context of growth factors (Figure 1). Fifth, the different cells contribute an important role, not yet functionally tested in this context of ectoenzyme. The result determines the balance between activation and suppression of cell growth. This happens either for the tumor or for immune defense.

In light of these considerations, plasma cells (and their malignant counterpart) and bone niches are good testing grounds for assessing the presence of a connection between ectoenzymes and neuropeptides (57) (Figure 2). The system is closed and nucleosides represent additional signals to those led by cytokines/chemokines and other conventional regulators: ATP and NAD^+ operating *in loco* may complement the physiological regulatory systems of plasma cells. The first observation in immunohistochemistry performed on osteomedullary biopsy obtained from patients with myeloma support the

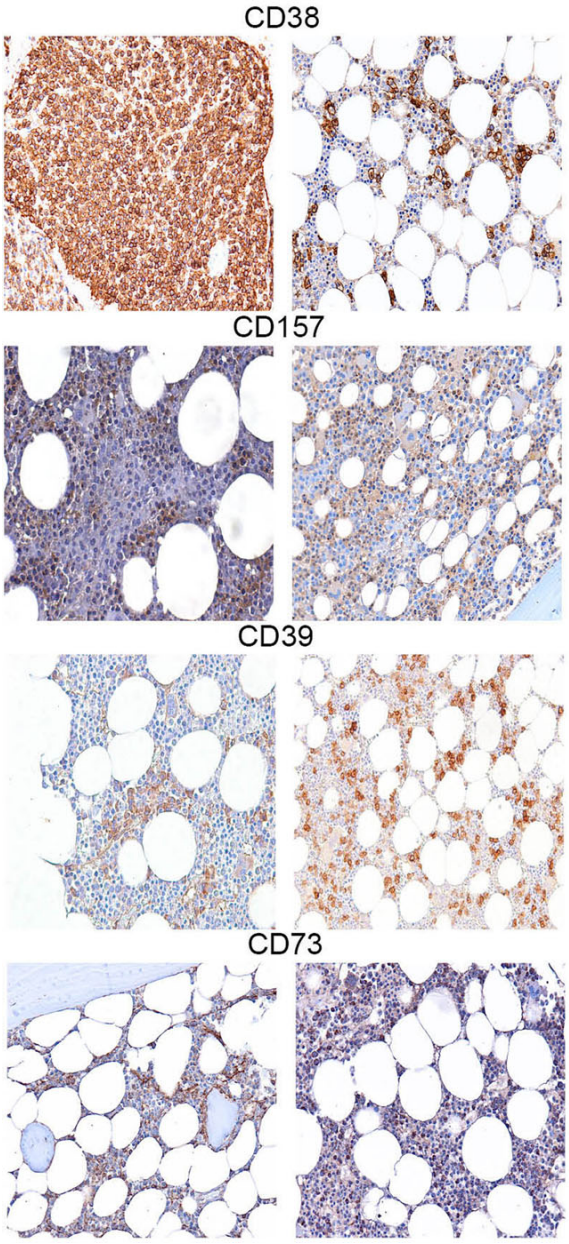

Figure 2. Distribution of CD38, CD157, CD39 and CD73 in human osteomedullary biopsies from multiple myeloma patients assessed by immunohistochemistry. CD38: expression in high density (left) and low density (right) myeloma cells. CD157: expression by monocytoid elements. CD39: expression by vessel endothelial cells and myeloma cells. CD73: expression by osteoblasts.

view that ectoenzymes may contribute to the indicate crosstalks taking place in the model.

8. ROLE OF CD38 AS AN ESCAPE PATHWAY

Tumor and leukemic cells have developed multiple mechanisms to impair the antitumor immune response. One such mechanism relies on the ability of cancer cells or - more generally - of the tumor microenvironment to generate ADO, a major molecule inducing suppression of response (58). ADO is constitutively present in the extracellular media at a very low concentration, but its concentration increases in many metabolically stressful conditions (59). ADO produced in the tumor microenvironment may influence cancer growth via direct binding to specific receptors expressed at the surface of tumor cells, acting as a growth factor. ADO receptors are also expressed by CLL (51), myelomas (60) and melanomas (61). ADO also acts on a variety of immune cells (dendritic cells, macrophages, neutrophils, NK cells and T lymphocytes), where it impairs negative effects (62).

The dominant pathway leading to the production of ADO outside the cells occurs through the sequential actions of CD39 (ectonucleoside triphosphate diphosphohydrolase 1, ENTPD1) and CD73 (ecto-5'-nucleotidase, NT5E). We have considered the hypothesis of an alternative pathway of ADO production, in which CD38 works in conjunction with other ectoenzymes to generate activation or suppression of immune responses according to the environment. The main feature of this alternative pathway is to metabolize NAD^+ present in the microenvironment, acting as a CD38 substrate and with production of ADPR and cADPR. Beside this, PC-1 (also known as CD203a) is an ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP-1), which converts ATP, NAD^+ or ADPR to AMP, in turn metabolized by CD73 to ADO (Horenstein A.L. *et al*, 2013 submitted). The consequence is that CD38 would be a component of one of the multiple strategies adopted by tumor to evade the immune response. This unconventional ectoenzyme network will be able to provide the generation of local tolerance in different disease models, such as the BM microenvironment in pathology (*e.g.,* myeloma or CLL). Other models are currently under study, such as recurrent pregnancy loss (63) and melanoma (Morandi F. *et al.*, 2013 submitted). Conceivably, CD38/PC-1/CD73 pathway may tip the balance from activation to anergy and suppression (Figure 3).

9. CD38 AS A THERAPEUTIC TARGET

Targeted immunotherapy based on mAbs (murine, humanized or human) specific for relevant tumor antigens has become a feasible and highly promising approach in hematological malignancies, mainly because can be combined with conventional treatments to further increase the potency of anti-tumor effects. CD38 is a particularly attractive target on malignant plasma cells at all stages of disease and in CLL patients with a poor clinical prognosis or refractory to therapies (64). As such, this molecule is a promising target for antibody therapy also in different tumors.

Improvement of anti-CD38 mAbs with strong cytolytic potential have been reported by 3 major groups (GenMab now Johnson & Johnson, Sanofi and Morphosys). These companies are in early-stage trials in myeloma therapy (65). One of the human mAb specific for

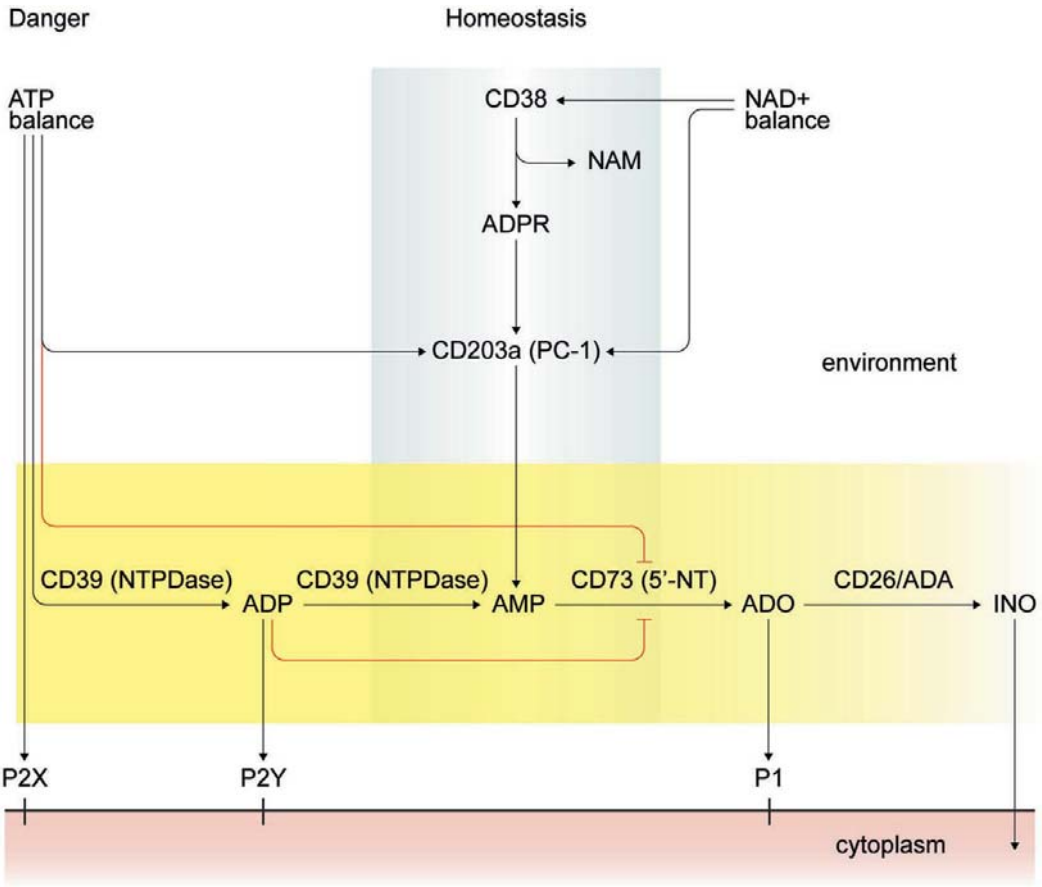

Figure 3. Canonical and alternative pathway of adenosine production. Black lines= positive actions. Red lines= negative actions. NAM: nicotinamide; INO: inosine; P2X: purinergic receptor 2X; P2Y: purinergic receptor 2Y; P1: purinergic receptor 1;

CD38 produced, Daratumumab, is in a phase I/II safety and dose finding study for MM therapy. Daratumumab is reported *in vitro* to kill either fresh MM or myeloma-derived cell lines by antibody-dependent cell-mediated cytotoxicity and by complement-dependent cytotoxicity (66). *In vivo*, Daratumumab has therapeutic effects at low doses in a SCID mouse model xenografted with a human myeloma (67). These results confirm that CD38 may be a crucial therapeutic target for the treatment of MM. The same target may also find clinical applications in $CD38^+$ CLL patients, whose clinical prognosis is frequently extremely poor or in patients with poor response to therapy. Murine anti-CD38 mAb as $F(ab')_2$ or Fab could also be used for arming drugs or nanoparticles with payloads varying according to the therapeutic strategies adopted, although this consideration lies outside the scope of this study.

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Abbreviations: Aa: amino acid; ADO: adenosine; ADPR: adenosine diphosphate ribose; AMP: adenosine monophosphate; ART: ADP ribosyl transferase; ATP: adenosine 5'-triphosphate; ATRA: all-*trans* retinoic acid; BM: bone marrow; BOM: osteomedullary biopsy; CLL: chronic lymphocytic leukemia; cADPR: cyclic adenosine diphosphate ribose; HPC: hematopoietic progenitor cell; Ig:

immunoglobulin; IP_3: inositol triphosphate; mAb: monoclonal antibody; LN: lymph node; NAD^+: nicotinamide adenine dinucleotide; $NAADP^+$: nicotinic acid adenine dinucleotide phosphate; $NADP^+$: nicotinamide adenine dinucleotide phosphate; OT: oxytocin; OTR: oxytocin receptor; PARP: poly ADP-ribose polymerase; RA: retinoic acid; RAR: retinoic acid receptor; RARE: retinoic acid response element; SNP: single nucleotide polymorphism.

Key Words: Bone marrow, Microenvironment, Multiple myeloma, CLL, Ectoenzyme, Review

Send correspondence to: Fabio Malavasi, Laboratory of Immunogenetics, Department of Medical Sciences, University of Torino Medical School, Via Santena 19, 10126 Torino, Italy. Tel: 39-011-696-1734; Fax: 39-011-696-6155; E-mail: fabio.malavasi@unito.it