ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
MARROW

Perspective

Unraveling the contribution of ectoenzymes to myeloma life and survival in the bone marrow niche

Valeria Quarona,[1] Valentina Ferri,[2] Antonella Chillemi,[1] Marina Bolzoni,[2] Cristina Mancini,[6] Gianluca Zaccarello,[1] Ilaria Roato,[1] Fabio Morandi,[4] Danilo Marimpietri,[4] Giuliano Faccani,[5] Eugenia Martella,[6] Vito Pistoia,[4] Nicola Giuliani,[2] Alberto L. Horenstein,[1] and Fabio Malavasi[1,3]

[1]Laboratory of Immunogenetics, Department of Medical Sciences and CeRMS, University of Torino, Torino, Italy. [2]Hematology and Blood and Marrow Transplantation (BMT) Center, University of Parma, Parma, Italy. [3]Transplantation Immunology, Città della Salute e della Scienza, Torino, Italy. [4]Laboratorio di Oncologia, Istituto Giannina Gaslini, Genova, Italy. [5]Section of Clinical Neurophysiology, CTO Hospital, Torino, Italy. [6]Anatomia e Istologia Patologica, Azienda Ospedaliero-Universitaria di Parma, Parma, Italy

Address for correspondence: Fabio Malavasi, Laboratory of Immunogenetics, Department of Medical Sciences, Via Santena 19, 10126 Torino, Italy. fabio.malavasi@unito.it

The bone marrow provides a protected environment for generating a vast array of cell types. Bones are thus a dynamic source of structural components and soluble factors used either locally or at a distance from their site of production. We discuss the role of ectoenzymes in the bone niche where human myeloma grows. Selected ectoenzymes have been tested for their ability to promote production of substrates involved in signaling, synthesis of growth factors and hormones, and modulation of the immune response. Because of the difficulty of simultaneously tracking all these activities, we narrow our focus to events potentially influencing synthesis of adenosine (ADO), an important regulator of multiple biological functions, including local immunological tolerance. Our working hypothesis, to be discussed and partially tested herein, is that CD38, and likely BST1/CD157—both NAD^+-consuming enzymes, are active in the myeloma niche and lead a discontinuous chain of ectoenzymes whose final products are exploited by the neoplastic plasma cell as part of its local survival strategy. Coadjuvant ectoenzymes include PC-1/CD203a, CD39, and CD73, which control the production of ADO. Results discussed here and from ongoing experiments indicate that the myeloma niche hosts the canonical, as well as alternative, pathways of ADO generation. Other possibilities are presented and discussed.

Keywords: ectoenzymes; CD38; mesenchymal stem cells; MSC; osteoblasts; osteoclasts; adenosine

Preferred citation:
Quarona, V., V. Ferri, A. Chillemi, *et al.* 2014. Unraveling the contribution of ectoenzymes to myeloma life and survival in the bone marrow niche. In "MARROW", ed. by M. Zaidi. *Ann. N.Y. Acad. Sci.* xxxx: 1–13.

Premise

Ectoenzymes comprise a large group of cell surface molecules endowed with pleiotropic functions. Considered an oddity until about 15 years ago, ectoenzymes now represent over 4% of known leukocyte surface molecules, a number that attests to their relevance in the economy of cell life. As the name indicates, ectoenzymes are membrane proteins that exert their catalytic function on the external surface of the cell membrane. A second function frequently attributed to ectoenzymes is their ability to modulate homotypic or heterotypic cell adhesion, a feature generally used for migration through tissues. Also, characteristic of ectoenzymes is their strict conservation in vertebrate phylogeny, which further highlights their evolutionary importance.[1]

doi: 10.1111/nyas.12485

Interest in ectoenzymes is twofold: while they are the main research interest of many basic scientists, clinicians tend to view them as useful markers for routine clinical investigations. Among the more familiar markers are CD38 and CD69 (used to monitor cell activation), CD26, CD73, CD39 (markers of immunity and inflammation), and prostate-specific membrane antigen (PSMA, a marker of neoplastic differentiation in the prostate). However, the use of ectoenzymes to monitor disease contributed to a paradigm shift in which the molecules ceased to be considered simply as biomarkers, and instead were seen as components of multistep enzymatic pathways with potential pathogenetic valences.[2,3]

Another feature characterizing ectoenzymes is the field of action of their products. Indeed, when the first surface molecules were recognized for their ability to transform an extracellular substrate, it was surprising that the final products of the reaction were bound for a cytoplasmic destination. In this sense, ectoenzymes represent a bridge between the environment, such as the blood stream or more secluded niches, and the intracellular milieu.[4]

The concept of a *microenvironment* began to gain attention when several human diseases were reported to display different traits according to their tissue localization. A good example is the leukemic cell, which tends to leave the circulation to expand in more protected sites. Chronic lymphocytic leukemia (CLL) cells, for example, are known to seek out a lymph node or spleen. Mobilization in the opposite direction, with leukemic cells reentering the circulation, was recently observed following the introduction of Bruton's tyrosine kinase (Btk) inhibitors in clinics.[5] This traffic to and from tissues is of extreme interest in medicine, and the molecules involved in the regulation of this traffic have become therapeutic targets.

Another feature of ectoenzymes is their unexpected ability to operate as components of a chained network of events, which involve substrates and products not necessarily derived from ectoenzymes located on the same cell. This mainly occurs when different cells coexist in a discrete environment and where substrates may be freely exchangeable from the same cells in different activation states or belonging to different lineages. This view led to the definition of a *discontinuous network*, which delayed an exact understanding of the real role played by ectoenzymes in physiology and a correct reading of the modifications caused by (or in inducing) diseases.[6]

Microenvironments are of potential interest for basic scientists and for clinical investigators. The bone marrow (BM) hosts a multipotent factory in the most protected sites of the body, a factory that produces cells with a vast array of differentiation potentials. Furthermore, bones have evolved beyond the role of simple mechanical element, becoming a dynamic source of structural components as well as soluble factors used locally or at a distance from the production site.[7]

Most of the steps required for optimal BM organization are now known. Still undefined is the functional role played by ectoenzymes in this context, even if the expression of some of them are relevant in the differentiation of the individual populations.

It is reasonable to assume that the role of ectoenzymes is more complex than currently appreciated, and that they are essential components governing the complicated BM cell society. We selected a panel of ectoenzymes analyzed for their ability to promote the production of substrates involved in signaling, synthesis of growth factors and hormones, and modulation of the immune response. Because of the difficulty of simultaneously tracking all of these activities, we narrowed our attention to events potentially influencing synthesis of adenosine (ADO), an important regulator of multiple biological functions, including local immunological tolerance.

To verify whether the ectoenzyme activities are functionally articulated and connected, preassessment was conducted in biological systems, where local tolerance is required, or where tolerance is one of the actions mediated by ADO. The first model tested was that of maternal–fetal interaction, where immunity controls tolerance using HLA-G products, their receptors, and soluble factors. The contributing role of ectoenzymes in maintaining homeostasis between two genetically different individuals was confirmed.[8]

These and other results were exploited in evaluating the potential roles of ectoenzymes in the context of multiple myeloma (MM), a neoplastic transformation of plasma cells. MM prevalently develops within the BM; some molecular aspects of the tumor transformation are known, as are some of the interactions taking place between myeloma and different cell components of the BM environment. Therapy

has improved dramatically in recent years, but the disease remains fatal.

Our working hypothesis is that CD38, and likely BST1/CD157—both NAD^+-consuming enzymes, are active in the myeloma niche and lead a chain of ectoenzymes whose final products are exploited by the neoplastic plasma cell as part of its local survival strategy. This hypothesis was suggested by the finding that CD38 and CD157 are both present in the niche together with CD31, a nonsubstrate CD38 counterreceptor.[9] Other coadjuvant ectoenzymes are PC-1/CD203a, CD39, and CD73, which control the production of ADO. To validate this working hypothesis, we commence by reviewing what is currently known about the expression of ectoenzymes in the context of the BM.

CD38 family

CD38 was first identified as a cell activation marker and later characterized as a receptor and a signaling molecule.[9] CD38 and CD157 were successively shown to be ectoenzymes by virtue of a marked similarity with the soluble enzyme ADP ribosyl cyclase purified from *Aplysia*, a mollusk distant 780 million years from *Homo sapiens*.[10] Belonging to a family of NAD^+-consuming enzymes, CD38 and CD157 are also endowed with the capacity to produce cyclic ADP ribose (cADPR), ADP ribose (ADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP). CD38 was then reported to be involved in the pathogenesis of different diseases, prevalently affecting the hematopoietic lineage (reviewed in Ref. 9). Results obtained with *Cd38*$^{-/-}$ mice indicated that CD38 plays a key role in the release of oxytocin (OT), a hormone involved in the control of various aspects of human behavior in addition to parturition and lactation.[2] ADO is conventionally produced through the concerted action of CD39, an ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1) that initiates the conversion of ATP to ADP and AMP, the substrate of CD73. ATP plays a central role in cell homeostasis and may vary quantitatively from tissue to tissue, depending also on the presence of specific P_2 receptors. The results of these multiple interactions forge an elastic and tunable regulatory system. An alternative route to ADO was recently identified in a human T cell line model, where CD38 operates along with PC-1/CD203a to generate ADO, bypassing the contribution of CD39.[11]

Our aim is to investigate whether the CD38/PC-1 (CD203a)/CD73 pathway is operational in the BM.[12] The role of CD38 in normal and malignant plasma cells has already received much attention, since both cell types express the highest surface density recorded for this protein. Indeed, that a terminally differentiated cell should express an activation marker at such high density was considered an exception in the scheme developed by the CD International Workshops on human cell surface molecules.

PC-1/CD203a

PC-1 was selected as a companion ectoenzyme in the pathway directed by CD38 in virtue of their overlapping features. Indeed, PC-1, or plasma cell-1, was first described in mouse models. In addition, PC-1 expression is detected in immunocompetent organs (thymus, lymph node, tonsil, and spleen), where it flanks CD38 in areas hosting crucial events in the life cycle of lymphocytes (Fig. 1).

Further interest in PC-1 was generated when the molecule was observed to play a role as an ectoenzyme in plasma membrane fractions isolated from liver and other tissues. This opened the way to the concept of multifunctional molecules: the enzymatic activity was reported as able to hydrolyze nucleotide pyrophosphate and phosphodiester bonds, and dual effects were carried out by the same enzyme. Eventually, these and other observations led to identification of plasma cell antigen Pca-1, later designated as PC-1.[13,14] Convergence of the worlds of enzymologists and immunologists did not occur until 1991, when ecto-phosphodiesterase was purified from mouse plasmacytoma cells and proved to be identical to PC-1.[15] Human PC-1 maintains the same enzymatic functions described in mouse.[15]

The family of ectonucleotide pyrophosphatases/phosphodiesterases (ENPPs) consists of seven structurally related ectoenzymes. ENPPs were only known to hydrolyze inorganic pyrophosphate (PPi) or phosphodiester bonds in (di)nucleotides and their derivatives.[16] However, other extracellular molecules with PPi or phosphodiester bonds have recently been reported to be ENPP substrates.[17] Current evidence suggests that ENPPs play multiple physiological roles, including in recycling of nucleotides, modulation of purinergic receptor signaling, regulation of extracellular PPi levels, stimulation of cell motility, regulation of insulin receptor signaling, and activation of ectokinases.[17] ENPPs

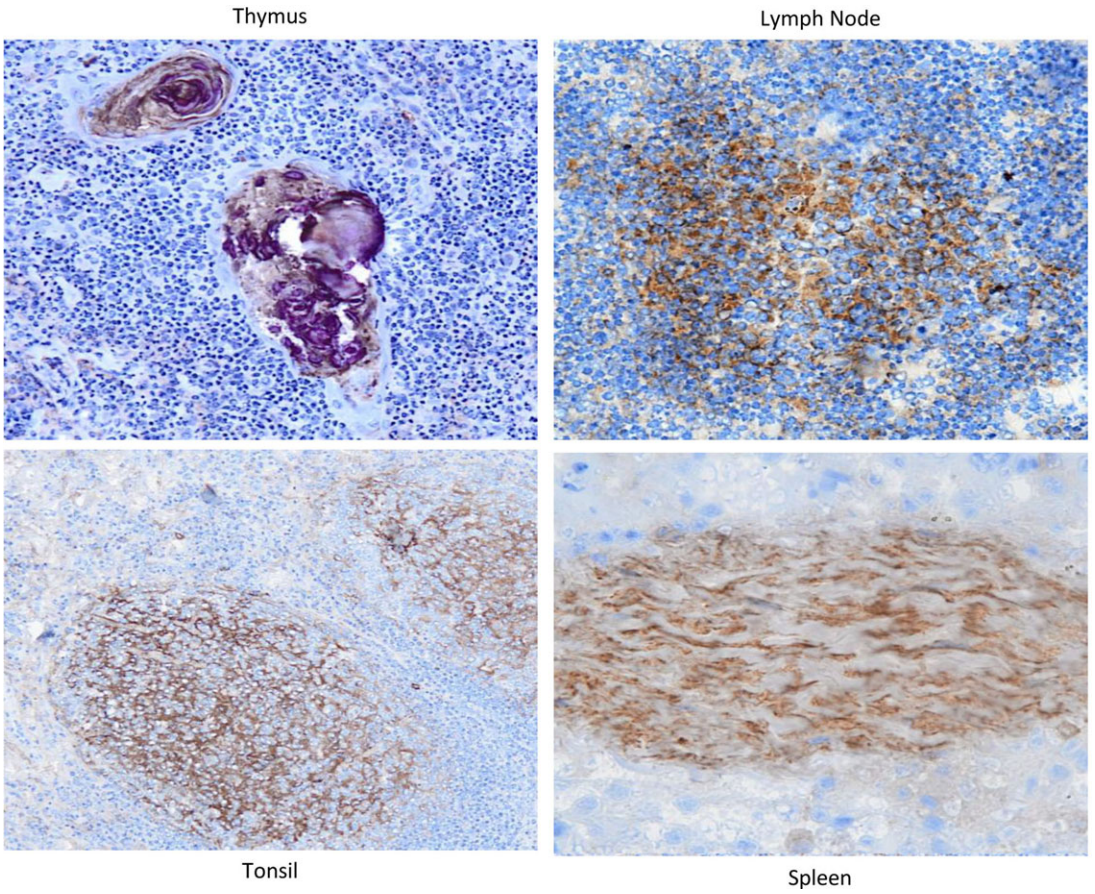

Figure 1. **Initial attempt to link enzymatic activities and lymphocyte functions in tissues and organs, where compartmentalization is instrumental in monitoring differentiation. Analysis of PC-1/CD203a expression in the major lymphoid organs (thymus, spleen, and lymph node) suggests that it plays an important function in these organs within the context of the complex events taking place prevalently in germinal centers. This function has yet to be elucidated in depth. The PC-1/CD203a protein is localized on the surface of stromal cells in the samples of normal spleen analyzed; the thymus was less informative, where PC-1/CD203a is apparently localized within Hassall's corpuscles. Results on PC-1/CD203a will be part of a large body of data reviewed in Ref. 69.**

are reported to be aberrantly expressed in several pathologies; however, the underlying mechanism(s) in physiology and pathology largely remain to be determined.[13,18]

CD39

The purinergic system is an evolutionarily conserved signaling pathway that finely tunes immune cell functions, such as cell–cell interaction, cytokine and chemokine secretion, shedding of surface antigen, removal of intracellular pathogens, and generation of reactive oxygen species.[19] Purinergic mediators, mainly ATP and ADO, are released into the extracellular space in response to metabolic alterations or stress and function simultaneously as sensory and efferent signals to shape immune responses. The ectoenzymatic activities of CD39 and CD73 play strategic roles in calibrating the duration, magnitude, and chemical nature of purinergic signals delivered to immune cells through the conversion of ATP/ADP to AMP and AMP to ADO.[20]

CD39 is an integral membrane protein that hydrolyzes ATP (and ADP, albeit less efficiently), in a Ca^{2+}- and Mg^{2+}-dependent manner, to yield AMP.[21] CD39 is constitutively expressed on the cell surface in spleen, thymus, lung, and placenta. CD39 is also localized on endothelial cells and in immune cell populations, such as B cells, natural killer (NK) cells, dendritic cells (where it is regulated by IL-27; see Ref. 22), Langerhans cells, monocytes,

macrophages, mesangial cells, neutrophils, and regulatory T and B cells.[23,24] CD39 expression is regulated by several proinflammatory cytokines, oxidative stress, and hypoxia through different transcription factors.[25,26] Expression of CD39 is upregulated in several solid tumors (colorectal cancer, head and neck cancer, and pancreatic cancer) as well as in CLL,[20] suggesting that this enzyme is also involved in the development and progression of malignancies.[27] CD39 also exists as soluble molecule that is functionally active.[28]

CD73

The second step in the metabolism of purine nucleotides is catalyzed by CD73, which dephosphorylates extracellular AMP to ADO. CD73, a 70 kDa dimer anchored to the plasma membrane through a glycosylphosphatidyl inositol (GPI) link, also exists in a soluble form that is functionally active.[29] CD73 is expressed by leukocytes from peripheral blood, spleen, lymph nodes, thymus, and BM as well as colon, brain, kidney, liver, lung, and heart. CD73 expression is regulated by proinflammatory products, including interferons, TNF-α, IL-1β, TGF-β, and prostaglandins. CD73 expression also marks a step in thymic differentiation, induced by TCR ligation.[30] Hypoxic conditions increase surface expression of CD73.[31] These findings attracted the attention of basic scientists and clinicians who focused on the possibility that CD73 might direct an immunosurveillance escape mechanism, adopted by different tumor cells, especially in closed systems or during metastasization. This point was indirectly confirmed when observations from the clinical laboratory routine showed that some tumors were characterized by an ectopic expression of CD73. Results derived from animal models *in vivo* confirmed a direct role of CD73 in tumor growth and metastasization.[32,33] Further confirmation came from the *in vivo* use of anti-CD73 mAb therapy in mice transplanted with human tumors.[34]

Adenosine

Primarily a product of ATP metabolism, ADO exerts pleiotropic functions in the human body. Besides the effects on the central nervous and cardiovascular systems, ADO potently influences inflammation by generally depressing immune responses.[35] The modalities of action exerted by regulatory T and B lymphocytes, and probably by myeloid-derived suppressor cells (MDSCs),[36] include the production of ADO. Intracellular ADO, which derives from the hydrolysis of *S*-adenosyl homocysteine or from increased intracellular metabolism of ATP during cellular stress, is released prevalently through equilibrative transporters. Once outside the cell, ADO levels increase as a result of the extracellular catabolism of released adenine nucleotides (ATP, NAD^+).[37]

ADO initiates its biological effects via four adenosine receptor P_1 subtypes, namely, A1, A2A, A2B, and A3. A1 and A2A receptors possess high affinity for ADO, while the A2B and A3 receptors are characterized by lower affinities.[38] These receptors, members of the superfamily of G protein-coupled receptors (GPCRs), display a seven transmembrane α-helical structure, with an extracellular N-terminus and an intracellular C-terminus. The N-terminal domain influences trafficking of the receptor to the plasma membrane.[39,40] The C-terminus serves as phosphorylation site for protein kinases and enables receptor desensitization. Furthermore, the C-terminus and the third intracellular loop couple the receptors to G proteins.[41]

ADO receptors are classified on the basis of their differential coupling to adenylyl cyclase to regulate intracellular cyclic AMP (cAMP) levels. A2A and A2B are coupled to stimulatory G proteins (G_s), while A1 and A3 are coupled to $G_{i/0}$ inhibitory proteins. Therefore, A2A and A2B typically suppress cell responses by increasing cAMP production. By contrast, cAMP levels are decreased after cell activation promoted through A1 and A3 receptors.[19,41]

Hypothesis and initial confirmations

The preceding observations were pooled together to create a model according to which human myeloma supports, either directly or indirectly, the production of ADO. In this model, ADO is obtained from ATP and/or NAD^+, which undergo reaction by a chain of ectoenzymes that includes CD38 and CD157, as well as PC-1/CD203a, CD39, and CD73. The conventional CD39/CD73 pathway may operate independently or in synergy with the CD38-CD157/PC-1(CD203a)/CD73 pathway. According to this view, NAD^+ flows through an ectonucleotidase cascade and culminates, in the myeloma cell niche, in the production of ADO. In turn, ADO would bind to specific purinergic receptors expressed by mesenchymal stem cells (MSCs) and bone cells. The expected outcome would be the

Table 1. Immunophenotype of cell lines established from patients with plasma cell leukemia (BF01) or multiple myeloma (LP-1 and DL06)[a]

Cell line	CD38	CD157	PC-1/CD203a	CD39	CD73	CD31	OTR	HLA Class I	Secretion
BF01	+	−	−	+	−	+	+	+	κ
LP-1	+	−	−	−	−	+/−	+	+	λ
DL06	+	−	−	−	−	−	+	+	λ

[a]BF01 maintained a phenotype more similar to that of the mature plasma cell (HLA Class II$^-$, secreting κ chain). As expected, all lines expressed CD38, while CD39 was only detected in the line with a mature phenotype. The OTR is expressed by all the lines. Phenotype was obtained by staining cells with specific mAbs followed by FITC-labeled anti-mouse Ig.

delivery of relevant and specific biological signals. The assumption is that the CD38/PC-1(CD203a)/CD73 pathway serves to balance the local concentrations of NAD^+ and ADO, hence determining the hierarchical engagement of the P2 or P1 receptors. The main functional feature of this adenosinergic signaling axis is its difference from the canonical ectonucleotidase pathway ruled by CD39. Indeed, NAD^+ hydrolysis by CD38 generates adenosine diphosphate ribose (ADPR) (either directly or through a cADPR intermediate), which is subsequently converted to AMP and finally to ADO.[11]

Besides its immunosuppressive functions, ADO is also a growth factor for different cell types, including osteoblasts and osteoclasts.[42] It is also reasonable to expect that ADO may be instrumental in creating niches that disrupt (or significantly influence) the behavior of MSCs.

The presence of a chain of selected ectoenzymes was evaluated in the major populations of cells forming the myeloma niche. The first step was to check whether the components of the ectoenzymatic network were present in BM samples from human myeloma patients and in which cell populations, an approach not yet undertaken as a whole. Ectoenzymes were previously tested individually or as markers of single populations.

The next step was to evaluate whether the major cell populations of the myeloma niche express the panoply of related ectonucleotidases, either on the surface of the same cells (in *cis*) or on different, but adjacent cells (in *trans*). Lastly, we asked whether these enzymes are functionally competent to metabolize different nucleotides to generate ADO.

To support the working hypothesis, the ectoenzymes under study were also analyzed on a panel of human myeloma-like cell lines. This panel was complemented by establishing new cell lines derived from the most aggressive cases of myeloma and plasma cell leukemia. The basic idea was to reconstitute *in vitro* a niche-mimicking environment similar to that in which myeloma cells grow *in vivo.*

Analysis of the distribution of ectoenzymes in the major cell populations of the myeloma niche along with the final products obtained will be the matter of further detailed work. We report here the main conclusions coming from a narrative view of the role played by ectoenzymes in the myeloma niche. Myeloma cells are known to express high amounts of CD38. CD157 expression was constantly undetected. Also not expressed is PC-1/CD203a, the ectoenzyme responsible of the generation of AMP from ATP and/or ADPR. The lack of expression of human PC-1 in myeloma cells, at variance with results obtained in mice, still is an intriguing issue. Myeloma cells are surface CD39- and CD73-negative. CD31, one of the CD38 nonsubstrate ligands, is expressed by approximately 80% of myeloma plasma cells, confirming an observation made some years ago but still difficult to interpret.[43] Extending the analysis to the endothelial cells in the myeloma niche confirms the presence of CD31, a surface molecule also known as platelet endothelial cell adhesion molecule-1 (PECAM-1), also responsible for homotypic adhesion. These results were confirmed in human cell lines derived from myelomas or plasma cell leukemias, models for the expansion of these studies. The results are collected and summarized in Table 1.

MSCs are a heterogeneous population that proliferates *in vitro* as plastic-adherent cells; they have fibroblast-like morphology, forms colonies, and can differentiate into bone, cartilage, and fat cells. MSCs can be obtained from almost every type of connective tissue.[44] Those obtained from normal or myeloma BM may influence normal and neoplastic

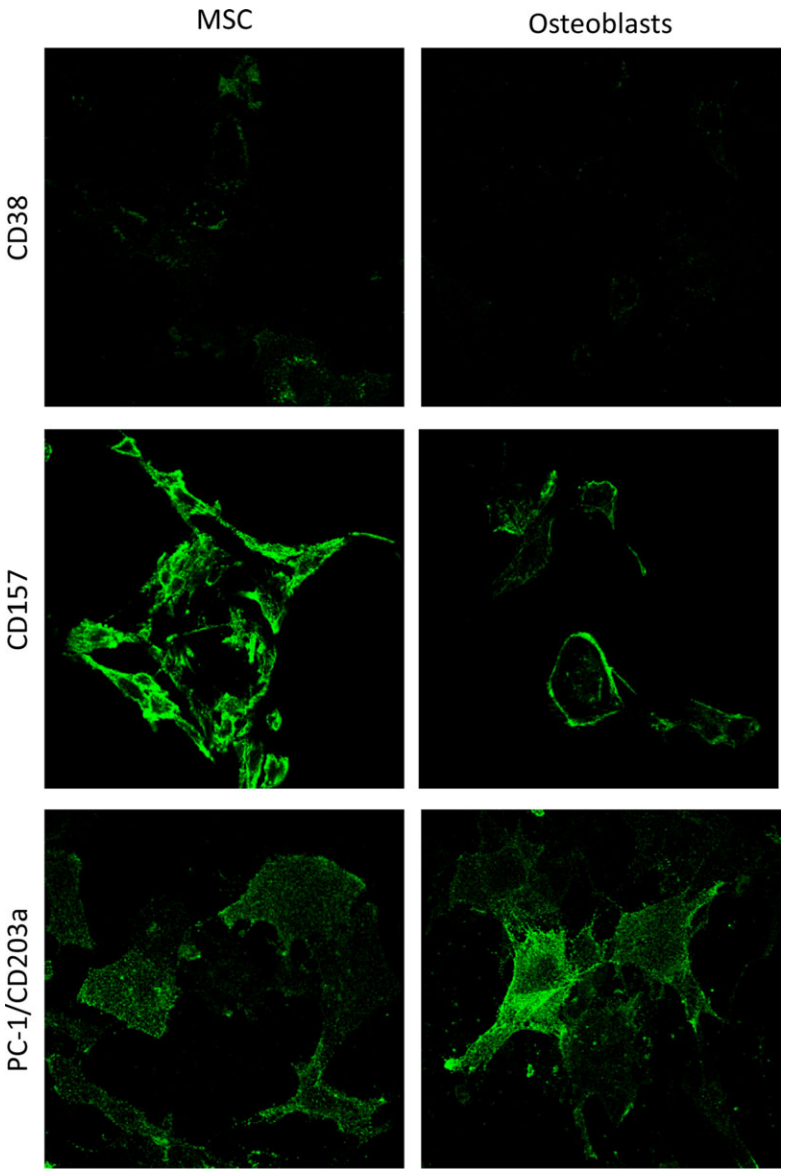

Figure 2. **Analysis of the expression of CD38, CD157, and PC-1/CD203a by MSCs during osteoblast differentiation. These three ectoenzymes were selected because they are responsible for driving the discontinuous ectoenzyme network leading to ADO production in the myeloma niche. CD38 and CD157 expression decreased significantly, while surface PC-1/CD203a markedly increased. MSCs were obtained from cultures derived from surgical samples of human knee, spinal column hemilamina, and slipped disk, stained with specific mAbs and then FITC-labeled anti-mouse Ig. Preparations were analyzed with an Olympus FV300 laser scanning confocal microscope equipped with blue argon (488 nm) and green helium neon (543 nm) lasers. Results were elaborated using FluoView 300 software (Olympus Biosystems, Hamburg, Germany).**

cell growth through signals conveyed by adhesion, by exosomes or by soluble factors. The dynamics of MSCs during myeloma transformation could either proceed through the normal steps of the MSC life or, alternatively, myeloma might reshape its niche by exploiting the existing mechanisms according to its requirements.

Osteoblastic differentiation is characterized by stability of surface CD73 (one of the reference markers of the population). Indeed, CD73 expressed

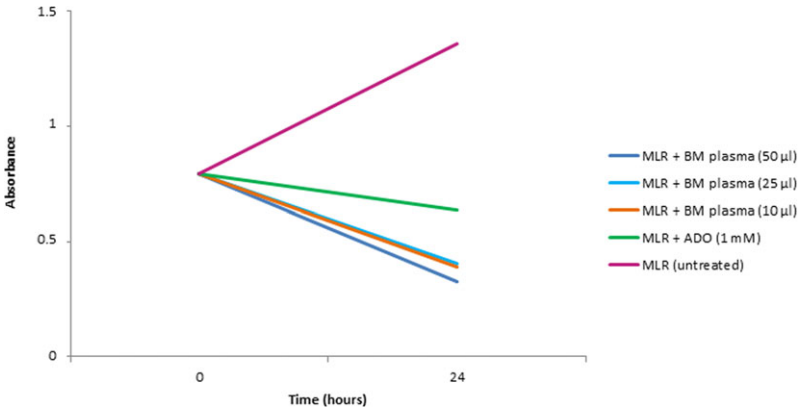

Figure 3. **The functionality of the ADO produced in BM plasma of myeloma patients was confirmed after monitoring the effects induced in mixed lymphocyte reactions (MLRs). MLRs were obtained by cocultivating peripheral blood mononuclear cells (PBMCs) obtained from 3 healthy donors (1 male, 2 females). The cultures were added with freshly obtained BM plasma samples (amounts ranging from 50 to 10 µl) with known ADO levels. Controls included synthetic ADO and normal plasma. Proliferation was monitored by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma, Milano, Italy), and results were analyzed with a BioRad Microplate Reader, Model 680 (BioRad, Milano, Italy).**

by osteoblasts was maintained at high density. In contrast, CD38 (and prevalently CD157) significantly decreases their surface expression after differentiation to osteoblasts, although this is balanced by an increase in PC-1/CD203a (Fig. 2). This molecule is found on surface osteoblasts as an established regulator of tissue mineralization by increasing extracellular levels of PPi derived from ATP metabolization.[45] CD39 was constantly undetected on the surface of differentiated or cultured MSCs.

The conclusions of our observations are that MSCs are equipped with ectoenzymatic machinery, potentially leading to the production of ADO. This may occur via the conventional CD39/CD73 pathway, but most likely the discontinuous CD38/PC-1/CD73 network is a suitable system for producing ADO. The abundant expression of CD73 on the cell surface suggests that the 5′-ectonucleotidase activity is simultaneously exploited for AMP dephosphorylation and final demolition of ATP.

Our understanding of the purinergic machinery involved in the conversion/signalling sequence of extracellular nucleotides[46] was recently advanced by the observation that ADO can be obtained from the pyridine nucleotide NAD^+.[11] The relevant characteristics of the ADO-producing network (CD38/PC-1(CD203a)/CD73) are that it is functional and its components may be spatially located on different (*trans*) but interacting cells. The latter event may be facilitated in restricted cellular microenvironment, such as the myeloma niche.

As BM plasmatic fluid provides a direct physical connection linking all the different cell components of the myeloma niche, its composition was analyzed in some detail to validate the hypothesis that myeloma cells exploit the products of selected ectoenzymes to create a protective microenvironment.

After first demonstrating the existence of this novel ectoenzymatic pathway by analysis of the expression and distribution of the ectoenzymes, the next step of the working hypothesis was to show that ADO is generated extracellularly in a myeloma niche containing cells expressing CD38, PC-1/CD203a, and CD73. This adenosinergic pathway bypasses the canonical CD39 pathway in the formation of AMP, the substrate of the ADO-generating CD73/5′-nucleotidase ectoenzyme.

Plasma fluids present in the myeloma niche were used to demonstrate part of the working hypothesis. Initial results demonstrate that ADO is present in the plasma samples, where the cells expressing the main ectoenzymatic players involved in the generation of the nucleoside are exposed (Horenstein, A.L., 2014, unpublished). ADO generated by AMP hydrolysis may accumulate in BM plasma, where its destiny is either to be uptaken by specific P1 cell receptors or to be inactivated at the cell surface by the ADA/CD26 complex, which converts ADO to

Table 2. Effects mediated by ADO and its receptors in human osteoblasts and osteoclasts[a]

Receptor	Activation	Block	Osteoblasts	Osteoclasts
A_1			↑late stage proliferation	↑differentiation[b]
	+	+	—	↑bone density
A_{2A}			↑late stage differentiation[c]	↓differentiation[b]
	+	+	—	↓bone density
A_{2B}			↑early stage differentiation	—
	+	+	—	↓bone density

[a]Modified from Ref. 70.
[b]Differentiation of myeloid cell into osteoclasts
[c]Differentiation of MSC into osteoblasts

inosine. In addition, ADO may also be internalized by nucleoside transporters.[47,48] Quantitative evaluation of ADO is still to be defined, also considering the nature and the extremely short half-life of ADO *in vivo*. The plasma fluids containing ADO were added to a mixed lymphocyte reaction (MLR) consisting of cocultivated PBMCs from healthy individuals;[49] the plasma fluids were able to inhibit alloreactivity and MLR proliferation (Fig. 3). This likely happens through the regulation of cAMP consequent to activation of adenylyl cyclase.[50]

These observations support the conclusion that the myeloma niche is equipped with a set of ectoenzymes, which include two dedicated pathways leading to the production of ADO through continuous and discontinuous modalities. An important issue to answer is whether there is a correlation between quantitative ectoenzyme expression and ADO concentration in BM plasma. Also critical is the fate in this environment of ADO, characterized by a very short half-life *in vivo*. This is a direct function of the complexity of the ADO receptors, their tissue distribution, and modulation. The main characteristics of the ADO receptors family are summarized in Table 2.

Conclusions

Here, we provide evidence suggesting that the CD38/PC-1(CD203a)/CD73 ectoenzymatic network is active in the myeloma niche, where it may lead to the production of functional ADO. The effect seems to be specific for myeloma. This finding is an important step in evaluating the contribution of ectoenzymes active in the production of ADO *in vivo*. Other key questions remain to be answered before designing a dependable model, where CD38 is adopted by myeloma as a picklock for evading immune defenses.

An appealing hypothesis is that myeloma cells usurp the normal organization of bone, replacing it with a niche that maximizes local growth and protection from immune defenses. In line with this hypothesis is the observation that myeloma and surrounding cells are endowed with an ectoenzymatic pathway potentially leading to ADO production. The CD38/CD157/PC-1(CD203a)/CD73 pathway appears to be highly efficient in the bone niche. The molecular circuit proposed here relies upon proteins that do not need to be ectopically expressed, a major difference from other models of immune evasion observed in breast or colon cancers, which are dependent on *de novo* expression of CD73.[32,33,51] Unlike these other tumor models, the myeloma niche is a closed system, which defines an environment that protects the neoplastic cells and enables exchange of ectoenzyme substrates and products.

ADO levels observed in the BM plasma samples analyzed seem to be variable. This may reflect either variations in the number of molecules of surface enzymes or the fact that they are shed in biological fluids. Genetic polymorphisms differentially influence surface expression of the components of the ectoenzyme network. Expression of *Cd38* is regulated by a single nucleotide polymorphism (SNP) located in intron 1 (rs6449182; C>G variation) and this sequence is a binding site for the E2A transcription factor.[52] The same transcription factor is involved in osteoclast maturation and survival in homeostatic bone remodeling, at least in murine models.[53]

Less is known about the regulation of CD157. Also, worth investigating is whether different

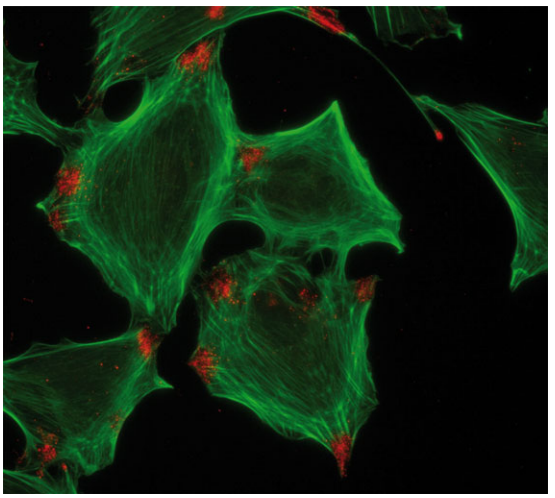

Figure 4. Besides myeloma and myeloma cell lines, the oxytocin receptor (OTR) is also expressed by human osteoblasts. These were stained with the CB-OTR mAb (in red), while actin microfilaments are in green (courtesy of A. Zallone, University of Bari, Italy). The roles of OT remain to be defined in detail in both cell lineages.

genotypes are associated with a similar release of CD38 and CD157 in soluble form or as exosomes (mini-cells where a set of proteins is maintained intact and functional).[54] Other components of possible heterotypic cross-talk not mediated by cell adhesion are soluble CD39 and CD73, presently under analysis.

Likely more relevant in the context of myeloma is that quantitative changes in ectoenzyme expression may derive from external regulation, which is amplified in the myeloma niche. IL-6, IL-7, and TNF-α are the soluble factors that most directly (positively or negatively) influence CD38 as expressed by different tissues and myeloma. Another possibility is that ADO is differentially consumed by surrounding or infiltrating cells. These issues are under scrutiny, considering the intrinsic complexity of simultaneous dealing of different functionally connected cells. Figure 4 depicts the hypothetical model considered in our discussion. Another point to be determined is the fate of ADO, a key factor in the survival of myeloma cells and/or resistance to drugs.

Not considered here, but tightly linked to the focus of this discussion, are interactions between ectoenzymes and their products with lymphocytic and myeloid populations that have immunosuppressive characteristics. There is now broad consensus that MDSCs are significantly increased in the BM and periphery of MM patients. In addition, MDSCs induces the growth of MM cells, while suppressing T cell–mediated immune responses.[55] The effects exerted by MDSCs appear to be variably linked with disease status, although they invariably increase with disease progression.[56] Another feature reported for this cell population that is difficult to fit into a model is their ability to transform into osteoclasts.[57]

In conclusion, MDSCs are more numerous and stronger in MM patients than in age-matched controls. Indeed, MDSCs increase with age.[56] The relationship between MDSCs and T_{reg} cells is the subject of limited and also conflicting reports.[58] One recent report showed that MDSCs purified from MM patients are able to upregulate T_{reg} cells more efficiently than age-matched controls; further, this effect is cell–cell contact dependent.[56] In any case, MDSCs are potent inhibitors of T cell proliferation. Understanding the role of NK cells in the MM environment is another challenge.

An additional area of potential interest that has not yet been investigated is the role of oxytocin in myeloma. This link stems from the report that CD38 regulates the release of OT,[2] a hormone with multiple activities.[59] It is still not known whether OT is involved in the growth of myeloma and/or bone cells, or if it influences the local immune response. The production of OT is not limited to the neurohypophysis, and its action is mediated by interaction with a specific OT receptor (OTR) that is widely distributed in different tissues. An ad hoc produced mAb (CB-OTR) has made it possible to extend the analysis of OTR expression in the myeloma niche.[60] As part of the initial phase of a new project, we have observed that normal plasma cells and myeloma as well as osteoblasts and osteoclasts express OTR (Fig. 4).

Furthermore, BM osteoblasts produce abundant OT, suggesting that locally released OT may be an autocrine regulator of bone formation and mass.[61] OT produced by osteoblasts in response to estrogen stimulates the release of OT, which, in turn, amplifies the action of estrogens. Physiologically, OT is not referred to as a downstream mediator of the action of estrogen on bone. The OT autocrine circuit is thought to play a role in coordinating the bone-forming activities of neighboring osteoblasts.[62,62] Indirect support that the hormone plays a role

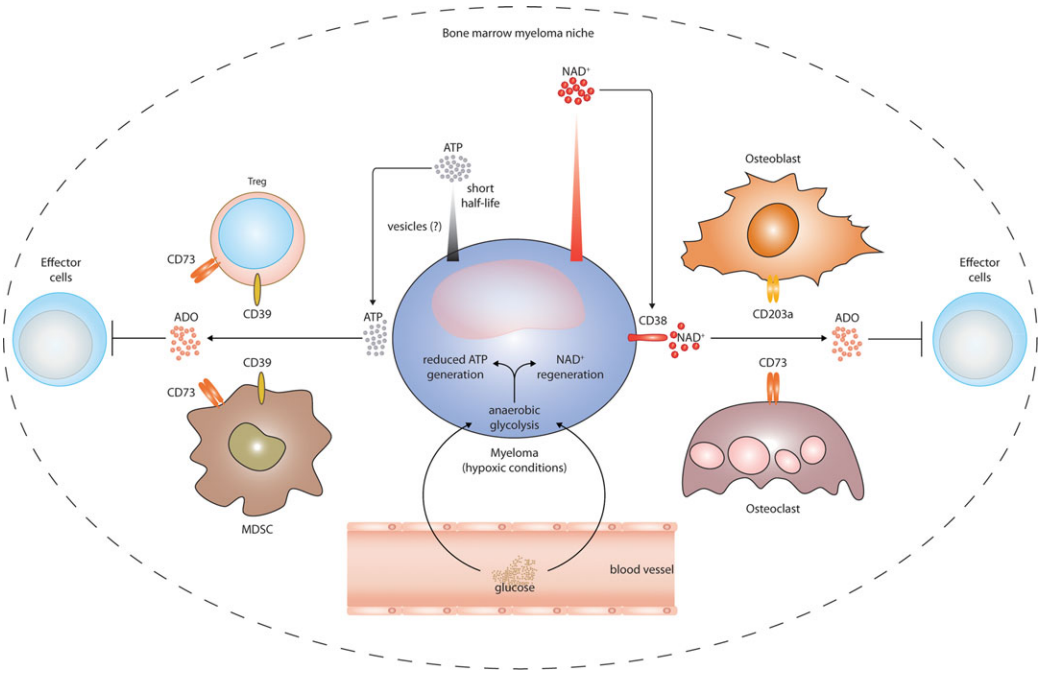

Figure 5. Schematic model summarizing key events controlled by ectoenzymes in the myeloma niche and that lead to the local production of ADO. The hypoxic conditions trigger anaerobic glycolysis, with reduced production of ATP and simultaneous increase in NAD^+ generation. ATP is the substrate for the canonical CD39/CD73 pathway. NAD^+ is the substrate activating the alternative pathway, where the CD38 substrate is converted to the final product, ADO, via PC-1/CD203a and CD73. The effects exerted by ADO on T_{reg} cells and MDSCs are partially speculative.

in the BM environment comes from findings that *Cd38*$^{-/-}$ and *Bst*$^{-/-}$ (CD157 deficient) models show decreased levels of OT.[63,64] Data on the relationship between OT and ADO are extremely scanty. However, a recent report indicates that ADO stimulates or modulates the release of OT, at least in the hypothalamus.[65]

The model we present is one step in an individualized strategy adopted by myeloma cells to evade the intricate network of immune defenses by subverting normal physiological mechanisms for its own needs in pathology (Fig. 5).

A hypothesis relevant for translational medicine is that ADO levels in the myeloma niche might be an early indicator of an aggressive form of the disease. Consequently, ADO levels could become a valuable prognostic marker in myeloma. This issue can be evaluated in BM niches recently presented in humanized mice.[66] Finally, the local presence and functions of ADO lends support to the use of anti-CD38 mAbs in myeloma therapy.[67] In this case, the mAb would exert direct cytotoxic functions on cells expressing the molecule, simultaneously depressing or blocking the enzymatic activities of CD38 and related ectoenzymes to produce ADO.[68]

Acknowledgments

This work was supported by grants from PRIN (Ministry of Education, University, and Research) and FIRB (Fondo per gli Investimenti della Ricerca di Base) (both in Rome, Italy); Fondazione CRT and Fondazione Ricerca Molinette (both in Torino, Italy); Contributi "5 × 1000 dell'IRPEF-Finanziamento della ricerca sanitaria"; and from Finanziamento Ricerca Corrente, Ministero Salute (contributo per la ricerca intramurale) to Istituto Giannina Gaslini, and AIRC Grant IG 13003 (Milano, Italy). Antonella Chillemi and Valeria Quarona are students in the PhD Program in Biomedical Sciences and Oncology, University of Torino. Valentina Ferri is a student in the PhD Program in Cell and Molecular Biology, University of Insubria. Thanks are given to Prof. R. Albera (Department of Surgical Sciences, University of Torino) and Dr. E. Aidala (Department of Pediatric Surgery, Regina Margherita Hospital,

Torino) for providing surgical samples of tonsils and thymuses. Thanks are also given to Dr. Enza Ferrero and Dr. Laura McLean for a stylistic revision of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Goding, J.W. & M.C. Howard. 1998. Ecto-enzymes of lymphoid cells. *Immunol. Rev.* **161:** 5–10.
2. Jin, D. *et al.* 2007. CD38 is critical for social behaviour by regulating oxytocin secretion. *Nature* **446:** 41–45.
3. Malavasi, F. *et al.* 2011. CD38 and chronic lymphocytic leukemia: a decade later. *Blood* **118:** 3470–3478.
4. Malavasi, F. *et al.* 2006. CD38 and CD157 as receptors of the immune system: a bridge between innate and adaptive immunity. *Mol. Med.* **12:** 334–341.
5. Hendriks, R.W., S. Yuvaraj & L.P. Kil. 2014. Targeting Bruton's tyrosine kinase in B cell malignancies. *Nat. Rev. Cancer* **14:** 219–232.
6. Quarona, V. *et al.* 2013. CD38 and CD157: a long journey from activation markers to multifunctional molecules. *Cytometry B Clin. Cytom.* **84:** 207–217.
7. Yin, T. & L. Li. 2006. The stem cell niches in bone. *J. Clin. Invest.* **116:** 1195–1201.
8. Cecati, M. *et al.* 2013. Contribution of adenosine-producing ectoenzymes to the mechanisms underlying the mitigation of maternal-fetal conflicts. *J. Biol. Regul. Homeost. Agents* **27:** 519–529.
9. Malavasi, F. *et al.* 2008. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. *Physiol. Rev.* **88:** 841–886.
10. Aarhus, R. *et al.* 1995. ADP-ribosyl cyclase and CD38 catalyze the synthesis of a calcium-mobilizing metabolite from NADP. *J. Biol. Chem.* **270:** 30327–30333.
11. Horenstein, A.L. *et al.* 2013. A CD38/CD203a/CD73 ectoenzymatic pathway independent of CD39 drives a novel adenosinergic loop in human T lymphocytes. *Oncoimmunology* **2:** e26246.
12. Chillemi, A. *et al.* 2014. CD38 and bone marrow microenvironment. *Front Biosci. (Landmark Ed).* **19:** 152–162.
13. Takahashi, T., L.J. Old & E.A. Boyse. 1970. Surface alloantigens of plasma cells. *J. Exp. Med.* **131:** 1325–1341.
14. Abney, E.R., W.H. Evans & R.M. Parkhouse. 1976. Location of nucleotide pyrophosphatase and alkaline phosphodiesterase activities on the lymphocyte surface membrane. *Biochem. J.* **159:** 293–299.
15. Buckley, M.F. *et al.* 1990. Plasma cell membrane glycoprotein PC-1. cDNA cloning of the human molecule, amino acid sequence, and chromosomal location. *J. Biol. Chem.* **265:** 17506–17511.
16. Kato, K. *et al.* 2012. Crystal structure of Enpp1, an extracellular glycoprotein involved in bone mineralization and insulin signaling. *Proc. Natl. Acad. Sci. U. S. A.* **109:** 16876–16881.
17. Goding, J.W. 2000. Ecto-enzymes: physiology meets pathology. *J. Leukoc. Biol.* **67:** 285–311.
18. Goding, J.W., B. Grobben & H. Slegers. 2003. Physiological and pathophysiological functions of the ecto-nucleotide pyrophosphatase/phosphodiesterase family. *Biochim. Biophys. Acta* **1638:** 1–19.
19. Junger, W.G. 2011. Immune cell regulation by autocrine purinergic signalling. *Nat. Rev. Immunol.* **11:** 201–212.
20. Antonioli, L. *et al.* 2013. CD39 and CD73 in immunity and inflammation. *Trends Mol. Med.* **19:** 355–367.
21. Heine, P. *et al.* 2001. The C-terminal cysteine-rich region dictates specific catalytic properties in chimeras of the ectonucleotidases NTPDase1 and NTPDase2. *Eur. J. Biochem.* **268:** 364–373.
22. Mascanfroni, I.D. *et al.* 2013. IL-27 acts on DCs to suppress the T cell response and autoimmunity by inducing expression of the immunoregulatory molecule CD39. *Nat. Immunol.* **14:** 1054–1063.
23. Dwyer, K.M. *et al.* 2007. CD39 and control of cellular immune responses. *Purinergic Signal.* **3:** 171–180.
24. Saze, Z. *et al.* 2013. Adenosine production by human B cells and B cell-mediated suppression of activated T cells. *Blood* **122:** 9–18.
25. Eltzschig, H.K. *et al.* 2009. Central role of Sp1-regulated CD39 in hypoxia/ischemia protection. *Blood* **113:** 224–232.
26. Chalmin, F. *et al.* 2012. Stat3 and Gfi-1 transcription factors control Th17 cell immunosuppressive activity via the regulation of ectonucleotidase expression. *Immunity* **36:** 362–373.
27. Bastid, J. *et al.* 2013. ENTPD1/CD39 is a promising therapeutic target in oncology. *Oncogene* **32:** 1743–1751.
28. Yegutkin, G.G. *et al.* 2012. Metabolism of circulating ADP in the bloodstream is mediated via integrated actions of soluble adenylate kinase-1 and NTPDase1/CD39 activities. *FASEB J.* **26:** 3875–3883.
29. Heuts, D.P. *et al.* 2012. Crystal structure of a soluble form of human CD73 with ecto-5′-nucleotidase activity. *Chembiochem* **13:** 2384–2391.
30. Coffey, F. *et al.* 2014. The TCR ligand-inducible expression of CD73 marks gammadelta lineage commitment and a metastable intermediate in effector specification. *J. Exp. Med.* **211:** 329–343.
31. Thompson, L.F. *et al.* 2004. Crucial role for ecto-5′-nucleotidase (CD73) in vascular leakage during hypoxia. *J. Exp. Med.* **200:** 1395–1405.
32. Stagg, J. 2012. The double-edge sword effect of anti-CD73 cancer therapy. *Oncoimmunology* **1:** 217–218.
33. Zhang, B. 2012. CD73 promotes tumor growth and metastasis. *Oncoimmunology* **1:** 67–70.
34. Terp, M.G. *et al.* 2013. Anti-human CD73 monoclonal antibody inhibits metastasis formation in human breast cancer by inducing clustering and internalization of CD73 expressed on the surface of cancer cells. *J. Immunol.* **191:** 4165–4173.
35. Antonioli, L. *et al.* 2013. Immunity, inflammation and cancer: a leading role for adenosine. *Nat. Rev. Cancer* **13:** 842–857.
36. Waldron, T.J. *et al.* 2013. Myeloid derived suppressor cells: Targets for therapy. *Oncoimmunology* **2:** e24117.
37. Deussen, A., M. Borst & J. Schrader. 1988. Formation of S-adenosylhomocysteine in the heart. I: An index of free intracellular adenosine. *Circ. Res.* **63:** 240–249.

38. Fredholm, B.B. *et al.* 2011. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors—an update. *Pharmacol. Rev.* **63:** 1–34.
39. Baldwin, J.M. 1994. Structure and function of receptors coupled to G proteins. *Curr. Opin. Cell Biol.* **6:** 180–190.
40. Schoneberg, T., A. Schulz & T. Gudermann. 2002. The structural basis of G-protein-coupled receptor function and dysfunction in human diseases. *Rev. Physiol. Biochem. Pharmacol.* **144:** 143–227.
41. Sheth, S. *et al.* 2014. Adenosine receptors: expression, function and regulation. *Int. J. Mol. Sci.* **15:** 2024–2052.
42. Takedachi, M. *et al.* 2012. CD73-generated adenosine promotes osteoblast differentiation. *J. Cell Physiol.* **227:** 2622–2631.
43. Vallario, A. *et al.* 1999. Human myeloma cells express the CD38 ligand CD31. *Br. J. Haematol.* **105:** 441–444.
44. Uccelli, A., L. Moretta & V. Pistoia. 2008. Mesenchymal stem cells in health and disease. *Nat. Rev. Immunol.* **8:** 726–736.
45. Nam, H.K. *et al.* 2011. Ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1) protein regulates osteoblast differentiation. *J. Biol. Chem.* **286:** 39059–39071.
46. Eltzschig, H.K., M.V. Sitkovsky & S.C. Robson. 2012. Purinergic signaling during inflammation. *N. Engl. J. Med.* **367:** 2322–2333.
47. Grenz, A. *et al.* 2012. Equilibrative nucleoside transporter 1 (ENT1) regulates postischemic blood flow during acute kidney injury in mice. *J. Clin. Invest.* **122:** 693–710.
48. Pastor-Anglada, M. *et al.* 2007. Concentrative nucleoside transporters (CNTs) in epithelia: from absorption to cell signaling. *J. Physiol. Biochem.* **63:** 97–110.
49. Takahashi, H.K. *et al.* 2007. Effect of adenosine receptor subtypes stimulation on mixed lymphocyte reaction. *Eur. J. Pharmacol.* **564:** 204–210.
50. Hasko, G. *et al.* 2008. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat. Rev. Drug Discov.* **7:** 759–770.
51. Lau, W.M. *et al.* 2013. Enpp1: a potential facilitator of breast cancer bone metastasis. *PLoS One* **8:** e66752.
52. Saborit-Villarroya, I. *et al.* 2011. E2A is a transcriptional regulator of CD38 expression in chronic lymphocytic leukemia. *Leukemia* **25:** 479–488.
53. Long, C.L. *et al.* 2012. E proteins regulate osteoclast maturation and survival. *J. Bone Miner. Res.* **27:** 2476–2489.
54. Zumaquero, E. *et al.* 2010. Exosomes from human lymphoblastoid B cells express enzymatically active CD38 that is associated with signaling complexes containing CD81, Hsc-70 and Lyn. *Exp. Cell Res.* **316:** 2692–2706.
55. Gorgun, G.T. *et al.* 2013. Tumor-promoting immune-suppressive myeloid-derived suppressor cells in the multiple myeloma microenvironment in humans. *Blood* **121:** 2975–2987.
56. Favaloro, J. *et al.* 2014. Myeloid derived suppressor cells are numerically, functionally and phenotypically different in patients with multiple myeloma. *Leuk. Lymphoma* [Epub ahead of print, May 12].
57. Zhuang, J. *et al.* 2012. Osteoclasts in multiple myeloma are derived from Gr-1^{+}CD11b^{+} myeloid-derived suppressor cells. *PLoS One* **7:** e48871.
58. Guichelaar, T. *et al.* 2013. Human regulatory T cells do not suppress the antitumor immunity in the bone marrow: a role for bone marrow stromal cells in neutralizing regulatory T cells. *Clin. Cancer Res.* **19:** 1467–1475.
59. Ebstein, R.P. *et al.* 2011. Are retinoids potential therapeutic agents in disorders of social cognition including autism? *FEBS Lett.* **585:** 1529–1536.
60. Colaianni, G. *et al.* 2012. Bone marrow oxytocin mediates the anabolic action of estrogen on the skeleton. *J. Biol. Chem.* **287:** 29159–29167.
61. Colaianni, G. *et al.* 2011. Regulated production of the pituitary hormone oxytocin from murine and human osteoblasts. *Biochem. Biophys. Res. Commun.* **411:** 512–515.
62. Colaianni, G. *et al.* 2014. The oxytocin-bone axis. *J. Neuroendocrinol.* **26:** 53–57.
63. Higashida, H. *et al.* 2011. CD38 gene knockout juvenile mice: a model of oxytocin signal defects in autism. *Biol. Pharm. Bull.* **34:** 1369–1372.
64. Lopatina, O. *et al.* 2014. Anxiety- and depression-like behavior in mice lacking the CD157/BST1 gene, a risk factor for Parkinson's disease. *Front Behav. Neurosci.* **8:** 133.
65. Burnstock, G. 2014. Purinergic signalling in endocrine organs. *Purinergic Signal.* **10:** 189–231.
66. Lux, A. *et al.* 2014. A humanized mouse identifies the bone marrow as a niche with low therapeutic IgG activity. *Cell Rep.* **7:** 236–248.
67. Laubach, J.P. *et al.* 2014. Daratumumab granted breakthrough drug status. *Expert Opin. Invest. Drugs* **23:** 445–452.
68. Chillemi, A. *et al.* 2013. Anti-CD38 antibody therapy: windows of opportunity yielded by the functional characteristics of the target molecule. *Mol. Med.* **19:** 99–108.
69. Quarona and Malavasi *et al.* 2014. In preparation.
70. Mediero, A. & B.N. Cronstein. 2013. Adenosine and bone metabolism. *Trends Endocrinol. Metab.* **24:** 290–300.