Department of Molecular Medicine

PhD program in "Innovation in Immuno-mediated and Hematological Disorders" – XXXIV Cycle

Curriculum: Immunology and Immunopathology

Immunostimulatory activity and regulation of extracellular 2'3'-cGAMP in human multiple myeloma

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1. Abstract

Multiple myeloma (MM) is a multi-step malignancy arising from the clonal proliferation of antibody-secreting cancerous plasma cells in the bone marrow. The progression of the disease, from the benign condition known as monoclonal gammopathy of undetermined significance (MGUS) to frank MM, is in part due to the gradual loss of immunosurveillance caused by pathological alterations of the bone marrow microenvironment, which affect most cell populations, including mesenchymal stromal cells (BMSCs) and Natural Killer (NK) cells. A promising frontier in cancer immunotherapy is represented by the cytosolic DNA-sensing cGAS-STING pathway, a crucial defense mechanism of the innate immunity. The second messenger cGAMP, endogenous activator of the STING protein, is released by tumor cells and uptaken by immune or stromal cells, which respond by secreting type I Interferons (IFNs) and other cytokines that can orchestrate a potent antitumor response. However, cGAMP is degraded by extracellular hydrolases like ENPP1, overexpressed in certain types of cancer and associated with metastasis development, which can therefore protect tumor cells from STING-mediated tumor clearance.

During my Ph.D. I demonstrated the ability of MM cell lines to secrete cGAMP in the extracellular environment upon stimulation with the genotoxic drug doxorubicin. In addition, I investigated how NK cells and BMSCs respond to the treatment with exogenous cGAMP, showing that NK cells from healthy donors increase their cytotoxicity, whereas BMSCs from MM patients increase the expression of *IFN-β* and other cytokines. I further demonstrated that the MM cell line SKO-007(J3) undergoes apoptosis when challenged with high doses of cGAMP, while a lower-dose stimulation results in the increase of senescence-associated β-galactosidase activity.

Secondly, I collected evidence for a role of the ectoenzyme CD38, an important marker and therapeutic target of MM, in the enzymatic degradation of cGAMP. Inhibition of CD38 on Dox-treated MM cell lines causes the accumulation of cGAMP in the conditioned medium (CM), as measured

through competitive ELISA. Moreover, stimulation of patients-derived BMSCs with CM from CD38-inhibited MM cells increases the expression of *IFN-β* and other STING-activated genes. Ultimately, in collaboration with Dr. Annamaria Sandomenico (Institute of Biostructure and Bioimaging, National Research Council, Napoli, Italy), I accumulated biochemical data that further corroborate the hypothesis of cGAMP being substrate of CD38. In particular, incubation of human recombinant CD38 in the presence of increasing concentrations of cGAMP induces quenching at 345 nm, which corresponds to the maximum of fluorescence emission of tryptophan residues, indicating a direct interaction between CD38 and cGAMP. Further, time-course measurements of fluorescence recovery suggest that natural cGAMP is actively transformed by the enzyme. Finally, reverse phase HPLC and ESI-TOF mass spectrometry show the complete disappearance of cGAMP after incubation with recombinant CD38, indicating that the enzyme can degrade cGAMP into different chemical species.

2. Introduction

2.1 – Multiple myeloma

2.1.1 - Epidemiology and clinical presentation

Multiple myeloma (MM) is a hematological neoplasia that derives from the abnormal proliferation of malignant plasma cells (PCs) of clonal origin within the bone marrow. The terminally differentiated, isotype-switched plasma cells secrete high levels of monoclonal immunoglobulins (defined as M protein), either complete or fragmented (light or heavy chains). The M protein accumulates abundantly in the serum, as revealed by the peculiar protein electrophoresis diagram showing a prominent spike in the γ-globulins region, and in the urine, where it is termed Bence Jones protein (Ig light chains in the urine). The accumulation of M protein in the tissues is the major driver of the pathogenesis and clinical manifestations of MM.

With a median age at diagnosis of approximately 66-70 years[1], MM is considered a disorder of the elderly; however, its incidence is steadily increasing since 1990, even in the younger population[2]. MM is a multi-step disease that lies within the spectrum of plasma cell dyscrasias, which includes asymptomatic conditions as the monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM), both considered as precursor stages of MM. In fact, almost all cases of MM develop from the MGUS, but only rarely MGUS progresses to malignant MM. Such risk of progression is estimated to be approximately 1% per year[3].

The MGUS is a benign condition characterized by < 10% monoclonal PCs in the bone marrow aspirate, levels of serum M protein < 3 g/dL (IgG, IgD and IgA; or >30 g/dL in the case of IgM-MGUS) and absence of clinical symptoms typically associated with MM: anemia, bone lesions, kidney failure, hypercalcemia. Its frequence in the population is estimated around 3% in people of age > 50 and around 6,5% in people of age 75-80, making MGUS the most frequent type of gammopathy. Instead,

SMM is defined by the presence of a number > 10% and < 60% of malignant PCs and > 3 g/dL of M protein in the absence of clinical symptoms associated with MM[4].

Regarding MM, the most frequent clinical manifestations are anemia, which aggravates with increasing tumor mass, and bone lesions, frequently accompanied by severe pain. Other common symptoms are the reduction of kidney function (until kidney failure in severe cases), caused by the accumulation and precipitation of light chains in the distal tubules and their obstruction[5], and increased susceptibility to infections due to marrow dysfunction.

2.1.2 - Pathogenesis of multiple myeloma

The slow accumulation of oncogenic mutations and chromosomal abnormalities, together with the gradual decline of immune surveillance, are believed to be the major causes behind the MGUS-SMM-MM progression. The deletion of chromosome 13 is the most frequent karyotypic abnormality in MM, followed by hyperdiploidy, translocations t(11;14) and t(4;14), and deletion of 17p[6]. The immunoglobulin heavy chain region on chromosome 14 is particularly important for the pathogenic consequences of primary chromosomal translocations, frequently dysregulating the genes *MMSET, CCND1* and *FGFR3.* Translocations involving the heavy chain locus and other cytogenetic abnormalities are associated with poor prognosis compared to a normal karyotype[6].

Secondary events often implicated in the disease progression are karyotypic alterations involving *MYC* and *IRF4*, activating mutations on *KRAS*/*NRAS* and *BCL2*, inactivation of *TP53* (located on 17p) and *CDKN2A*[6].

2.1.3 - A diseased microenvironment

The complex BM niche in which MM proliferates consists of numerous cell populations, including BM mesenchymal stromal cells (BMSCs), fibroblasts, adipocytes, osteoblasts, osteoclasts and endothelial, immune and hematopoietic cells, embedded in the extracellular matrix (ECM) and surrounded by a liquid phase enriched with cytokines, growth factors, enzymes and extracellular vesicles. Moreover, MM niche is frequently characterized by hypoxia, which further neovascularization and acidification of the tumor microenvironment (TME)[7].

During the progression of MM, peculiar interactions between malignant PCs and between PCs and other populations are established, mediated by either soluble cues or by direct contact between surface proteins. The altered expression of adhesion molecules on MM cells, especially CD138 (syndecan-1), CD38, CD56, CD29, ICAM1 and CD49d results in abnormal interactions with the cells of the BM microenvironment, inducing the secretion of cytokines and growth factors that foster tumor growth and survival. Among these, IL-6 is believed to be crucial for the proliferation of malignant PCs[8]. IL-6 secretion by MM cells and other populations in the BM microenvironment, especially BMSCs, osteoblasts and myeloid cells, is sustained by autocrine and paracrine feedback loops.

The interaction between CD49d (VLA4) and CD106 (VCAM1), expressed by endothelial cells and BMSCs, is critical for the homing in the bone marrow and for the acquisition of resistance to chemotherapeutic drugs[9]. Necessary for the homing is also the binding of the chemokine SDF1α to its receptor CXCR4, expressed by MM cells[10].

In the diseased microenvironment, BMSCs, osteoclasts, osteoblasts and endothelial cells secrete several growth factors and cytokines that sustain tumor growth and local inflammation, including insulin-like growth factor 1 (IGF1), vascular endothelial growth factor (VEGF), B-cell activating factor (BAFF), fibroblast growth factor (FGF), stromal cell-derived factor 1α (SDF1α), and tumor necrosis factor-α (TNFα), which are further upregulated by MM cells adhesion to ECM proteins and BMSCs[11]. Finally, the secretion of the inflammatory C-C chemokines MIP-1α and MIP-1β by MM cells enhances osteoclastic bone resorption, possibly by acting on BMSCs via CCR5, upregulating RANK ligand. The interaction between RANK and its ligand then promotes the differentiation of osteoclasts precursors into bone-resorbing osteoclasts[12].

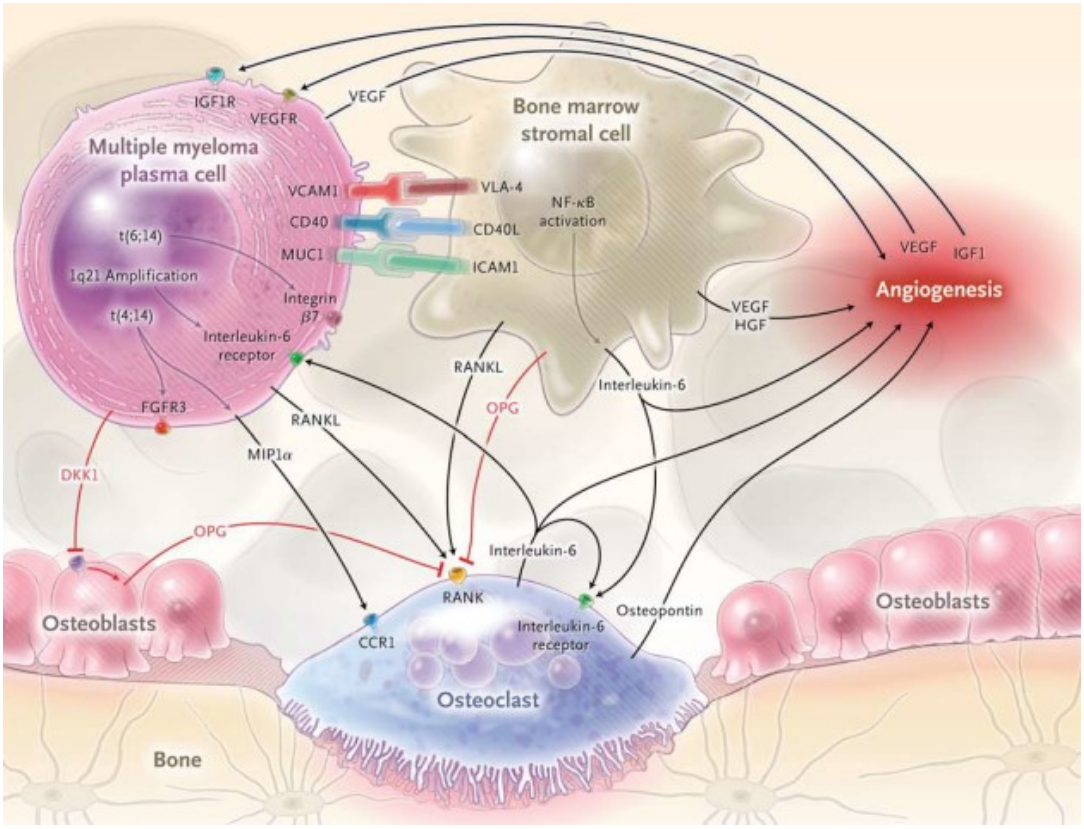


Figure 1: Schematic overview of the principal interactions, mediated by cell-cell contacts and soluble factors, between malignant plasma cells, bone marrow stromal cells, osteoclasts and osteoblasts in the multiple myeloma niche. CCR1: chemokine receptor 1, CD40L: CD40 ligand, VEGF: vascular-endothelial growth factor, FGFR3: fibroblast growth factor receptor 3, HGF: hepatocyte growth factor, ICAM1: intercellular adhesion molecule 1, IGF1: insulin-like growth factor 1, OPG: osteoprotegerin, MIP1α: macrophage inflammatory protein 1α, MUC1: cell-surface–associated mucin 1, NF-κB: nuclear factor κB, RANK: Receptor activator of nuclear factor κB. From [1].

2.1.4 - Interaction between myeloma cells and bone marrow mesenchymal stromal cells

BMSCs are fibroblast-like cells characterized by multipotent stemness, capable of differentiating into osteoblast, adipocytes, or chondrocytes under appropriate stimuli.

The International Society of Cellular Therapy (ISCT) established minimum criteria to allow comparison of MSCs between different studies: adherence to plastic under standard culture conditions, expression of CD105, CD73 and CD90 (≥95%) and lack of hematopoietic cell surface markers (CD34, CD45, CD14/CD11b, CD79a/CD19 and HLA-DR)[13].

Under physiological conditions, BMSCs regulate bone homeostasis and hematopoiesis; however, in MM they establish a complex cross-talk with malignant PCs and acquire a phenotype that supports tumor growth, angiogenesis and bone resorption[13].

Adhesion of MM to BMSCs triggers a series of pathways that cause the upregulation of cell cycle-regulatory and anti-apoptotic proteins[14]. Moreover, the interaction between MM cells and BMSCs induces secretion of IL-6 by BMSCs by activating NF-κB; in turn, IL-6 stimulates MM cells to secrete vascular endothelial growth factor (VEGF)[15]. BMSC-MM cell contact is also mediated through the interaction Notch-Notch ligand, which triggers the secretion of IL-6, VEGF, and insulin-like growth factor (IGF-1), promoting MM cell proliferation and drug resistance[16]. The chemokine IL-8 (CXCL8) is a crucial mediator of the pathological crosstalk between BMSCs and MM cells, inducing resistance to bortezomib[17] and osteoclastogenesis[18]; at the same time, however, BMSCs-produced IL-8 upregulates the expression of PVR, ligand of the NK cell activating receptor DNAM-1, on MM cells[19].

2.1.5 - Immune dysregulation in multiple myeloma

As in many types of malignancies, also in MM the TME is characterized by a chronic state of sterile inflammation, accompanied by profound immunosuppression[20].

The use of immunomodulatory drugs (IMiDs), namely thalidomide, lenalidomide, and pomalidomide, has encountered some success in the management of MM, but only in a subset of patients. In addition, the employment of monoclonal antibodies (mAbs) such as daratumumab (anti-CD38) and elotuzumab (anti-SLAMF7) had a significant impact on the extension of life expectancy of MM patients without, however, resulting in complete remission in many cases.

The incomplete efficacy of these regimens is due to the severely compromised antitumor immunity. The immune dysregulation is probably involved also in the progression from MGUS to MM[20]. Indeed, the tumor immune microenvironment (TIME) shows substantial alterations already during the MGUS stage, with increased numbers of NK cells, Tregs and nonclassical CD16+ monocytes, indicating an active response of the immune system against the transformed clone[21]. Still, in the later stages of disease, several phenotypical alterations occur, negatively affecting the response of most immune cell types.

NK cells, which are critical for early immune surveillance and for the response to mAbs therapy, are progressively crippled during tumor progression. Nonetheless, it is still unclear whether the progression of the disease is a consequence of NK cell dysfunction or rather a cause. Several studies reported that NK cells from patients with MGUS and newly diagnosed MM preserve their functions, whereas the advanced disease is accompanied with signs of a deeper impairment[22]. For instance, NK cells downregulate the expression of the activating receptors NKG2D, NKp30 and DNAM-1[23], while increasing the expression of PD-1, thereby reducing their cytotoxicity towards PD-L1-expressing MM cells. Interestingly, lenalidomide can directly downregulate the expression of PD-L1 by MM cells[24]. The reduced expression of NKG2D on NK cells is possibly due to the secretion of the soluble form of its ligand MICA, which can downregulate NKG2D on NK cell surface and whose concentration is directly correlated with tumor progression[25,26].

The dysfunction of NK cells is also manifested as a decrease in CD16+ subsets, which are mainly involved in antibody-dependent cytotoxicity (ADCC) and are crucial for the efficacy of mAb-based therapies[27].

MM cells in advanced disease appear to be intrinsically resistant to NK cell-mediated killing compared to early disease, even when challenged against healthy-donors NK cells. The *de novo* expression of PD-L1 and the sustained expression of MHC-I molecules are believed to be the main causes behind this phenomenon[28,29].

Importantly, the use of low-dose chemotherapy, in particular genotoxic agents like doxorubicin and melphalan, induces in human and murine MM cells a state of highly immunogenic senescence, in which the expression of NKG2D and DNAM-1 ligands is strongly increased, rendering MM cells more susceptible to recognition and killing by NK cells *in vitro* and *in vivo*[30,31].

Tumor-induced T cell dysfunction deeply impacts the prognosis of MM. CD8+ T cells in the tumor site express markers associated with T cell exhaustion (PD-1, CTLA-4, 2B4, CD160) and senescence (CD57, lack of CD28), showing impaired function and lower proliferative capacity[32].

Finally, several works reported increased numbers of Tregs, which impair cytotoxic T cells, NK cells and dendritic cells function by secreting IL-10 and TGF-β, in the diseased BM of MM patients, suggesting an important contribution of Tregs to the establishment of an immunosuppressive

TME[21,33,34,35]. There is also evidence that MM cells can directly induce Tregs through the expression of ICOS-L and direct contact[36]. Interestingly, it has been reported that the use of IMiDs can inhibit the proliferation and function of Tregs in MM patients[37].

Myeloid-derived suppressor cells (MDSCs), identified by the immunophenotype CD11b(+) CD14(-) HLA-DR(-/low) CD33(+) CD15(+), are also believed to negatively impact anti-myeloma immune response, since a few reports described their abnormal abundance in peripheral blood and BM of MM patients, resulting in impaired T cell response[38,39].

2.1.6 - State of the art in the therapy of multiple myeloma

As of 2021, the standard frontline therapy for newly diagnosed MM patients eligible for transplantation consists of an induction therapy, followed by autologous stem cell transplantation (ASCT) and maintenance therapy. Nevertheless, all patients eventually relapse and die within 7-10 years[40], making MM essentially an incurable disease.

The induction therapy has the goal to minimise the tumor burden, with the hope that the residual cancerous cells can be kept under control by the immune system after the reconstitution post-ASCT. Currently, the drugs are used in various combination of three agents, including alkylating agents (melphalan, cyclophosphamide), corticosteroids (dexamethasone, prednisone), immunomodulatory drugs (lenalidomide, thalidomide, pomalidomide), proteasome inhibitors (bortezomib, carfilzomib), monoclonal antibodies (anti-CD38 daratumumab and isatuximab, anti-SLAMF7 elotuzumab) and HDAC inhibitors (panobinostat). The preferred initial therapy for ASCT-eligible and non-eligible patients is the combination bortezomib, lenalidomide and dexamethasone (Velcade, Revlamid, dexamethasone; VRd). An important alternative for specific categories of patients is represented by the regimen daratumumab, lenalidomide and dexamethasone (DRd). Maintenance therapy usually consists of lenalidomide or bortezomib alone, or in a combination of both at lower doses. The use of anthracyclines like doxorubicin, albeit infrequently, is still included in some multi-agent combinations for aggressive or refractory MM[41].

The overall effect of these drug regimens on the status of the immune system is extremely difficult to assess, and indeed several studies show contradictory results. For instance, alkylating agents,

genotoxic drugs and proteasome inhibitors can induce immunogenic cell death (ICD) and senescence in MM cells, which boost tumor antigen presentation and recognition by NK cells[42,30]; still, the antiproliferative effects of chemotherapy are known to negatively impact both innate and adaptive immunity[43].

2.2 – The cGAS-STING pathway

2.2.1 - Biological function of the cGAS-STING pathway

The recent groundbreaking advancements in tumor immunotherapy encouraged a deeper investigation of newly discovered signalling pathways of the innate immunity. Among the most studied, possibly because of its involvement in vastly different pathological conditions, is the cGAS-STING pathway. The signal that triggers the cGAS-STING pathway is the presence of double-stranded DNA in the cytoplasm; indeed, cytosolic dsDNA is highly immunogenic, representing both a pathogen-associated (PAMP) and a damage-associated molecular pattern (DAMP), in the first case during viral or bacterial replication in the cytoplasm, in the latter when nuclear or mitochondrial integrity is compromised. The identification of homologs of the proteins cGAS and STING in most animal phyla, including invertebrates[44], indicates that DNA sensing is an ancient and fundamental function of the immune system.

The cGAS-STING pathway is pivotal for the defence against microbes. DNA viruses including adenovirus, papillomavirus and vaccinia virus, but also retroviruses like HIV can trigger STING signalling, although the mechanism for the latter is still unknown. In addition, different species of Gram+ and Gram- bacteria, including *L. monocytogenes, L. pneumophila, F. tularensis, S. pyogenes, M. tuberculosis* induce a type I IFN response through STING[45].

The overall mechanism by which the pathway operates has been dissected: cytosolic DNA binds and activates cGAS, which in turn synthesises the second messenger 2’3’-cGAMP. This molecule diffuses to the ER and activates the adaptor protein STING, which then recruits the kinase TBK1 and consequently the transcription factor IRF3. Phosphorylation of IRF3 by TBK1 results in its

activation and migration into the nucleus, where it regulates the expression of numerous target genes, including type I interferons (IFNs).

Since STING signalling is triggered by cytoplasmic DNA, the activation status of the pathway is negatively regulated by cytoplasmic endonucleases, especially TREX1 and Dnase2. In the absence of TREX1 or Dnase2, mice succumb to a severe STING-mediated autoinflammatory disease, but they can be rescued by concomitant STING inactivation. Mutations in TREX1 are also associated with human autoinflammatory diseases like systemic lupus and Aicardi-Goutières syndrome[45].

2.2.2 - The sensor protein cGAS

The enzyme cyclic guanosine monophosphate–adenosine monophosphate (cyclic GMP – AMP) synthase (cGAS) is a virtually ubiquitous cytosolic DNA sensor, in contrast to the other DNA sensor TLR9, which is mainly expressed by immune cells and is localized in the ER and endosome[46]. cGAS contains a nucleotidyltransferase domain and two DNA-binding domains. In the absence of DNA, cGAS is hold in an autoinhibited state; however, upon DNA binding, it forms a 2:2 complex and catalyzes the synthesis of the second messenger 2'3'-cyclic GMP-AMP (2'3'-cGAMP) from ATP and GTP[47]. The cGAS dimer binds DNA through electrostatic interactions between positively charged residues and the negatively charged sugar-phosphate backbone of DNA, which explains the lack of sequence-specificity of cGAS-DNA interaction[48]; nevertheless, length is critical for the extent of cGAS activation, with long dsDNA molecules being more potent activators than shorter fragments[49]. The formation of the cGAS-DNA 2:2 complex brings the two DNA molecules in a roughly parallel arrangement, leading to the subsequent binding of other cGAS dimers along the "ladder", in a strongly cooperative manner[50].

cGAS is subject to several post-translational modifications (PTMs) that regulate its stability, enzymatic activity and DNA binding. So far, events of phosphorylation, ubiquitylation, acetylation, glutamylation, and sumoylation have been reported in literature[51].

For example, during normal mitosis, in which the nuclear envelope breakdown brings the DNA in contact with the cytoplasm, cGAS activity is restrained by the complex CDK1-Cyclin B, which

phosphorylates the enzyme at S305 (in humans), making it unresponsive to self-DNA and inhibiting its ability to synthesize 2'3'-cGAMP[52].

Several recent works demonstrated, surprisingly, that manganese ions (Mn^{2+}) can potently increase the sensitivity and accelerate the catalytic activity of cGAS by binding to the enzyme. Moreover, at higher concentrations Mn^{2+} can directly activate cGAS, independently of dsDNA, triggering a non-canonical synthesis of 2'3'-cGAMP. Mn^{2+} was found to be released from intracellular membrane-enclosed organelles upon viral infection with VSV, HCV-1 and vaccinia virus, amplifying DNA-sensing and antiviral response[53-55].

2.2.3 - The second messenger cGAMP and its regulation

The second messenger 2'3'-cGAMP (cyclic guanosine monophosphate–adenosine monophosphate, from now on simply cGAMP) is a soluble cyclic dinucleotide (~674.4 $gmol^{-1}$) with two negative charges at physiological pH. cGAMP contains unique mixed phosphodiester bonds, one canonical between 3'-OH of AMP and 5'-phosphate of GMP, and one non-canonical between 2'-OH of GMP and 5'-phosphate of AMP[56]. To date, its sole function is believed to be the activation of the adaptor protein STING, since the stimulation of STING-KO THP-1 cells with exogenous cGAMP results in a gene expression profile that is indistinguishable from untreated cells[57]. Compared to other cyclic dinucleotides (CDNs) of bacterial origin and isomers like 3'3'-cGAMP or 3'2'-cGAMP, the endogenous cGAMP has the highest affinity for STING [58-61].

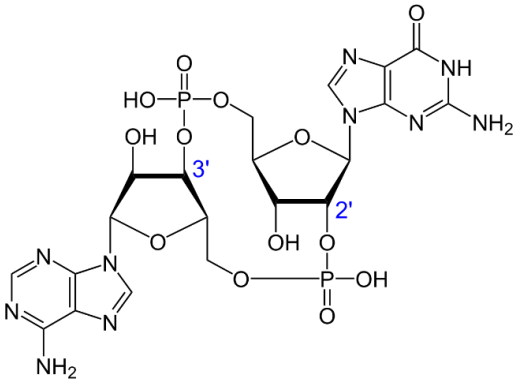


Figure 2: Chemical structure of 2'3'-cGAMP (or cGAMP), endogenous second messenger synthesized by the dsDNA sensor cGAS and potent activator of STING. At physiological pH, the molecule has two negative charges on the phosphate groups.

It was originally believed that the function of cGAMP was limited to the cell producing it. Being cGAMP a negatively charged molecule, its passive diffusion across the plasma membrane is not plausible. More recently, however, it was proven that cGAMP-mediated paracrine communication is a crucial process, especially in the context of viral infections and tumor immune surveillance. Indeed, fully functional cGAMP can pass to nearby cells through gap junction composed of connexin 43[62] and can be even carried by viral particles, including lentivirus and herpesvirus virions[63].

Perhaps more significantly, cGAMP can move across the plasma membranes through different types of channel proteins. The first, identified by CRISPR screening, is SLC19A1 (also known as reduced folate transporter) and acts mainly as a direct importer of cGAMP and other CDNs. Folinic acid and methotrexate, whose uptake by the cell is mediated by the same channel, are competitive inhibitors of extracellular cGAMP[64]. Similarly, the previously uncharacterized channel protein SLC46A2 is the main extracellular cGAMP importer in human monocytes and macrophages[65]. Much more widespread transporters of cGAMP are the volume-regulated anion channels (VRACs) LRRC8. These multimeric channels are composed of six subunits (the obligatory LRRC8A, and LRRC8B, -C, -D, -E) with an overall structure similar to connexins. Depending on the composition of subunits, they transport a variety of other substrates, including Cl- ions and organic compounds like taurin, inositol, glutamate, cisplatin, blasticidin S etc.; nevertheless, in order to transport cGAMP and other CDNs across the membrane, the subunits LRRC8A and LRRC8E must be included in the structure of the channel. Crucially, LRRC8 channels are bidirectional cGAMP transporters, the first identified[66,67]. It is important to note that extracellular cGAMP, uptaken through all the

aforementioned channels, has to interact with the STING protein of bystander cells in order to exert its function; no other signalling pathways are known to date.

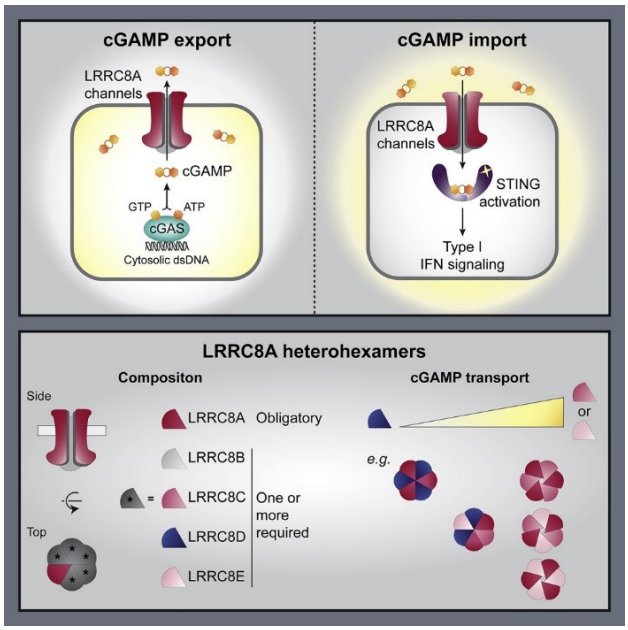


Figure 3: LRRC8 hexameric channels (also known as Volume-Regulated Anion Channels, VRACs) are the first known bidirectional transporters of cGAMP that allow its passage across the plasma membrane. The presence of the subunit LRRC8A is obligatory for preserving the functions of the channel; the inclusion of the subunits LRRC8C or LRRC8E in the hexamer allows transport of cGAMP and other CDNs, whereas LRRC8D inhibits their transport. From [67].

Given its powerful inflammatory activity in several cell types, the concentration of cGAMP must be regulated in order to rapidly turn off the STING signalling once the stress response is resolved. It is possible that cGAMP efflux is a mechanism to achieve this type of regulation; however, it appears that cGAMP activity is mainly restricted through enzymatic degradation. Remarkably, DNA viruses of the family *Poxviridae* evolved a specific mechanism to hydrolyze and inactivate cGAMP through the so-called poxvirus immune nucleases, or poxins[68].

The enzyme ecto-nucleotide pyrophosphatase/phosphodiesterase 1, or ENPP1 (EC: 3.6.1.9), is the first identified mammalian enzyme able to hydrolyze cGAMP into AMP and GMP[69]. ENPP1 (also known as CD203a or PC-1) is a type II transmembrane glycoprotein that was originally described as an important regulator of bone mineralization for its ability to hydrolyze ATP and GTP generating inorganic pyrophosphate (PPi), which inhibits bone and cartilage mineralization. Accordingly, loss-of-function mutations of ENPP1 result in inherited syndromes characterized by ectopic calcifications[70]. ENPP1 is localized on the plasma membrane and ER lumen, but it is also found in

the serum as a secreted/shed protein[71]; its enzymatic activity strictly requires Ca^{2+} and Zn^{2+} ions and its affinity to cGAMP is comparable to that of ATP[69].

Kato and colleagues, after crystallizing mouse ENPP1 in complex with cGAMP, proposed a mechanism of reaction for cGAMP enzymatic hydrolysis[72]. The crucial event is the nucleophilic attack of the phosphorus atom in the 2'-5′ phosphodiester linkage by the -OH of the threonine residue 238 (Thr238), resulting in the production of a pApG intermediate. This intermediate is then flipped over in the enzymatic pocket, exposing the second phosphodiester bond to another nucleophilic attack by Thr238, which finally breaks down pApG into AMP and GMP.

2.2.4 - Activation of the adaptor protein STING and downstream signalling

STING (stimulator of interferon genes, also known as TMEM173) is a small protein of ~40 kDa discovered via expression cloning by H. Ishikawa and G. N. Barber in 2008[73] and described in its ability to induce a potent type I interferon immunity following expression. Gain-of-function mutations of STING cause a severe autoinflammatory disorder termed STING-associated vasculopathy with onset in infancy (SAVI)[74]. The structure of the protein, recently resolved by Cryo-EM[75], comprises four transmembrane helices that anchor it to the endoplasmic reticulum (ER), a ligand-binding domain (LBD), which mediates the interaction with cGAMP, and a C-terminal tail (CTT) responsible for its autoinhibition and for the recruitment of downstream kinases[48]. In the absence of its ligand cGAMP, STING forms inactive homodimers on the surface of ER. The STING dimer is the functional unit capable of binding cGAMP and the others CDNs; its shape can be compared to a butterfly, with the central crevice between the wings being the site where a single molecule of cGAMP can settle in. Following the binding event, displacement of the CTT and conformational changes on the sides of the LBD allow the dimers to arrange into tetramers and higher-order oligomers through side-by-side contacts, stabilized by disulfide bonds[75,76]; at the same time, STING oligomers leave the ER and reach the Golgi apparatus, where they recruit Tank Binding Kinase 1 (TBK1) through the CTT. It appears that STING migration to Golgi is necessary for the activation of TBK1, since disruption of vesicles trafficking with brefeldin A impairs STING signalling[78]. In addition, palmitoylation of STING on Cys88/91 at the Golgi is also required for its function[79].

After activation, TBK1 phosphorylates STING on several residues of the CTT, creating a docking site for the transcription factor IRF3. The docking of IRF3 allows TBK1 to phosphorylate and activate the transcription factor, which then dimerizes and enter the nucleus, leading to the transcription of type I Interferons (I-IFNs) and other cytokines[48].

Besides IRF3, STING can also activate NF-κB through TBK1 and IKK, with a mechanism that is probably independent of the CTT[80,81,73]. In fact, activation of NF-κB seems to be a more ancient function of STING, since the CTT, which is the motif required for IFN response, is only present in vertebrates, whereas the appearance of STING precedes the evolution of animals[44]. Moreover, disruption of a single phosphorylation site of the CTT (Ser366 in humans) completely abrogates IRF3 recruitment and I-IFN expression without affecting NF-κB signalling[81].

For its ability to relay the signal to both IRF3 and NF-κB, activation of the STING pathway could potentially result in the expression of several hundreds of genes. Particularly relevant in the context of tumor immunology are type I IFNs (IFN-α1, IFN-β, IFN-λ1) and the chemokines CXCL10, CCL5, CCL2, which are target of IRF3, and the cytokines IL-1A/B, IL-2, IL-6, IL-10, IL-15, IL-17, IL-21, which are regulated by the NF-κB complex[46,82].

Type I IFNs signal in autocrine and paracrine fashion by interacting with the heterodimeric interferon receptor, composed of IFNAR1 and IFNAR2 (or with the homodimer of IFNAR1, which has particularly high affinity for IFN-β), triggering the recruitment and activation of the kinases Jak1 and Tyk2, which in turn phosphorylate the transcription factors STAT1 and STAT2. The heterodimer STAT1/STAT2 migrates to the nucleus and associates with IRF9, forming the transcriptional complex IFN-stimulated gene factor 3 (ISGF3) which leads to the expression of numerous interferon-stimulated genes (ISGs)[83].

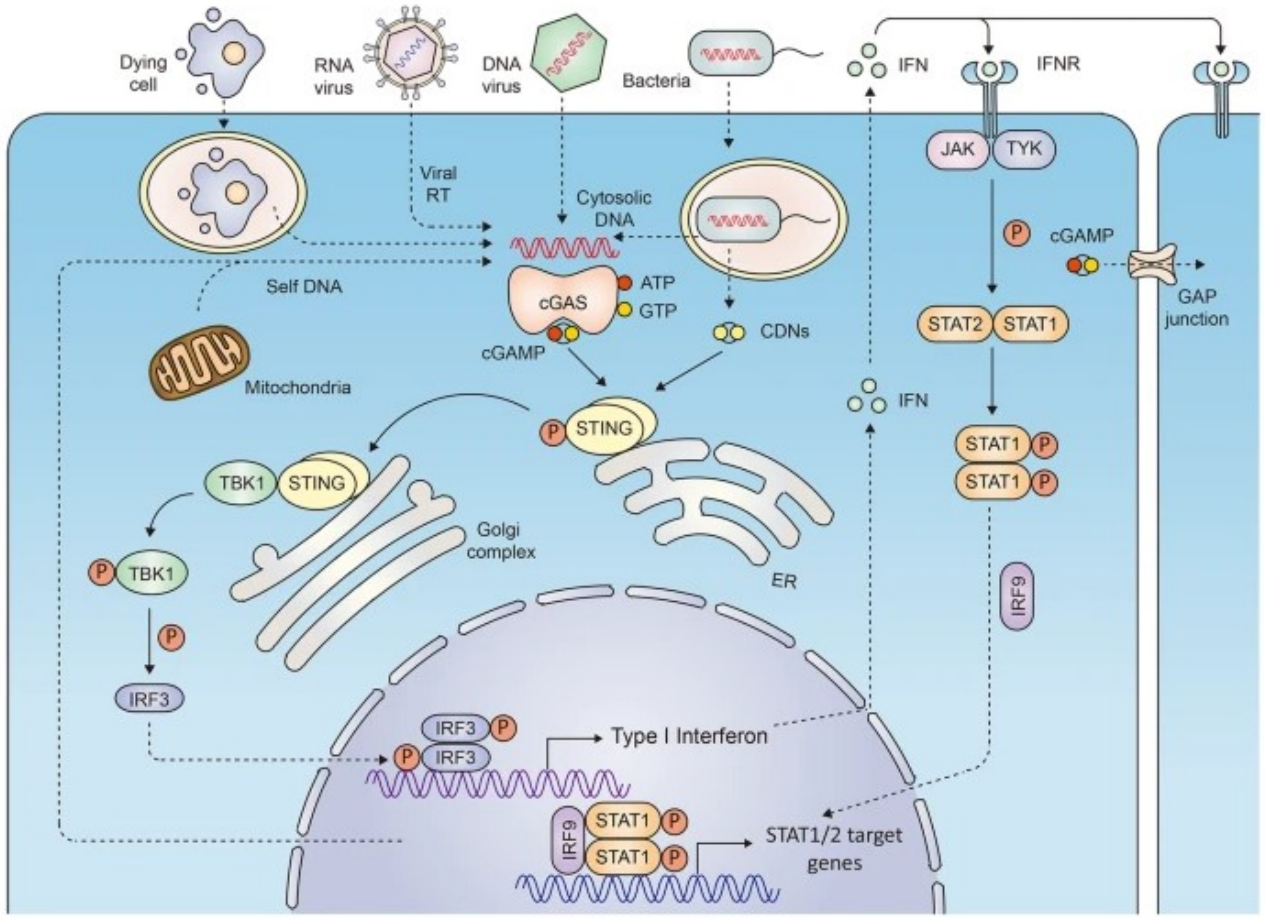


Figure 4: Scheme of the cGAS-STING pathway. Viral, bacterial, self-DNA (nuclear or mitochondrial) and DNA from engulfed cells can all be detected by cGAS when located in the cytoplasm. The transcriptional program activated by the pathway partially overlaps with the one mediated by type I IFNs and STAT1/2. From [84].

2.2.5 - Activity and regulation of extracellular cGAMP in the context of cancer immunology

Previously considered as limited to antiviral immunity, the STING pathway is now deemed one of the most promising frontiers of cancer immunotherapy, and STING agonists have been tested (or are currently being tested) in dozens of clinical trials[84,46], with the hope of translating in the clinical practice their remarkable antitumor activity demonstrated in animal models[85]. However, the comprehension of the pathophysiological role of the endogenous STING agonist, cGAMP, in the interaction between the immune system and cancer is far from complete.

The first strong evidence of an important role of cancer-secreted cGAMP in stimulating anti-tumor immune responses came from the work of Marcus and colleagues[86]. Using STING-deficient mice models and cGAS-KO cancer cell lines, the authors demonstrated that cGAMP released by the

tumor, by acting on IFN-β-producing cells in the microenvironment, triggers a potent cytotoxic response mediated by NK cells. In this work, expression of cGAS by non-tumor cells was dispensable for tumor rejection, implying that malignant cells are the main source of extracellular cGAMP[86].

A recent work demonstrated that cancer cells continuously export cGAMP and that ionizing radiation (IR), by causing chromosomal missegregation and accumulation of cytoplasmic DNA, amplifies the phenomenon. The authors further demonstrated *in vivo* that cGAMP exported by cancer cells and sensed by host STING is critical for the efficacy of IR in inducing immune-mediated tumor rejection, especially by promoting conventional DC infiltration and tumoral antigens cross-presentation[87].

From this body of evidence, it appears that the active release of cGAMP is disadvantageous for the tumor. However, paradoxically, cancerous cells might secrete cGAMP as a mean to defend themselves from cytotoxic lymphocytes. Indeed, cGAMP might be toxic for T cells in certain circumstances, and it was reported that intratumoral STING-KO CD8+ T cells, albeit recruited at the tumor site in lesser number, show better tumor control and reduced cell death compared to STING-WT CD8+ T cells[81]. It is likely that in T cells overstimulation of STING can culminate in apoptosis, in contrast to macrophages, which can readily turn off the signal by degrading STING[88].

Another hypothesis is that some tumors develop a mechanism to export cGAMP in order to avoid self-STING activation, which could lead to the recruitment of immune cells at the tumor site. However, there is evidence that tumor cells characterized by elevated CIN can switch from a type I IFN-predominant to a NF-κB-predominant STING signalling to simultaneously evade immune surveillance (mediated by IFNs), while activating non-canonical NF-κB-driven pro-metastatic programs[89,90].

Finally, degradation of extracellular cGAMP by ENPP1 may be crucial in the pathogenesis of a subset of tumors. Indeed, ENPP1 expression by chromosomally unstable breast cancer cells strongly promotes metastasis *in vivo* by inducing an immunosuppressive microenvironment[89].

The pathophysiological relevance of cGAMP paracrine signalling seems not to be limited to cancer. Recently, it was reported that transfer of cGAMP from non-hematopoietic cells to bystander immune cells is a crucial mediator of autoinflammatory disease in Trex1-deficient mice; moreover, cell-to-cell

cGAMP transmission led to lupus erythematosus-like skin pathology upon UV exposure in the same model[91].

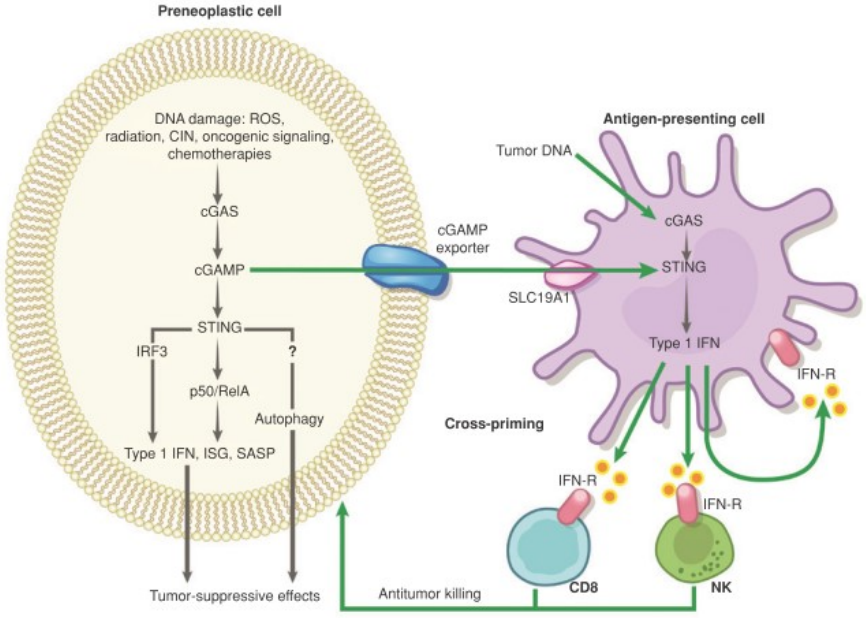


Figure 5: A simplified model of cGAMP-mediated paracrine signalling. cGAMP exported by preneoplastic or neoplastic cells is uptaken by bystander IFN-producing APCs (like pDCs or macrophages) through the channel SLC19A1, stimulating the release of type I IFNs, which in turn activate cytotoxic cells. From [186].

2.2.6 - The cGAS-STING pathway in multiple myeloma

To date, there are only few reports connecting MM and the cGAS-STING pathway. Recently, it was demonstrated how the proteasome inhibitor bortezomib induces a type of immunogenic cell death (ICD) through the activation of cGAS-STING pathway and production of type I IFNs in MM mouse models[92]. Another group, by using *in vivo* mice models, showed that the STING agonist 3'3'-cGAMP can trigger mitochondria-mediated apoptosis of MM cells independently of type I IFNs, because of the inefficient degradation of STING after its activation in B cell-derived malignant cells[93].

Clearly, more experimental evidence will be required to understand whether the contribution of the cGAS-STING pathway may be relevant for the pathogenesis of multiple myeloma.

2.3. – Natural Killer cells

2.3.1 - General features of Natural Killer cells

Natural Killer (NK) cells are innate lymphoid cells (ILC) originally characterized for their ability to kill cancer cells *in vitro* in the absence of a priming event[94], in contrast to CD8+ T cells which require antigen presentation by APCs.

NK cells respond to germline-encoded ligands expressed by other cells of the organism, integrating antagonistic stimuli from an ample spectrum of activating and inhibitory surface receptors. Stressed, transformed and virus-infected cells are the main target of NK cell-mediated cytotoxicity, since such pathological conditions increase (or induce *de novo*) the expression of activating ligands and/or decrease that of inhibitory ligands, rendering the target cells sensitive to the engagement and killing by the NK cell.

Besides their cytotoxic activity, NK cells are also capable of producing cytokines upon stimulation, in particular IFN-γ and TNF-α, but also the chemokines MIP-1α/β and CCL5[95], the growth factor GM-CSF[96], and possibly others.

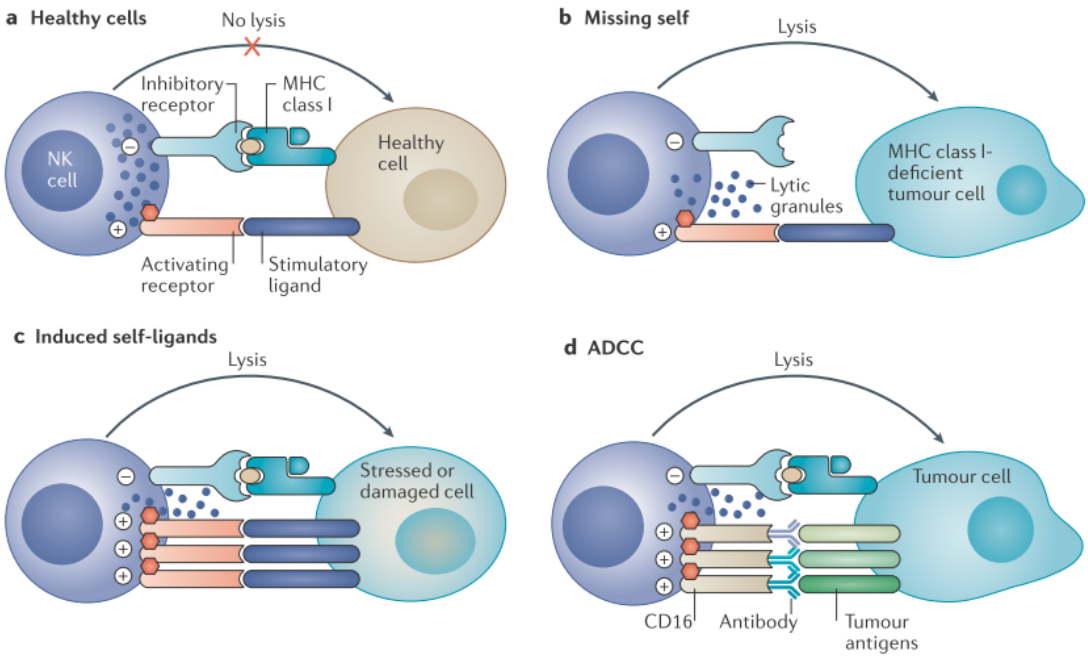


Figure 4: Interactions between NK cell and target cells. a) Healthy cells express normal levels of MHC class I molecules, which engage several types of inhibitory receptors on NK cell's surface, preventing autoreactivity. b) Neoplastic cells often downregulate MHC class I to escape adaptive immunity; consequently, they become sensitive to NK cell-mediated

cytotoxicity, which is no longer restrained by inhibitory receptors signalling ("missing self" recognition). c) Stressed or damaged cells overexpress activating ligands, whose signal overcomes that of inhibitory receptors and results in the killing of the damaged cell. d) Target cells opsonized by antibodies are targeted via CD16 (FcγRIII), an activating receptor that binds to the Fc portion of IgG, triggering NK cell's cytotoxic response. The phenomenon is known as Antibody-Dependent Cellular Cytotoxicity (ADCC). From [187].

2.3.2 - Localization and development

NK cells comprise about 5% - 20% of the lymphocytes in the peripheral blood[97] and are widespread throughout the entire organism, in both lymphoid and non-lymphoid tissues, including bone marrow (BM), lymph nodes (LNs), tonsils, skin, gut, liver, kidney, lungs and uterine decidua[98]. Their turnover in the human bloodstream is estimated around two weeks[99], making them relatively short-lived compared to T cells.

Human NK cells develop and mature mainly in the BM but also in secondary lymphoid tissues like spleen, LNs and tonsils. As with all leukocytes, NK cells derive from the multipotent hematopoietic stem cell (HSC) and the subsequent CD45RA+ lymphoid-primed multipotential progenitor (LMPP). LMPP then differentiates into the common lymphoid progenitor (CLP), which has the potential to generate Pro-B, Pre-T, NK cell progenitor (NKP) and the other ILCs. A subset of the early CLPs, defined as pre-NK cell precursor (Pre-NKP) starts expressing the IL-2 receptor β-chain, becoming NKP, with immunophenotype CD3ε−CD7+CD127+. This event highlights the irreversible commitment into the NK lineage. During the maturation, cells acquire the expression of activating receptors as NKG2D, NKp46, NKp30 and the inhibitory NKG2A. The later transition between immature NK cells and mature NK cells is marked by the expression of CD56 (NCAM); in the end, immature NK cells downregulate CD56 to become $CD56^{dim}$ and start expressing of CD16 (FcγRIII). For this reason, $CD56^{bright}$ are considered less mature than $CD56^{dim}$ NK cells[97].

NK cells development strictly requires the signalling through the common gamma chain (γ_c; CD132), a receptor subunit necessary to respond to IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21[97]. All the receptors comprising γ_c signal through the JAK/STAT pathway and are crucial for regulating NK cell proliferation, homeostasis and function.

In humans, NK cells are generally defined as CD3-/CD56+ cells; however, they can be further categorized in two main subsets on the basis of CD56 expression: $CD56^{dim}$ represent the majority (around 90%) of circulating NK cells; they express CD16 (FcγRIII) and perforin and are considered mostly cytotoxic. On the other hand, $CD56^{bright}$ are less frequent in the blood and are mainly found in lymph nodes and tonsils; they express variable levels of CD16 and are specialized in cytokine production[95], having modest or no ability to kill target cells.

2.3.3 - Regulation of NK cell's function

Regulation of NK cell function is primarily mediated by cytokines and cell-cell contacts.

Cytokines such as type I IFNs, IL-2, IL-12, IL-15 and IL-18 can potently activate NK cell's effector functions; in particular, IL-2 and IL-15 are considered the main proliferative and pro-survival factors involved in NK cell biology; further, they can upregulate the expression of perforin and granzyme B, enhancing their cytotoxic potential[100]. In contrast, TGF-β is the main immunosuppressive cytokine for NK cells, reducing their cytotoxicity, proliferation and ability to produce IFN-γ[101].

Chemokines regulate NK cells migration towards the sites of injury or infection, and towards lymphoid and non-lymphoid tissues. Multiple chemokine receptors are expressed at various levels by different NK cells subsets, making their biology particularly intricated[102]. The major chemokine receptor involved in NK cell migration in tumor sites and during viral infection is CXCR3[103,104], which is specific for the chemokines CXCL9, CXCL10 and CXCL11. In addition, peripheral blood NK cells express CXCR1 and CXCR2 as CXCL8 (IL-8) receptor[105] and CX3CR1 as CX3CL1 (fractalkine) receptor[106].

Type I IFNs are critical for NK cell response to viral infections and are among the most potent NK cell-activating cytokines. Albeit most cell types can produce I-IFNs, plasmacytoid dendritic cells (pDC) are considered the major producers *in vivo*. I-IFNs can strongly stimulate perforin-dependent cytotoxicity against cells infected by viruses and, in combination with IL-12, induce abundant IFN-γ secretion[107]. Further, I-IFNs increase the expression and trans-presentation of IL-15 by DCs and non-immune cells[108].

NK cells largely interact with dendritic cells (DC), establishing a bi-directional crosstalk. DCs act during the priming of the NK cell, enhancing their cytolytic activity, whereas NK cell can promote DC maturation and secretory activity by releasing TNF-α[109]. In addition, NK cells can kill DCs via NKp30 in inflamed sites as a mean to control the recruitment of immature DC in the tissue[110].

DC-produced IL-12 and IL-15 in the lymph node are essential for promoting cytokine production, proliferation and survival of contacting $CD56^{bright}$ NK cells, which can migrate to the lymph node thanks to the expression of CCR7 and L-selectin[110,111]. In turn, NK-produced IFN-γ in the LN provides an early stimulus for T helper 1 polarization[112]. On the other hand, NK cells in the LN might exert a sort of "quality control" of mature DCs. This putative control function might be based on the ability of NK cells to kill mDCs that do not express sufficient levels of MHC class I molecules, which are therefore not able to efficiently activate T cells[110].

2.3.4 - NK cell receptors

NK cells are equipped with a vast array of activating and inhibitory receptors that ensue a prompt response against stressed cells while sparing normal cells and preventing autoreactivity[113].

Infected or transformed cells frequently reduce or turn off the expression of MHC class I molecules as a mean to hide from T cell-mediated adaptive immunity. Consequently, NK cells evolved a mechanism to rapidly activate themselves when they do not sense MHC-I proteins on the interacting cell. Such mechanism, termed "missing self" recognition, is based on inhibitory receptors of the KIR (Killer cell immunoglobulin-like receptors) and LIR (Leukocyte Ig-like receptor) family, and on the CD94/NKG2A heterodimer, all of which recognize classical and non-classical HLA class I molecules. KIRs receptors are specific for the highly polymorphic HLA-A, HLA-B and HLA-C, whereas CD94/NKG2A is specific for the less polymorphic HLA-E. Similarly to HLA-I proteins, also KIRs are polymorphic and this could affect their interaction with MHC-proteins. Both types of receptors signal through ITIMs (Immunoreceptor tyrosine-based inhibition motives) localized on the cytoplasmic tail. ITIMs mediate the inhibitory signal by the phosphorylation-dependent recruitment of a tyrosine phosphatase with Src homology (SH)2 domains, SH region 2 domain-containing phosphatase (SHP)-1 or SHP-2[114]. During NK cells development in secondary lymphoid organs, a process termed

NK cell “education” guarantees that all the circulating NK cells are equipped with at least one inhibitory receptor for HLA; the cells lacking these receptors become anergic or are deleted. Interestingly, activatory receptors of the KIR family, lacking ITIM motives, have also been identified, albeit their biology is less known.

In the absence of inhibitory signals or when stimulated with activatory interactions, NK cells kill the target cell and/or secrete cytokines. Among the activating receptors are the natural cytotoxicity receptors (NCRs), namely NKp46, NKp44 and NKp30. These receptors recognize different ligands, including virus-derived molecules (e.g. hemoagglutinin from influenza virus, which interacts with NKp46 and NKp44) and intracellular proteins, which are exposed on the surface in response to stress cues or malignant transformation (e.g. the nuclear protein PCNA, ligand of NKp44). Further, B7-H6 is an activating ligand of NKp30, frequently expressed by tumor cells but absent on healthy cells. Tumor cells can in turn evade NK cells attack by secreting the soluble form of B7-H6, which decrease the expression and the function of NCRs.

NKG2D is another crucial activating receptor, whose expression is shared by NK and T cells. Its ligands are MICA/B (MHC class I chain-related protein A/B) and ULBP1-3 (UL16-binding protein 1-3), structural homologs of HLA proteins that are expressed by stressed, infected or transformed cells, and which are frequently shedded by tumors as a mechanism to evade NKG2D-mediated surveillance.

Particularly important for $CD56^{dim}$ NK cell-mediated response to therapeutic antibodies is CD16 (FCγRIII), the low affinity receptor of Fc portion of IgG, essential for the killing through antibody-dependent cellular cytotoxicity (ADCC).

NK cells also express the Toll-like Receptors TLR3, TLR7 and TLR8, whose engagement (e.g. with Poly(I:C) that stimulates TLR3) can result in cytotoxicity or IFN-γ production in the presence of IL-12-producing accessory cells[115].

Other surface proteins mainly act as co-receptors. Among these, DNAM-1, 2B4 and NKp80 can amplify the activation mediated by NCRs and NKG2D[113].

Inhibitory immune checkpoints have been described not only on T cells but also in NK cells. These molecules are especially relevant for tumor immune escape and can be expressed *de novo* by NK

cells in pathological conditions. For example, PD-1+ NK cells have been found in patients with Kaposi sarcoma, ovarian carcinoma and Hodgkin lymphoma[116-118]. NK cells expressing PD-1 at the tumor site have been described as bearing an exhausted phenotype *in vivo*[119]. Other inhibitory checkpoints include TIGIT and CD96 (tactile); their ligands, frequently overexpressed by tumor cells, are respectively CD155 (poliovirus receptor) and nectin-2[120]. Finally, TIM-3 is also associated with reduced function of NK cells in cancer patients and is being evaluated as a future target of cancer immunotherapy[121].

2.3.5 - Killing by NK cells

The killing event is a complex mechanism that entails the formation of the activating "immunological synapse" between the NK cell and its target, the remodeling of actin cytoskeleton and the release of lytic granules in a polarized fashion. After the contact with the target cell, adhesion molecules as LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) segregate to the so-called peripheral supramolecular activation cluster (pSMAC)[122,123] leading to the establishment of a tight conjugate between the cells. In contrast, the activating receptors cluster at the center of the immunological synapse, a region called the central supramolecular activation cluster (cSMAC)[122,124], where the integration of multiple signals from different activating receptors and co-receptors is critical for triggering the killing event. Following the establishment of multiple receptor-ligand interactions that turn on activatory signalling pathways, the ordered rearrangement of microtubules and actine filaments (which is strictly necessary for an efficient killing) allows the polarized release of lytic granules into the synapse. The granules contain several proteins, including perforin, granzymes, granulysin, FasL, TRAIL and antimicrobial peptides[125]. These molecules can induce target cell's death through several mechanisms.

Perforin is a pore-forming protein, constitutively expressed at high levels by $CD56^{dim}$ NK cells. Once released, it can insert into the membrane of the target cell and oligomerize, forming ring-shaped pores. The pore is believed to be essential for the killing, as it allows the delivery of serine-proteases known as granzymes into the target cell's cytoplasm. In humans, there are five types of granzymes (A, B, H, M, K), which are synthesized as pro-enzymes and are processed to their active forms by

cathepsin C and H inside the granules[126,127]. When delivered into the cytoplasm of the target cell through the perforin-formed pores, granzymes can induce death by apoptosis. However, the validity of this mechanism is debated, and an alternative model in which granzymes are uptaken by an endocytosis-like process has been proposed[128]. Granzyme-induced apoptosis can be either caspase-dependent or caspase-independent; granzyme B, for example, is able to directly cleave and activate several caspases[129]. On the other hand, granzymes B and K can process the pro-apoptotic protein Bid, leading to mitochondrial outer membrane permeabilization, release of cytochrome C, and activation of the mitochondrial apoptotic pathway[130].

The current model of action of granulysin, a cytolytic and proinflammatory 9-kDa protein, is the induction of apoptosis through the increase of intracellular calcium, decrease of intracellular potassium and direct damage to mitochondria, which causes the release of cytochrome C and apoptosis-inducing factor (AIF), culminating in DNA fragmentation[131].

As a result of the fusion of NK cell's lytic granules with the plasma membrane, the proteins FasL and TRAIL are exposed on the NK cell surface[125]. Engagement of the TRAIL receptor or Fas on the target cell surface by TRAIL or FasL causes the formation of receptor trimers and, subsequently, of the death-inducing signaling complex (DISC), which activates the caspases and initiates apoptosis[132,133].

2.3.6 - Immune surveillance and anti-myeloma activity of NK cells

Ample evidence indicates that NK cells contribute significantly to the immune surveillance and response to therapy in MM, albeit the progression of the disease is inevitably associated with a decline in their effector functions, as discussed above.

Early *in vitro* observations showed how NK cells can spontaneously kill malignant PCs from MM patients, as well as immortalized MM cell lines, expressing normal levels of HLA-I molecules[28]. In those experimental settings, it is evident that the overexpression of activating ligands could counterbalance the negative signals coming from MHC-I-specific inhibitory receptors. Successively, these findings were confirmed by several other works in which primary PCs were challenged against autologous and heterologous NK cells[134-136]. Since then, the mechanisms that render MM cells

susceptible to the killing by NK cells have been extensively studied. The overexpression of NCRs, DNAM-1 and especially NKG2D ligands by PCs is critical for their effective recognition and killing, and several pharmacological treatments, including P.I., IMiDs and DNA-damaging agents, can strongly induce MICA/B, ULBP1-3 (NKG2D ligands), PVR and Nectin2 (DNAM-1 ligands) on tumor cells, enhancing their susceptibility to NK cell killing[26,30,137]. In other models, the engagement of NCRs (especially NKp46) demonstrated to be strictly required for NK cell-mediated cytotoxicity[23,138]. From a clinical perspective, a higher count of peripheral blood NK cells after allo-HSCT is predictive of better overall survival and lower nonrelapse mortality in several hematological neoplasias, including multiple myeloma[139].

Lenalidomide can potently stimulate NK cells antitumor activity *in vitro* by lowering their activation threshold[140]; however, a one-year follow-up study in 10 MM patients contradicted these results[141], implying that the effects of lenalidomide on NK cells need to be further investigated. Moreover, the immunostimulatory activity of lenalidomide is strongly antagonized by dexamethasone, which is often used in combination in relapsed/refractory MM[142].

Proteasome inhibitors such as bortezomib can reduce the expression of MHC class I molecules on MM cells, sensitizing them to the killing by allogenic and autologous NK cells[143]. At the same time, P.I. can directly induce the expression of NKG2D and DNAM-1 ligands on MM cells through the activation of the DNA-damage response[30,144].

The success of mAb-based therapy is largely due to the ability of NK cells to kill through ADCC. Elotuzumab, a humanized IgG1 targeting SLAMF7, exerts a potent anti-myeloma activity mainly through the engagement of CD16 on NK cells. Even though it is not used as monotherapy, the combination Elotuzumab plus pomalidomide and dexamethasone is effective in patients with MM that was refractory to lenalidomide and a proteasome inhibitor[145]

2.4 – The ectoenzyme CD38

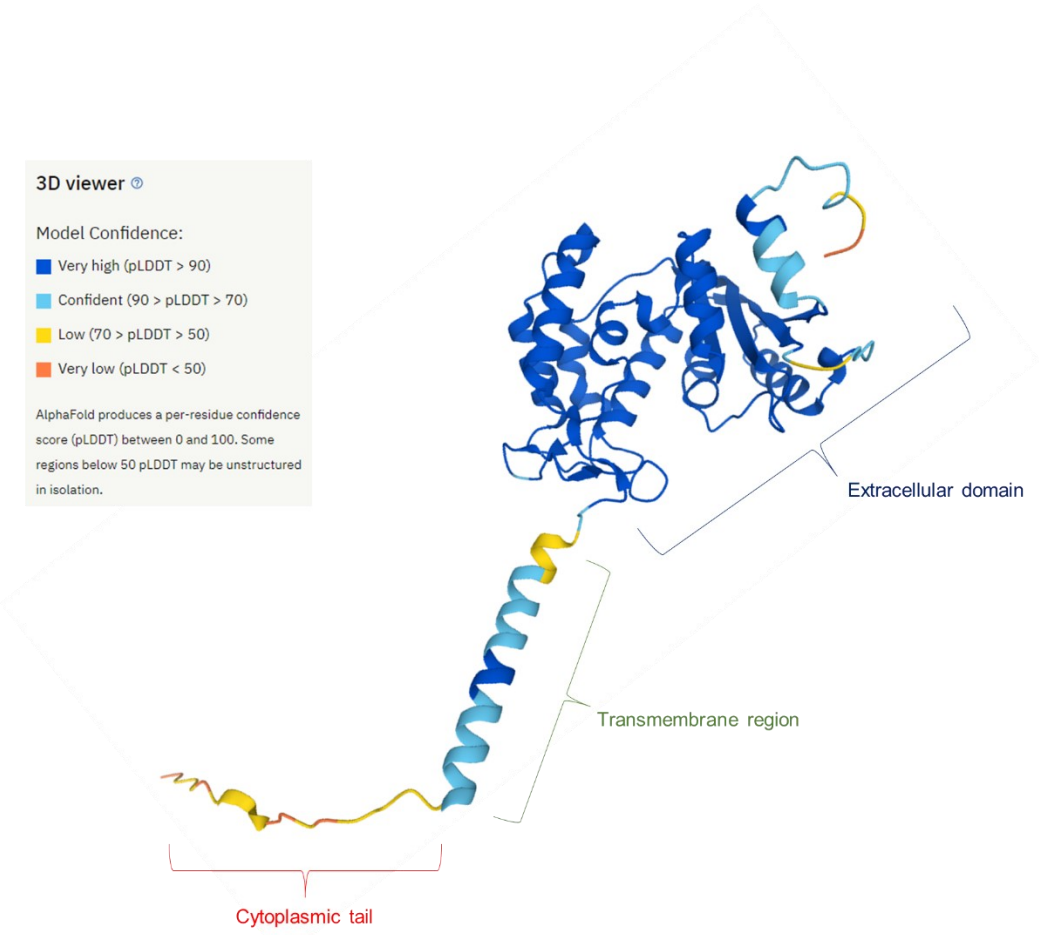
2.4.1 - Expression of CD38 in the immune system

CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1; EC: 3.2.2.6) is a multifunctional receptor and ectoenzyme widely expressed by immune cells and in several non-hematopoietic tissues. On immune cells, its expression is readily induced during inflammation in response to cytokines, interferons and LPS[146,147]. CD38 is also frequently expressed by hematological tumors; for example, it is a negative prognostic marker for chronic lymphocytic leukemia[148] and is expressed at high levels in several lymphomas; in addition, it is the most important target of monoclonal antibody-based therapy in multiple myeloma. The levels of CD38 among immune cells are variable, depending on cell's differentiation and activation status, and organism's age[149]. CD38 is strongly induced on the majority of peripheral blood mononuclear cells, of both lymphoid and myeloid lineage, upon *in vitro* and *in vivo* activation[150]. Interestingly, CD38-positive regulatory immune cells have been found to be highly immunosuppressive in some tumor models, in particular MDSCs in esophageal cancer[151], and a subset of Tregs in multiple myeloma[152].

2.4.2 - Structure and function of CD38

CD38 is a type II/type III transmembrane protein of 300 aminoacidic residues (~45 kDa). It exists in two opposite orientations on the plasma membrane, with the catalytic site facing the extracellular environment (type II CD38) or the cytoplasm (type III CD38)[153]. CD38 is also found on the membranes of ER and within the lysosomes; in particular, type II CD38 exposes the active site in the lumen of the organelles, whereas in type III CD38 the active site is oriented towards the cytosol[154].

Residues 1-21 (N-domain, indicated in red below) constitute a short cytoplasmic tail, whereas residues 22-42 form a helical transmembrane region (indicated in green). The extracellular domain is composed of residues 43-300 (C-domain, indicated in blue) and its tertiary structure is stabilized by six disulfide bonds. The C-domain contains both the catalytic site and the binding site for its counter-receptor.



[1]MANCEFSPVSGDKPCCRLSRR[22]AQLCLGVSILVLILVVVLAV[43]VVPRWRQQWSGPGTTKRFP
ETVLARCVKYTEIHPEMRHVDCQSVWDAFKGAFISKHPCNITEEDYQPLMKLGTQTVPCN
KILLWSRIKDLAHQFTQVQRDMFTLEDTLLGYLADDLTWCGEFNTSKINYQSCPDWRKDC
SNNPVSVFWKTVSRRFAEAACDVVHVMLNGSRSKIFDKNSTFGSVEVHNLQPEKVQTLEA
WVIHGGREDSRDLCQDPTIKELESIISKRNIQFSCKNIYRPDKFLQCVKNPEDSSCTSEI[300]

Figure 5: CD38 protein structure predicted by AlphaFold 2 (https://alphafold.ebi.ac.uk/entry/P28907), an AI-based network that can accurately predict 3D protein structures using solely the primary amino acid sequence and aligned sequences of homologues as inputs (Jumper, J., Evans, R., Pritzel, A. *et al.* Highly accurate protein structure prediction with AlphaFold. *Nature* 596**,** 583–589 (2021)). The structure is mostly equivalent to the one reconstructed through X-ray crystallography.

The protein can be secreted in fully active form, and it is found in the serum of multiple myeloma patients and in the supernatant of lymphoid and myeloid CD38+ tumor cell lines[155].

As a receptor, CD38 interacts with CD31/PECAM-1, mainly expressed by endothelial cells, facilitating the adhesion of CD38+ cells to the endothelium[156]. The binding event triggers a complex

signalling cascade whose biological role is still debated. Importantly, the receptor and enzymatic activity of CD38 are currently considered as totally independent functions[149].

The main enzymatic activity of CD38 described so far is the catabolism of NAD+ (nicotinamide adenine dinucleotide) and NADP (nicotinamide dinucleotide phosphate) into ADPR, cyclic ADPR (cADPR) and NAADP, which are all potent intracellular Ca^{2+}-mobilizing second messengers. In the case of NAADP, under the acidic conditions of endolysosomes (with an optimum at pH = 4) CD38 catalyzes the exchange of the nicotinamide group of NADP with nicotinic acid to generate NAADP[154,157].

CD38 is thought to be the major regulator of NAD+ levels in mammalian cells. Intriguingly, however, an unrelated, non-homologous enzyme called SARM1 (sterile α and Toll/interleukin-1 receptor motif-containing 1), which has a role in pathological axonal degeneration, shares the same catalytic activities with CD38, therefore representing another important NAD-degrading enzyme[154].

CD38 activity seems fundamental for age-related NAD+ decline, which is related to the appearance of metabolic abnormalities[158]; accordingly, the senescence-associated secretory phenotype (SASP), a fundamental feature of the senescent cell, has the capacity to induce CD38 expression in non-senescent cells[159].

The hydrolysis of NAD+ generates ADPR and nicotinamide; ADPR can then be cyclized into cADPR by CD38 itself, which is therefore both a cyclase and a hydrolase. However, CD38 is a very inefficient cyclase and it is estimated that it must degrade nearly 100 molecules of NAD+ to generate one molecule of cADPR[160]. The enzyme is also reported to act on NAD+ precursors like NMN (nicotinamide mononucleotide)[158].

An emerging role of CD38 is the involvement in the generation of adenosine, a metabolite with immunosuppressive function. In particular, CD38 catalyzes the first of a chain of reactions (which is believed to occur mainly in the extracellular environment) that transforms NAD+ into adenosine. First, CD38 generates ADPR, which becomes substrate of the second ectoenzyme ENPP1 (CD203a) that converts it into AMP. A third ectoenzyme, CD73 (5'-nucleotidase, 5'-NT), finally dephosphorylates AMP, generating adenosine. Extracellular ATP can also enter this pathway, becoming substrate of CD39, which can transform it into AMP[161]. However, since CD39 has an

optimum at alkaline pH, it is possible that its function of processing ATP is inhibited in the acidic MM niche[162].

The generation of adenosine through CD38/ENPP1/CD73 is deemed to be particularly relevant in multiple myeloma, in which all the enzymes are expressed conjointly in the same acidic microenvironment[162]. Indeed, MM cells express high levels of CD38 and variable levels of ENPP1; osteoblasts and BMSCs express ENPP1 and CD73. Microvesicles of various cellular origin from diseased BM aspirates can bear these ectoenzymes at elevated density on their surface and are therefore able to generate adenosine in the presence of NAD+ and ATP when tested *in vitro*[163]. Hypoxia, a general feature of MM and several other cancers, further amplifies the generation of adenosine by inducing the accumulation of extracellular ATP and acidification of the microenvironment[164].

Adenosine signals through adenosinergic receptors (also called P1 purinergic receptors), expressed by numerous cell types. For example, signalling through the adenosinergic receptor A2AR impairs the function of NK cells and CD8+ T cells[165]; furthermore, it inhibits T cell proliferation and cytokine production, while increasing surface expression of PD-1 and CTLA4[164].

2.4.3 - CD38-targeted therapy in multiple myeloma

Because of its abundant expression by MM cells, monoclonal antibodies targeting CD38 already entered or are currently being tested in MM therapy. The first approved for relapsed and refractory MM (but sometimes used in newly diagnosed MM as well) is called daratumumab, an IgG1κ human monoclonal antibody; its efficacy is believed to be linked mainly to immune-mediated mechanisms (antibody-dependent cellular cytotoxicity, ADCC; complement-dependent cytotoxicity, CDC; antibody-dependent cellular phagocytosis, ADCP) or direct apoptosis. Daratumumab is also reported to inhibit CD38 cyclase activity while enhancing hydrolase activity[166], although this action does not seem to contribute significantly to its therapeutic effect.

Daratumumab, apart from targeting MM cells, can affect in several ways other CD38+ cell populations. For example, it can reduce the frequency of CD38+ Tregs, a particular subset of Tregs

with enhanced immunosuppressive capacity[152]; unfortunately, daratumumab therapy frequently results in NK cells fratricide because of the high expression of CD38 by NK cells[167].

Contrary to daratumumab, the mAb Isatuximab (recently approved by EMA for relapsed and refractory MM in adults) strongly inhibits both CD38 enzymatic functions (hydrolase and cyclase) by targeting a different epitope of the protein[168].

Pharmacological modulation of CD38 enzymatic activity in cancer immunotherapy is an active area of research, but despite all the information collected so far on its complex biochemistry, its precise function in physiological and pathological conditions remains elusive.

3. Aim

In the last decade, the importance of the cGAS-STING pathway in antitumor innate responses has been established, and STING agonists already entered several clinical trials. However, only recently came the observation that the second messenger cGAMP, the endogenous activator of STING, can be exported by tumor cells and activate bystander immune and stromal cells in a paracrine manner. The immunostimulatory activity of this molecule is restrained by the ectoenzyme ENPP1, which can hydrolyze and therefore inactivate cGAMP in the extracellular environment. These phenomena have been demonstrated in *in vivo* models of solid cancers, but evidence of their occurrence in hematological malignancies like multiple myeloma (MM) is currently lacking. For this reason, the first aim of my Ph.D. research project was to investigate whether human MM cells are able to release cGAMP under basal conditions and upon drug treatment. Secondly, with the aim of understanding the possible contribution of extracellular cGAMP in anti-myeloma immune response, I studied how NK cells from healthy donors and BMSCs from MM patients respond to exogenous cGAMP, focusing on the killing ability of NK cells and cytokine production by BMSCs.

Finally, with the goal of increasing the stability, and thus the efficacy, of extracellular cGAMP, I sought to determine whether the inhibition of CD38, an ectoenzyme related to ENPP1 and overexpressed by MM cells, could have an impact on extracellular cGAMP levels. Provided preliminary *in vitro* data of a possible role of CD38 in the catabolism of cGAMP, I accumulated additional evidence at cellular and biochemical level aiming at demonstrating the previously unreported capacity of CD38 to degrade cGAMP.

4. Materials and methods

4.1 - Cell lines and clinical samples

The human multiple myeloma (MM) cell lines SKO-007(J3), RPMI-8226, ARK and the human NK cell line NKL were kindly provided by Prof. P. Trivedi ("Sapienza" University of Rome, Italy). THP-1 cells were kindly provided by Prof. John Hiscott (Istituto Pasteur Italia). All the cell lines were maintained in RPMI-1640 (Life Technologies, Gaithersburg, MD) supplemented with 15% Fetal Calf Serum (FCS, Gibco), 2 mM glutamine and 100 U/mL penicillin 100 µg/mL streptomycin at 37°C and 5% CO_2. NKL cells were maintained in complete medium enriched with recombinant human IL-2 (200 U/ml; PeproTech, London, UK). All the cell lines were maintained in culture for no more than 5 weeks and were periodically tested for the absence of mycoplasma contamination (ABM Mycoplasma PCR detection Kit; Applied Biological Materials Inc.).

Bone marrow samples from MM patients with different diagnoses were managed at the Department of Cellular Biotechnologies and Hematology, Institute of Hematology ("Sapienza" University of Rome, Italy). Informed consent in accordance with the Declaration of Helsinki was obtained from all patients, and approval was obtained from the Ethics Committee of the "Sapienza" University of Rome. BM mononuclear cells (BMMCs) and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient (Lympholyte, Euroclone); red blood cells were lysed with a buffer composed of 1.5M NH_4Cl, 100mM $NaHCO_3$, and 10mM EDTA.

Bone marrow stromal cells were isolated through plastic adhesion from untreated BMMCs in MEMα medium supplemented with 10% Fetal Calf Serum (FCS), 2 mM glutamine and 100 U/mL penicillin 100 µg/mL streptomycin at 37°C and 5% CO_2. In line with the International Society for Cellular Therapy (ISCT) recommendations, a combination of antibodies, including anti-CD45/APC-H7, anti-CD90/PeCy5, anti-CD105/APC, anti-CD146/BV395, anti-CD73/V450 PEA, anti-CD106/PE, was used to phenotypically characterize BMSCs by multiparameteric flow cytometry. The immunophenotype was analysed using a FACS LRSFORTESSA flow cytometer (BD Biosciences, San Jose, CA, USA).

Primary PCs were maintained at 37°C and 5% CO_2 in complete RPMI medium (or in medium without FCS for ELISA experiments) supplemented with 20 ng/mL human recombinant IL-3 and 2 ng/mL human recombinant IL-6 (PeproTech).

4.2 - Isolation of malignant PCs from MM patients and NK cells from healthy donors

Percentage of CD38/CD138 double-positive cells among BMNCs, representing malignant plasma cells, was determined by flow cytometry. CD138-positive malignant PCs were isolated from BMNCs by using anti-CD138 conjugated magnetic beads (CD138 MicroBeads, human, Miltenyi Biotec) according to the manufacturer's protocol. Similarly, NK cells (CD56+CD3-) were isolated from peripheral blood mononuclear cells through negative selection with a cocktail of biotin-conjugated monoclonal antibodies against antigens not expressed by NK cells and streptavidin-conjugated magnetic beads (NK Cell Isolation Kit, Miltenyi Biotec). A buffer composed of PBS w/o calcium and magnesium, 0.5% Bovine Serum Albumine, 2 mM EDTA was used for the magnetic separation. For all the subsequent experiment, the purity of PCs (CD38+CD138+) and NK cells (CD56+CD3-) was greater than 85%.

4.3 - Drugs and chemicals

Doxorubicin and melphalan were purchased from Sigma Aldrich, bortezomib and carfilzomib from Selleckchem. 2'3'-cGAMP and 2'3'-cG^{s}A^{s}MP were purchased from Invivogen, CD38 Inhibitor 78C (CAS 1700637-55-3) from Calbiochem, ENPP1 inhibitor C (CAS 2378640-92-5) from Cayman Chemical, NAD from Sigma-Aldrich.

4.4 - Flow cytometry and antibodies

For flow cytometry experiments, cells in PBS w/o Ca and Mg were incubated with antibodies directed against surface proteins for 30 minutes at 4°C. For the staining of intracellular proteins, cells were fixed and permeabilized with Fixation/Permeabilization kit (BD Cytofix/Cytoperm) according to the manufacturer's protocol and then incubated with antibodies for intracellular proteins for 30 minutes at 4°C. Acquisition was performed on a BD FACSCanto II cytometer.

The following antibodies were used for flow cytometry: anti-CD138/FITC (clone MI15, BD Biosciences); anti-CD38/APC (clone HIT2, BD Biosciences); anti-CD38/PE (clone HIT2, BD Biosciences); anti-perforin/BV421 (clone δG9, BD Biosciences); anti-γH2AX (Ser139)/FITC (clone 2F3, BioLegend); anti-granzyme B/FITC (clone GB11, BioLegend); anti-ENPP1/APC (polyclonal sheep IgG, R&D), polyclonal sheep IgG/APC (R&D). The following antibodies from BD Biosciences were used for validating the immunophenotype of BMSCs: anti-CD45/APCH7 (clone 2D1), anti-CD105/APC (clone 266), anti-CD90/PeCy5 (clone 5E10), anti-CD146/BV395 (clone P1H12), anti-CD106/PE (clone 51-10C9) and anti-CD73/V450 (clone AD2).

For the exclusion of dead cells, the following dyes were used: BD Horizon Fixable Viability Stain 780 (APC-H7); Zombie Green Fixable Viability Kit (BioLegend).

For the quantification of Senescence-associated β-galactosidase activity, the substrate C_{12}FDG (5-Dodecanoylaminofluorescein Di-β-D-Galactopyranoside) was used for flow cytometric experiments. Cells were incubated 1h at 37°C and 5% CO_2 with bafilomycin A1 100 nM (Sigma) in culture medium to induce lysosomal alkalinization at pH 6 and then for 1h with C_{12}FDG 33 μM (Invitrogen). The C_{12}FDG signal was measured on the FITC detector, and β-galactosidase activity was estimated using the median fluorescence intensity (MFI) of the population.

For the detection of apoptotic cells, APC Annexin V Apoptosis Detection kit with PI (propidium iodide) (BioLegend) was used according to the manufacturer's protocol. Early apoptotic cells are Annexin V-positive PI-negative, whereas late apoptotic and necrotic cells are double-positive.

All flow cytometry data were analyzed using FlowJo Software (BD Biosciences).

4.5 - Cytotoxicity assay with 7-aminoactinomycin D (7AAD)

SKO-007(J3) human MM cells were labeled with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester 2.5 μM (CFSE, Sigma Aldrich) in PBS for 10 minutes. Purified NK cells from healthy donors (effector) were cocultured with CFSE-labeled SKO-007(J3) cells (target) for 4 hours at 37°C and 5% CO_2 at different effector:target ratios. At the end of the coculture, cells were washed once with PBS 1% BSA and then stained with 7AAD 5 μg/mL (Sigma Aldrich) for 20 minutes at 4°C. At the end, the

percentage of dead target cells (CFSE and 7AAD double positive, respectively on FITC and PerCP detectors) was measured by flow cytometry.

4.6 - SDS-PAGE and Western Blot

For Western-Blot analysis, MM cell lines, purified patient-derived myeloma cells and purified NK cells from healthy donors were pelleted, washed once with cold PBS, resuspended in lysis buffer [2.5 mM EDTA, 2.5 mM EGTA, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1.0% Triton-X-100, 5 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, Protease Inhibitor Cocktail 1X (Sigma-Aldrich, St. Louis, Missouri, USA), Phosphatase Inhibitor Cocktail 3 1X (Sigma-Aldrich)] and then incubated for 30 minutes on ice. The lysates were centrifuged at 16,000 g for 20 min at 4°C and the supernatants were collected as whole-cell extract. Protein concentration was determined through Bradford Protein Assay (Bio-Rad). 10 to 40 µg of cell extracts were run on 10% denaturing SDS-polyacrylamide gels. Proteins were then electroblotted onto Amersham Protran nitrocellulose membranes (GE Healthcare Life Science), stained with Ponceau to verify that similar amounts of proteins had been loaded in each lane, and blocked with 5% BSA or non-fat dry milk in TBST buffer for 1h. Immunoreactive bands were visualized using horseradish peroxidase-linked/coupled donkey anti-rabbit (NA934V) or sheep anti-mouse (NA931V) IgG (Amersham, GE Healthcare Life Science) and the ECL substrate WESTAR ηC ULTRA 2.0 (Cyanagen).

Primary antibodies used: rabbit anti-human phospho NF-κB p65 (Ser536) (clone 93H1, Cell Signaling Technology), rabbit anti-human cGAS (clone D1D3G, Cell Signaling Technology), rabbit anti-human IκBα (C-21, Santa Cruz Biotechnology), rabbit anti-human p65/RelA (C-20, Santa Cruz Biotechnology), mouse anti-human β-Actin (clone AC-15, Sigma-Aldrich), rabbit anti-human PI3 Kinase p85, N-SH2 domain (ABS233, Merck Millipore).

The ImageLab software version 5.2.1 was used for image acquisition and densitometric analysis of the membranes using a ChemiDoc MP System (Bio-Rad, Hercules). Target protein levels were referred to β-Actin or p85, chosen to normalize protein expression. For phospho-p65, the signal was normalized against p85-normalized total p65.

4.7 - ELISA for 2'3'-cGAMP and IFN-β detection

To quantify extracellular cGAMP produced by MM cells, a commercial competitive ELISA kit was used (2'3'-cGAMP ELISA Kit, Cayman Chemical) according to the manufacturer's protocol. The conditioned medium, obtained in absence of FCS, was tested undiluted in duplicate or triplicate. Calculations were performed with the use of a ready-made spreadsheet provided by the manufacturer (available at www.caymanchem.com/analysis/elisa). The R^2 of the standard curve was always greater than 0.95.

IFN-β secreted in the CM by BMSCs was quantified using a DuoSet ELISA (R&D) according to the manufacturer's protocol.

4.8 - Reverse-transcription (RT) and Real time PCR

Total RNA was extracted with Trizol (Invitrogen) and chloroform/ethanol precipitation or with Total RNA mini kit (Geneaid). RNA concentration was measured with a Nanodrop spectrophotometer (ThermoFisher Scientific). When used for real time amplification of *IFN-β*, RNA was treated with DNase I (DNase I Amplification Grade, Sigma-Aldrich) before RT-PCR.

Total RNA (300 ng to 1 µg) was used for cDNA first- strand synthesis using oligo-dT, dNTPs and M-MLV Reverse Transcriptase with its 5X buffer (Promega, Madison, WI) in a 25 µL reaction volume.

Real time PCR was performed using SYBR Green (Master Mix: Power SYBR Green PCR Master Mix, Applied Biosystems) for amplification of *IFN-β* and *GAPDH* or TaqMan probes (Master Mix: 2x SensiFAST Probe Hi-ROX Mix, Meridian Bioscience) for all other genes.

TaqMan probes: *CD38*: Hs01120071_m1, *cGAS*: 00403553_m1, *CXCL10*: Hs00171042_m1, *IL-15*: Hs00174106_m1, *GAPDH*: Hs02758991_g1. Primers *IFN-β*: forward: TAGCACTGGCTGGAATGAG, reverse: GTTTCGGAGGTAACCTGTAAG.

The genes *CCL5*, *IRF7*, *IFI16*, *IFN-α1* and the housekeeping gene *GUSB* were amplified using a TaqMan Array 96-well FAST Plate, Custom format 16 (Applied Biosystems) with the master mix SensiFAST Probe Lo-ROX Kit (Bioline). TaqMan Probes: *GUSB* Hs99999908_m1; *CCL5* Hs99999048_m1; *IRF7* Hs01014809_g1; *IFI16* Hs00986764_m1; *IFN-α1* Hs04189288_g1.

Results were analyzed by subtracting, for each condition, the average Ct of the samples to the average Ct of housekeeping genes (GAPDH and GUSB) and were reported as fold change in respect to a reference condition, calculated with the method of $2^{-\Delta\Delta Ct}$.

4.9 - Quenching Fluorescence analysis

Fluorescence quenching measurements were performed a on Cary Varian Eclipse fluorescence spectrophotometer (Agilent) equipped with a Peltier thermostatted system (Thermo Fisher Scientific) using a 10-mm path length quartz cell with a Teflon stopper (quartz cuvette of 1 cm path length).

The recombinant human His-tagged CD38 protein (Sino Biological) at the fixed concentration of 1 μM (1 pmol/μL = 400 pmol in total) was titrated in 25 mM phosphate pH 7.5, 50mM NaCl, with increasing concentrations of ligands 2'3'-cGAMP, 2'3'-cG^{s}A^{s}MP and NAD dissolved in the same buffer. The Trp fluorescence spectra of rhCD38 in the absence and presence of ligands were acquired at 25.0 °C, using an excitation wavelength of 295.0 nm to avoid interference with tyrosine residues, and a fluorescence emission wavelength ranging from 300 nm to 500 nm. The excitation and emission slits were set at 5 nm; the scan rate was 120 nm/min; the data interval was 1.00 nm; the averaging time was set at 0.500s. The samples were allowed to equilibrate for 1 min prior to measurement. The emission spectra of the ligand solutions were acquired under the same conditions, using solutions up to 205 μM, in order to exclude interference from ligands intrinsic fluorescence.

4.10 - Analysis of fluorescence quenching data and determination of dissociation constant

The fluorescence values recorded at 345 nm were extracted, normalized to the intrinsic fluorescence of rhCD38, and then plotted as average trace against the ligand concentrations. The value of the dissociation constant (K_D) for different ligands were calculated by fitting fluorescence quenching data using a non-linear regression analysis on Prism 5.0 (Graph Pad Software).

4.11 - Recording of CD38 Tryptophan fluorescence changes over time

The fluorescence assays were performed on an EnSpire™ Multimode Plate Reader (PerkinElmer) using 96 wells black plate in 100 µL reaction volume. All assays were conducted using rhCD38 protein at the fixed concentration of 1 µM and mixed with 2'3'-cGAMP or 2'3'-cG^{s}A^{s}MP at 10 µM. The Trp fluorescence signal was recorded at different time points using excitation and emission wavelengths of 295 and 400–600 nm, respectively. Experiments were performed in triplicate and the resulting data averaged.

4.12 - RP-HPLC kinetics analysis

Reverse-phase HPLC analysis were conducted using a 5 µm X-Bridge C18 (50 x 21.2 mm) column (Waters). Standard dinucleotides and reaction mixtures, obtained mixing 50 pmol of CD38 to 5 nmol of dinucleotides in 50 µL of buffer, were analysed (5 µL) applying a linear gradient from 0% to 100% methanol in 25 minutes (buffer B) over buffer A (20 mM KH2PO4 and 5 mM tetrabutylammonium dihydrogen phosphate pH=6). Analyses were performed at 20 °C at a flow rate of 0.2 mL/min. Eluate was detected by UV absorption at λ=260 nm with a diode array detector.

4.13 – ESI-TOF mass spectrometry

Mass spectrometry analyses were performed with an Agilent 1290 Infinity LC System coupled to an Agilent 6230 time-of-flight (TOF) LC/MS System. The mass analyzer Agilent 6230 TOF-MS was set to operate in negative ion scan mode with mass scanning from 120 to 1000 m/z. Mass spectrometry data were acquired by direct infusion by means of a syringe pump working at flow rate of 5 µL/min. The instrument parameters were set as follows: drying gas temperature, 250°C; drying gas flow, 5 L/min; nebulizer, 20 psi; sheath gas temperature, 400°C; sheath gas flow, 11 L/min; VCap. 3.500; Vnozzle, 0 V; fragmentor, 150 V; skimmer, 65 V; octapole RF Vpp was 750. Data collection and integration were performed using the MassHunter workstation software (version B.05.00) and the Agilent MassHunter Qualitative software, respectively.

5. Results

5.1 - Doxorubicin induces MM cell lines to secrete cGAMP in the extracellular environment

cGAMP is mainly known as an intracellular second messenger acting in a cell-autonomous manner. However, it was recently demonstrated that tumor-derived cGAMP secreted in the TME can evoke a potent immune response; later, different types of channel proteins that can transport cGAMP across the plasma membrane were discovered. These findings raised great interest around the biological role and regulation of extracellular cGAMP [66,81,86,87,89].

For this reason, the ability of multiple myeloma cell lines to secrete cGAMP in the extracellular environment was investigated through a commercially available competitive ELISA. Treatment of multiple myeloma cell lines SKO-007(J3) and RPMI-8226 with the genotoxic drug doxorubicin (Dox) for 24 hours caused a pronounced release of cGAMP in the conditioned medium (**Figure 1B**), whereas other drugs and stimuli used in MM or cancer therapy, including melphalan, bortezomib, carfilzomib and ionizing radiation did not (**Figure 1A**). The time of stimulation was short enough to minimize cell death (**Figure 1B**), implying that cGAMP is actively produced and released by living cells rather than passively emanated by dead cells, as previously demonstrated[87]. Dox administration, however, did not cause significant release of cGAMP in ARK cells (**Figure 1B**) despite their relatively high expression of cGAS (**Figure 1D**), suggesting reduced export and/or lower stability of extracellular cGAMP in these cells.

MM cells basally express high levels of cGAS (**Figure 1E**). Interestingly, Dox treatment further increased the expression of the *cGAS* gene approximately by twofold in SKO-007(J3), RPMI-8226, ARK and primary plasma cells from MM patients, as evaluated by Real Time PCR (**Figure 1C**). A similar trend was observed also for the cGAS protein through western blot (**Figure 1D**). Collectively, these results suggest that Dox amplifies the production and secretion of cGAMP not only by inducing DNA damage, but also by regulating the amount of the sensor protein cGAS at the transcriptional level.

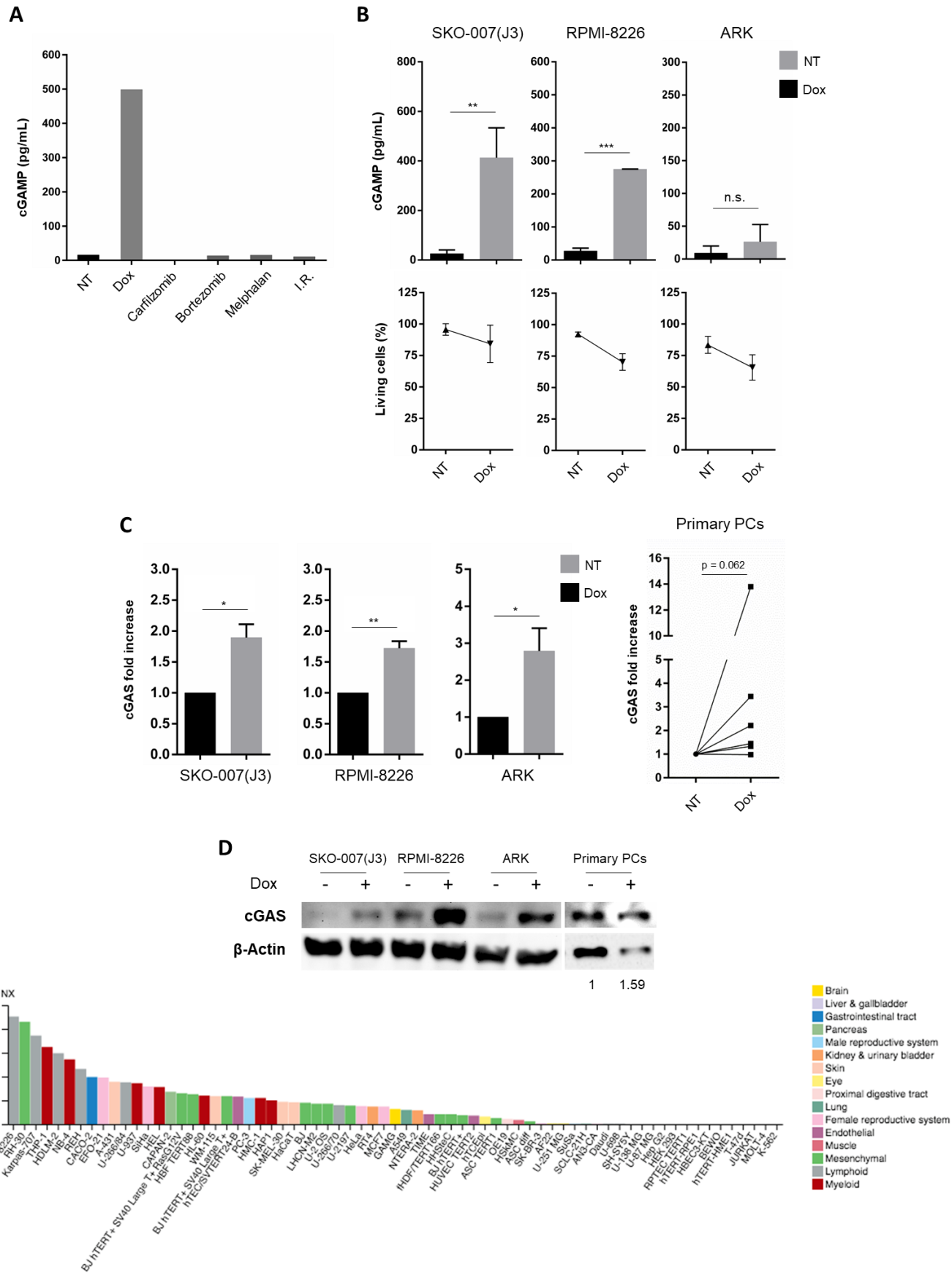

A
cGAMP (pg/mL)
NT
Dox
Carfilzomib
Bortezomib
Melphalan
I.R.
B
SKO-007(J3)
RPMI-8226
ARK
NT
Dox
cGAMP (pg/mL)
Living cells (%)
n.s.
C
cGAS fold increase
SKO-007(J3)
RPMI-8226
ARK
Primary PCs
p = 0.062
D
SKO-007(J3)
RPMI-8226
ARK
Primary PCs
Dox
cGAS
β-Actin
1
1.59
E
NX
Brain
Liver & gallbladder
Gastrointestinal tract
Pancreas
Male reproductive system
Kidney & urinary bladder
Skin
Eye
Proximal digestive tract
Lung
Female reproductive system
Endothelial
Muscle
Mesenchymal
Lymphoid
Myeloid

Figure 1: **Doxorubicin treatment induces the release of cGAMP in the extracellular environment and increases the expression of cGAS in MM cells**. A) SKO-007(J3) cells were treated for 24h with Dox 2 μM, carfilzomib 18 nM, bortezomib 2 nM, melphalan 1 μM and ionizing radiation (25 Gy); the medium was then replaced with serum-free medium; after another 24h, cGAMP in the supernatant was quantified through competitive ELISA (the total time of Dox stimulation was therefore 24h in the presence of the drug + 24h without the drug). B) Top: RPMI-8226 cells secrete cGAMP in the extracellular environment upon stimulation with Dox 1 μM, similarly to SKO-007(J3); however, ARK cells do not release significant amounts of cGAMP under the same conditions. Bottom: Dox treatment for 48h did not cause massive cell death, suggesting that cGAMP is exported by living cells. C) SKO-007(J3), RPMI-8226, ARK and primary MM cells (patients 11-16) upregulate the expression of *cGAS* upon 24h+24h Dox administration as measured through Real Time PCR. D) Western blot analysis of cGAS protein in total cellular lysate of SKO-007(J3), RPMI-8226, ARK and primary MM cells (patient 15). Error bars represent SD; statistical significance was calculated using two-tailed paired Student's T test; * $p \leq 0.05$, ** $p \leq 0.01$. E) Expression of *cGAS* mRNA across several cell lines of different origin. Multiple myeloma cells express *cGAS* at the highest levels. Source: The Human Protein Atlas (https://www.proteinatlas.org/ENSG00000164430-CGAS/cell+line).

Patient	Sex	Age	Clinical stage	PCs in BM
1	F	89	Smoldering	17%
2	M	59	Relapse	1%
3	F	69	MGUS	7%
4	M	85	Onset	26%
5	M	74	Onset	24%
6	F	77	Relapse	39%
7	F	76	MGUS	15%
8	F	83	Onset	43%
9	M	68	Extramedullary relapse	1%
10	F	69	MGUS	8%
11	F	69	Onset	38%
12	F	83	Onset	19%
13	M	63	Onset	30%
14	F	70	Onset	24%
15	F	82	Onset	36%
16	M	83	Relapse	17%
17	F	82	Onset	35%
18	M	76	Onset	2%

Table 1: Main clinical features of multiple myeloma patients whose BM samples were used in the present work.

5.2 - Bone marrow mesenchymal stromal cells (BMSCs) from MM patients respond to exogenous cGAMP by secreting IFN-β

Once demonstrated the ability of MM to secrete cGAMP, the following goal was to investigate whether this molecule is active in its soluble form on BMSCs, NK cells and MM cells.

Only the expression of a specific set of channel proteins (e.g. SLC19A1, LRRC8) allows a cell to import cGAMP in the cytoplasm, where STING resides. Nonetheless, most authors make use of digitonin-based permeabilizing buffers in order to force the double-negatively charged cGAMP inside the cell and provoke a detectable response. This method was not employed in the following experiments, being unnecessarily artificial.

To date, it is unknown whether BMSCs can uptake and respond to extracellular cGAMP. Nevertheless, there is evidence of the expression of ENPP1 by BMSCs[162], which could in principle restrain extracellular cGAMP activity on this cell type.

BMSCs from MM patients with different diagnoses were isolated by exclusion of non-adherent cells and validated using multiparametric flow cytometry as described in Materials and methods.

To verify their capacity to respond to extracellular cGAMP, BMSCs were treated with purified cGAMP for 16 hours; the expression of *IFN-β* was then evaluated by Real Time PCR (**Figure 2A**), whereas the secretion of the cytokine was measured by ELISA (**Figure 2B**). Untreated cells did not secrete measurable amounts of IFN-β; however, after treatment with cGAMP 500 nM, a strong transcriptional induction accompanied by protein secretion in the conditioned medium was observed. Nevertheless, the amount of IFN-β secreted upon treatment was highly variable among the four samples.

Treatment of BMSCs with higher concentration of cGAMP (5 μM) allowed to detect a significant upregulation of *CXCL10* and *IL-15* mRNA that was not observed at lower concentration, indicating that transcription of these genes requires a stronger activation of STING in BMSCs (**Figure 2C**).

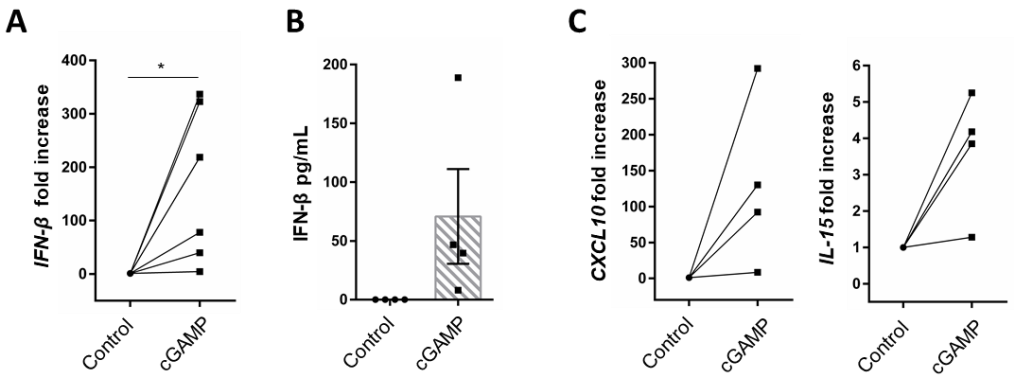


Figure 2: BMSCs from MM patients are responsive to exogenous cGAMP without prior permeabilization.

A) and B) Treatment with cGAMP 500 nM for 16h (in serum-free medium) induces the transcription of *IFN-β* gene and the secretion of the protein as evaluated by Real Time PCR and ELISA, respectively (patients 1,2,7,8). C) A higher concentration of 5 μM cGAMP is required to upregulate the transcription of *CXCL10* and *IL-15* in BMSCs (patients 3, 4, 17, 18). Error bars represent SD; statistical significance was calculated using two-tailed paired Student's T test; * $p \leq 0.05$.

5.3 - Natural killer cells from healthy donors increase their cytotoxic potential against MM cells upon cGAMP treatment

It has been reported that type 1 IFNs produced by stromal cells in response to cGAMP can activate NK cells[86]; however, the response of NK cells to cGAMP itself is not known. Considering that NK cells express relatively high levels of STING[169], a transcriptional response mediated by either IRF3 or NF-κB should be expected upon cGAMP treatment in these cells. Therefore, freshly purified and ex vivo-expanded NK cells from healthy donors were treated with cGAMP for 16 hours; the effect on their cytotoxicity was evaluated by a 4-hour co-culture with CFSE-marked MM cell line SKO-007(J3). At the end of the co-culture, cells were stained with 7-aminoactinomycin D (7AAD) and the percentage of killed CFSE-positive MM cells was measured by flow cytometry. Freshly purified NK cells show a dramatic increase in their cytotoxicity following treatment with 1 μM cGAMP (**Figure 3A, right**). Such effect is less evident for ex vivo-expanded NK cells (**Figure 3A, left**), possibly because of their basally activated state.

To investigate the mechanism behind this phenomenon, a flow cytometric panel was designed to evaluate the expression changes, following cGAMP treatment, of important activating receptors and

proteins involved in the killing: NKG2D, DNAM-1, NKp46, NKp30, CD16, perforin and granzyme B. $CD56^{dim}$ NK cells were selected for the analysis since they represent the most cytotoxic subset[95] (**Figure 3B**).

A subtle but reproducible augment in perforin and granzyme B content was revealed in cGAMP-treated NK cells (**Figure 3C**), whereas no similar trends were observed for the activating receptors (not shown).

Both perforin and granzyme B are known target genes of the NF-κB complex[171,172], which is activated downstream of STING together with IRF3. Upon cGAMP treatment, phosphorylation of p65 was revealed by western blot, coupled with a partial and very fast degradation of the inhibitor IκBα, suggesting the activation of the NF-κB pathway (**Figure 3D**).

Collectively, these results demonstrate that cGAMP can increase NK cells cytotoxicity possibly by upregulating the levels of perforin and granzyme B through NF-κB transcriptional regulation.

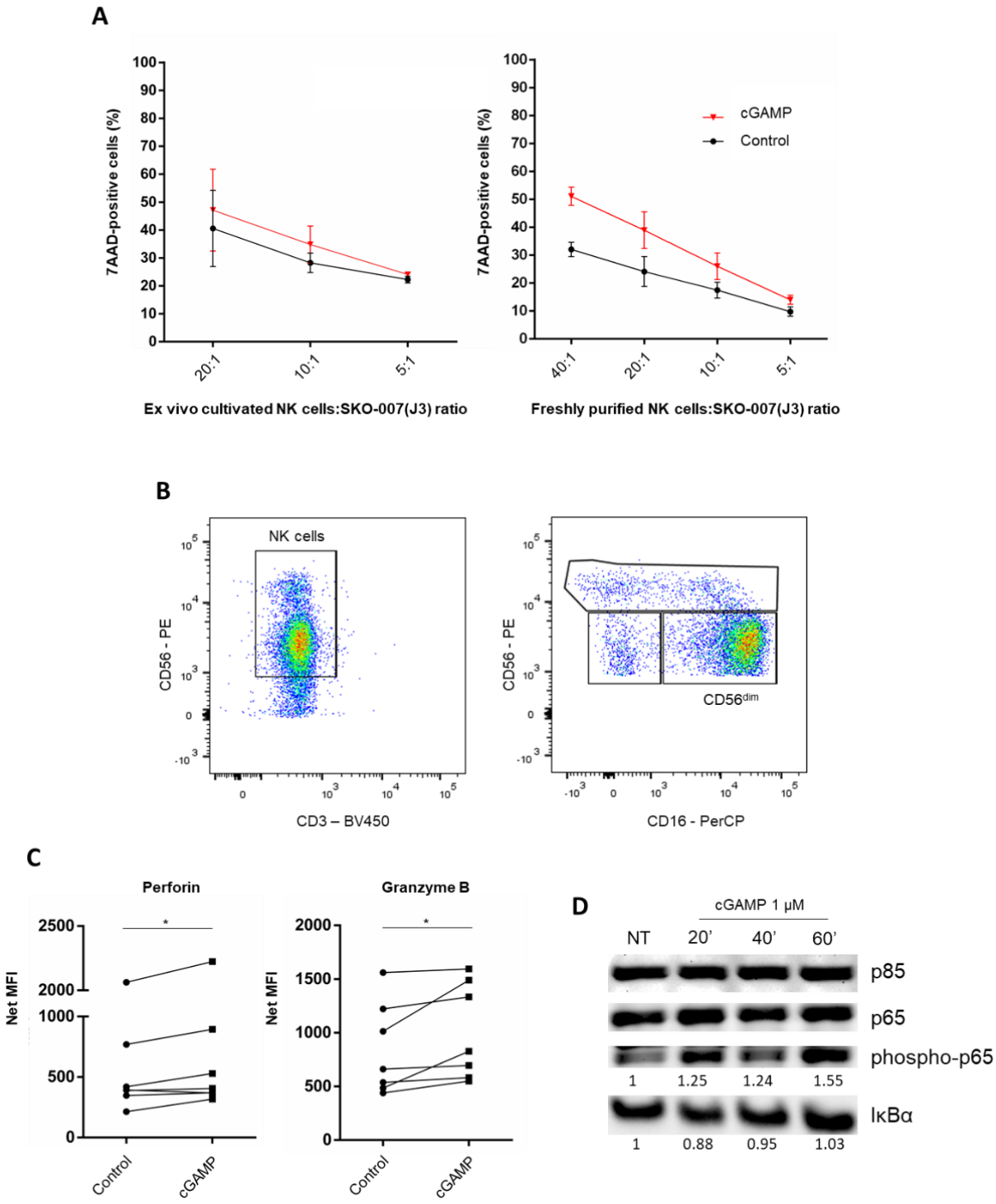


Figure 3: **NK cells increase their cytotoxicity towards MM cells in response to treatment with exogenous cGAMP possibly by increasing perforin and granzyme B content.** A) Coculture of SKO-007(J3) MM cell line with *ex vivo* cultivated (left) or freshly purified (right) NK cells from healthy donors (n=4), untreated or treated with cGAMP 1 µM for 16h. B) and C) Representative gating strategy for selecting $CD56^{dim}$ NK cells for cytofluorimetric analysis of intracellular perforin and granzyme B content. The conditions of treatment were the same used for the cytotoxicity assay. D) Western

blot analysis of total cellular lysates of primary NK cells from one healthy donor treated with cGAMP 1 μM for 20, 40 and 60 minutes. The fast degradation of IκBα together with the phosphorylation of p65 at Ser536 suggest a partial activation of NF-κB pathway. For phospho-p65, the signal was normalized against p85-normalized total p65.

Error bars represent SD; statistical significance was calculated using two-tailed paired Student's T test; * $p \leq 0.05$.

5.4 - MM cell line SKO-007(J3) undergoes apoptosis when challenged with high doses of cGAMP

It has been reported that STING activation with the agonist 3'3'-cGAMP (20 μM) triggers mitochondria-mediated apoptosis in mouse MM cells[93]. Whether the same molecule or the endogenous cGAMP can have a similar effect in human MM cells is not known.

Therefore, SKO-007(J3) cells were treated with cGAMP or the isomer 3'3'-cGAMP 20 μM for 48 hours; the percentage of apoptotic cells was then evaluated by flow cytometry with Annexin V/Propidium Iodide double staining. cGAMP-treated cells showed a substantial increase in the proportion of early apoptotic cells compared to untreated cells; 3'3'-cGAMP similarly increased the percentage of apoptotic cells, albeit to a lesser extent than cGAMP (**Figure 4A**). On the other hand, treatment with lower doses of cGAMP for 5 days (1 μM and 5 μM) did not cause apoptosis, but significantly increased β-galactosidase activity, a typical marker of cellular senescence (**Figure 4B**). Interestingly, this senescent-like phenotype induced by cGAMP was not associated with phosphorylation of histone H2AX (also indicated as γH2AX), indicating absence of DNA double-strand breaks (**Figure 4C**), in contrast to a recent report[172].

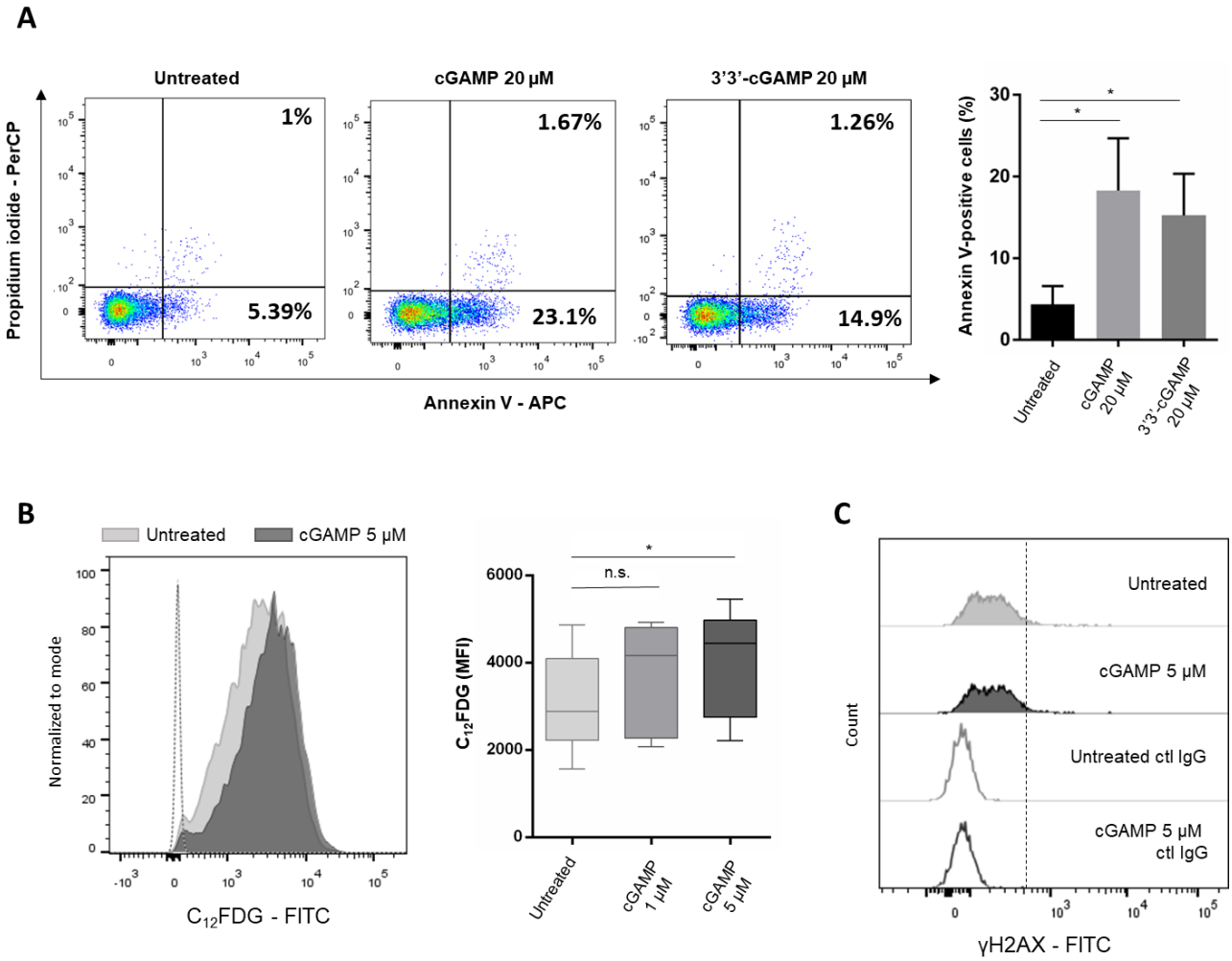


Figure 4: Treatment of SKO-007(J3) cells with high dose of cGAMP induces apoptosis and elevated SA-β Gal activity in absence of DNA damage. A) SKO-007(J3) cells were incubated with cGAMP or 3'3'-cGAMP 20 µM for 48h; the percentage of Annexin V-positive early apoptotic cells was then evaluated through flow cytometry. B) SKO-007(J3) cells were incubated with cGAMP 1 µM and 5 µM for 5 days; SA-β Gal activity was quantified by flow cytometry using the fluorogenic substrate C_{12}FDG (which is cleaved by β-galactosidase) and expressed as increase in mean fluorescence intensity (MFI). C) Under the same conditions, cGAMP-treated cells do not show greater γH2AX positivity compared to untreated cells, suggesting that the DNA Damage Response (DDR) is not involved in the increased β Gal activity.

The results shown are representative of at least three independent experiments, except for C) which represents a single experiment. Error bars represent SD; statistical significance was calculated using one-way ANOVA; * $p \leq 0.05$.

5.5 - Inhibition of CD38 causes accumulation of extracellular cGAMP

Although Dox treatment strongly induced the secretion of cGAMP, the amounts produced by MM cells are still far from the doses of exogenous cGAMP tested in the aforementioned experiments. Therefore, in order to find a strategy to further boost accumulation of extracellular cGAMP, inhibition of the ectoenzyme ENPP1 on Dox-treated MM cells was tested using the commercial inhibitor C (CAS number: 2378640-92-5). However, addition of ENPP1i at a concentration of 10 μM on Dox-treated SKO-007(J3) cells did not result in a significant increase in extracellular cGAMP (not shown). Accordingly, flow cytometric measurement of surface ENPP1 on SKO-007(J3), RPMI-8226 and ARK showed that these human MM cell lines do not express ENPP1 on the plasma membrane at detectable levels. Primary PCs from patients, on the other hand, express variable levels of ENPP1 on their membrane, ranging from undetectable to low (**Figure 5A**). It is worth noting that some authors reported a higher expression of ENPP1 on PCs from MM patients[163].

Since MM cells are known to express high levels of CD38 (**Figure 5D**), a hydrolase that recognizes several substrates structurally and chemically similar to those of ENPP1, it was tested whether CD38 inhibition may impact the stability of extracellular cGAMP.

Strikingly, when a commercial inhibitor of CD38 was used (CAS number: 1700637-55-3), significantly higher levels of extracellular cGAMP were detected through competitive ELISA in conditioned medium from Dox-treated SKO-007(J3) and ARK (**Figure 5B**). Addition of CD38i on untreated cells did not result in a significant increase of cGAMP levels, implying that the stimulus with Dox is strictly necessary to activate cGAS in these cells. Compared to ARK cells, SKO-007(J3) cells release higher levels of cGAMP and express much lower levels of CD38 after Dox treatment (**Figure 5D**). It is possible that these two observations are causally related.

The result obtained with the inhibitor suggested that CD38 could be directly implicated in the stability of extracellular cGAMP. Being CD38 a hydrolase, I raised the hypothesis that the enzyme may directly degrade cGAMP through hydrolysis. It is important to note that, since the inhibitor is cell-permeable, it was not possible to determine whether the degradation of cGAMP occurred inside the cell or in the extracellular environment.

To evaluate whether the treatment with doxorubicin can influence the expression of CD38 in MM cells, Real time PCR and flow cytometric measurements of CD38 surface levels were performed in SKO-007(J3), RPMI-8226, ARK and in primary PCs from MM patients. Untreated SKO-007(J3) express almost undetectable levels of CD38 on their surface; however, upon Dox treatment a strong transcriptional induction is accompanied by increased surface protein levels (**Figure 5C and D**). RPMI-8226 basally express moderate levels of CD38; treatment with Dox upregulates its transcription and protein levels, albeit to a lesser extent compared to SKO-007(J3). In ARK cells, curiously, the considerable upregulation of surface CD38 upon Dox treatment was not accompanied by a parallel upregulation of the gene at the transcriptional level, indicating that other mechanisms may regulate CD38 exposure on the membrane in these cells. Finally, primary PCs do not seem to respond to Dox treatment by altering CD38 expression, although the small dimension of the data sample may have impeded to observe any significant effect.

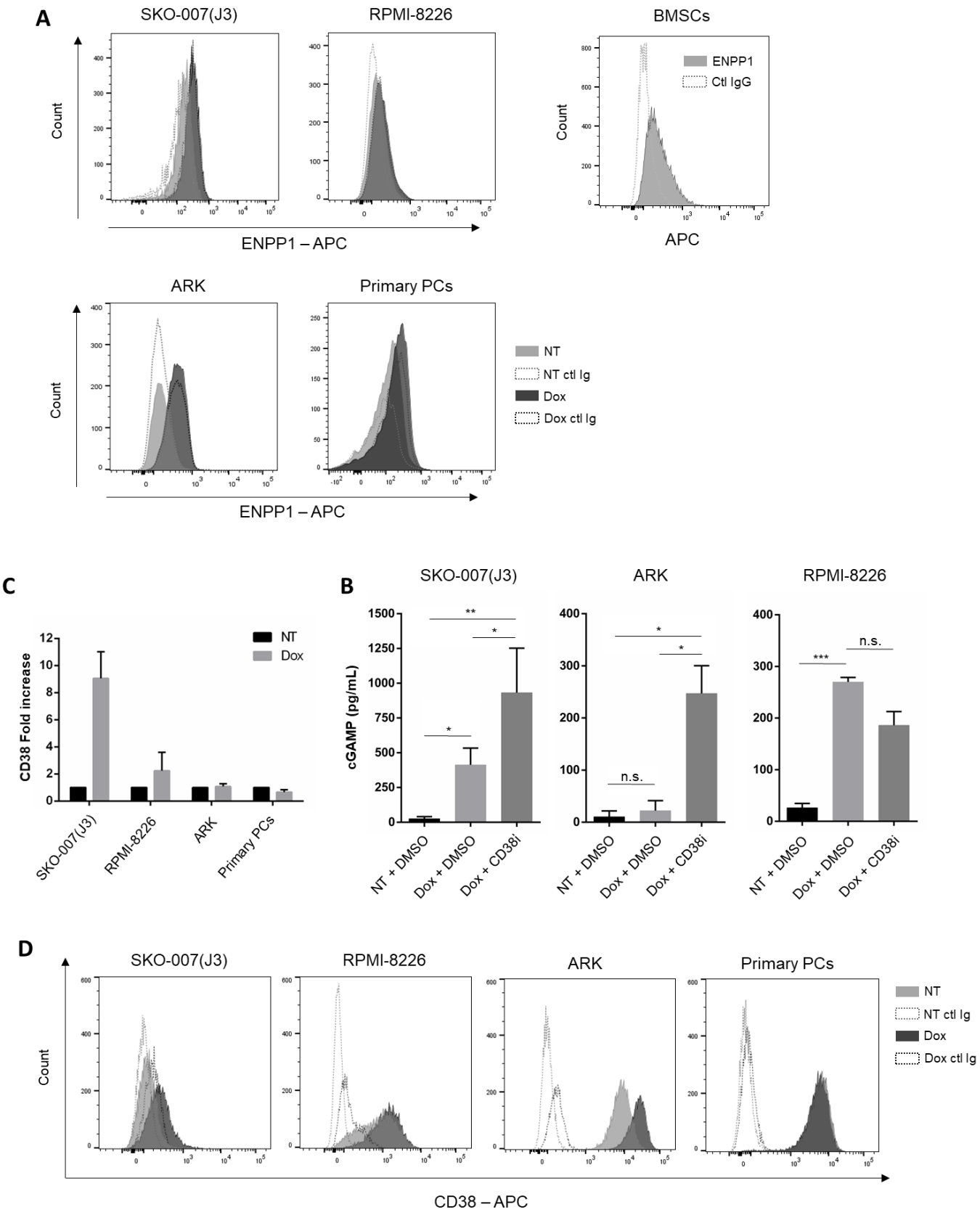

A
SKO-007(J3)
RPMI-8226
BMSCs
ENPP1
Ctl IgG
Count
ENPP1 – APC
APC
ARK
Primary PCs
NT
NT ctl Ig
Dox
Dox ctl Ig
C
CD38 Fold increase
NT
Dox
SKO-007(J3)
RPMI-8226
ARK
Primary PCs
B
cGAMP (pg/mL)
NT + DMSO
Dox + DMSO
Dox + CD38i
n.s.
D
CD38 – APC

Figure 5: inhibition of CD38 on Dox-treated MM cells causes accumulation of extracellular cGAMP. A) Surface expression of ENPP1 on untreated and Dox-treated SKO-007(J3), RPMI-8226, ARK and primary PCs from one representative MM patient. The amount of membrane ENPP1 on cell lines is barely detectable; primary PCs express variable levels of ENPP1, ranging from undetectable to very low; the patient shown above expressed the highest level of ENPP1 among those collected. The treatment with Dox did not influence the expression of surface ENPP1 on MM cells, although it increased autofluorescence or non-specific binding of the control IgG. Untreated BMSCs (from one representative MM patient), which are known to basally express ENPP1, were used as positive control. B) Addition of CD38i on Dox-treated SKO-007(J3) (CD38i 10 μM) and ARK (CD38i 20 μM) cells results in further accumulation of cGAMP in the conditioned medium compared to Dox alone. For unknown reasons, the same trend was not observed on RPMI-8226 cells. C) The variation of CD38 expression at transcriptional level upon Dox treatment was evaluated through Real Time PCR in SKO-007(J3), RPMI-8226, ARK and primary PCs (patients 11-16). D) Surface expression of CD38 on untreated and Dox-treated SKO-007(J3), RPMI-8226, ARK and primary PCs from one representative MM patient. Dox treatment substantially increased the expression of membrane CD38 on MM cell lines, but not on primary PCs of the patients tested.

Error bars represent SD; statistical significance was calculated using one-way ANOVA; * $p \leq 0.05$, ** $p \leq 0.01$

5.6 - Conditioned medium (CM) from CD38-inhibited SKO-007(J3) cells induces the transcription of IFN-β and other ISGs in BMSCs from MM patients

To verify whether the amount of extracellular cGAMP secreted by Dox-treated, CD38-inhibited MM cells is sufficient to exert a biological response, BMSCs from MM patients were incubated with CM from SKO-007(J3) for 16 hours. Subsequently, transcriptional induction of *IFN-β* and other genes induced by the STING pathway was measured through Real Time PCR. This method represents a previously validated biological read-out to reveal the presence of biologically active cGAMP in conditioned medium[66,87]. As negative control, CM was pre-treated with 0.5 U/mL snake venom phosphodiesterase (SVPDE) for 40 min at 37°C in order to degrade all the soluble cGAMP, as previously described[58]. Following incubation with CM from Dox-treated CD38-inhibited SKO-007(J3), BMSCs expressed higher mRNA levels of the STING-induced genes *IFN-β*, *CCL5*, *IRF7* and *IFI16* compared to BMSCs incubated with CM from only Dox-treated SKO-007(J3) (**Figure 6A and B**). Further, pre-treatment of the CM with SVPDE abrogated this effect, suggesting that cGAMP is

responsible for the transcriptional induction. For *IRF7* and *IFI16,* however, the small dimension of the data sample did not allow the trend to become statistically significant.

The *IFN-β* gene was by far the most sensitive to the incubation with CM, suggesting that the induction of *IFN-β* by cGAMP could depend on a threshold effect in BMSCs. Interestingly, *IFN-α1* was not induced by the CM (**Figure 6B**), nor by exogenous cGAMP administration at the doses tested (not shown).

These results demonstrate unambiguously that inhibition of CD38 on Dox-treated MM cells causes the accumulation of biologically active cGAMP in the extracellular environment; in addition, they suggest the ability of BMSCs from MM patients to respond to MM-derived cGAMP.

5.7 - cGAMP treatment increases the expression of surface CD38 on THP-1 and NKL cells, but not on MM cell lines

It has been demonstrated *in vitro* that exogenous administration of IFN-β can induce the transcription of *CD38* gene and upregulate surface protein levels in leukemic B cells[173]. However, whether STING activation by cGAMP can somehow regulate the expression of CD38, directly or indirectly via IFN-β, is unknown. Admitting that CD38 can negatively regulate the activity of cGAMP, the question becomes particularly relevant; indeed, if cGAMP can induce the expression of surface CD38, which, in turn, can reduce the concentration of extracellular cGAMP, this would represent a negative feedback mechanism to control extracellular cGAMP concentration (**Figure 6D**). Accordingly, treatment of monocytic THP-1 cells, which express high levels of STING[174], and NK leukemia cell line NKL with cGAMP (100 nM, 500 nM and 5 μM) for 48 hours resulted in a dose-dependent increase in CD38 surface levels. In contrast, on MM cell lines SKO-007(J3), RPMI-8226 and ARK the expression of CD38 was not influenced at any dose and time by the treatment (**Figure 6C**). This observation implies that the response to extracellular cGAMP differs substantially between these cell types and suggests that MM-derived cGAMP, once reached a relevant concentration in the microenvironment, might increase CD38 expression in paracrine, but not in autocrine, manner.

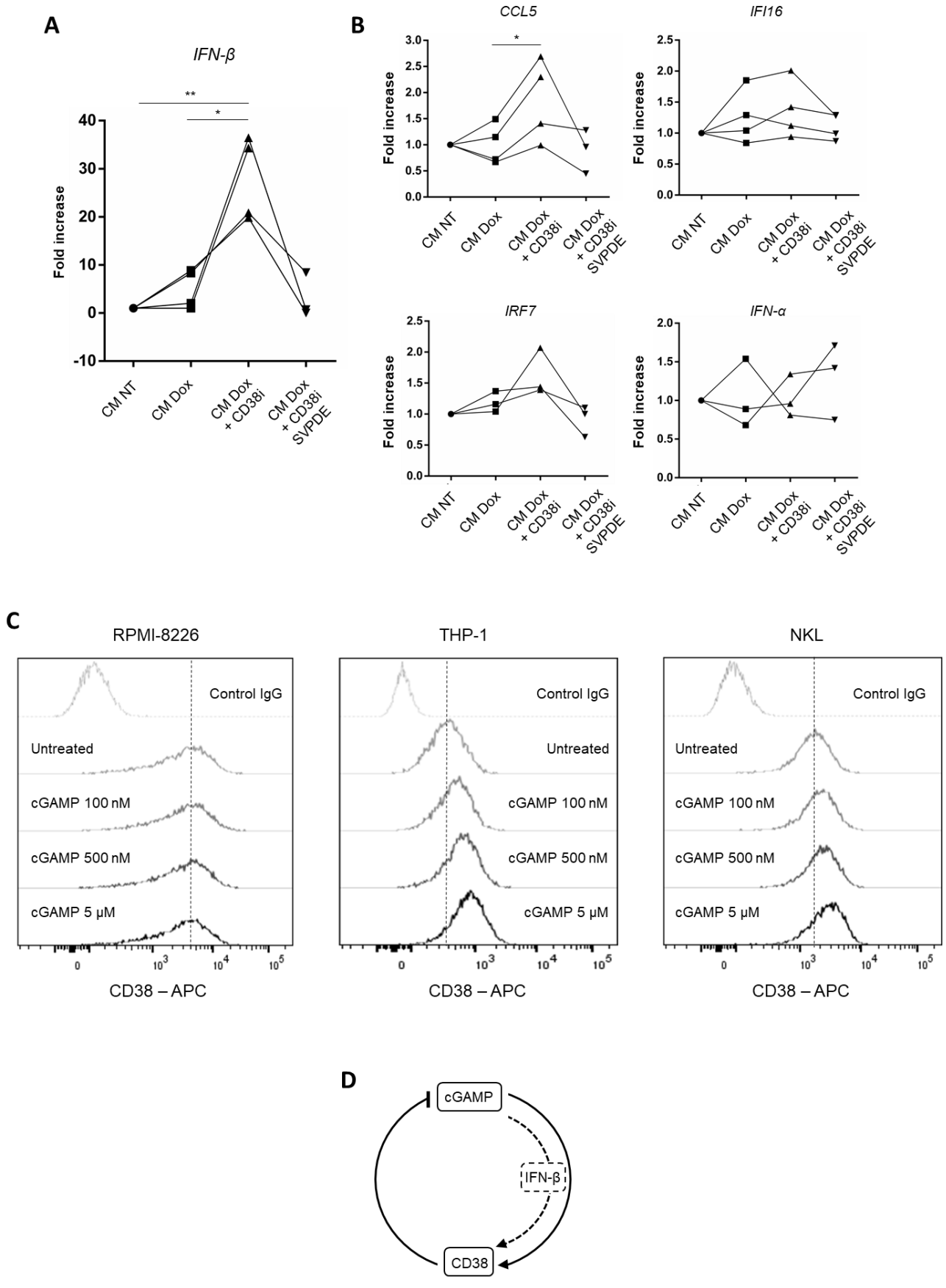

A
IFN-β
Fold increase
CM NT
CM Dox
CM Dox + CD38i
CM Dox + CD38i SVPDE
B
CCL5
IFI16
IRF7
IFN-α
C
RPMI-8226
THP-1
NKL
Control IgG
Untreated
cGAMP 100 nM
cGAMP 500 nM
cGAMP 5 µM
CD38 – APC
D
cGAMP
IFN-β
CD38

Figure 6: conditioned medium (CM) from Dox-treated, CD38-inhibited SKO-007(J3) induces the transcription of *IFN-β* and other STING-induced genes on BMSCs from MM patients. A) BMSCs from MM patients (5-10) were incubated for 16h with CM from SKO-007(J3); the transcription of several STING-induced genes was then quantified through Real Time PCR. The *IFN-β* gene was by far the most responsive to CM Dox + CD38i; pre-treament of the CM with SVPDE for 40 min at 37°C completely abrogated the effect, indicating that the induction is mediated by cGAMP. B) The transcription of the genes *CCL5*, *IRF7* and *IFI16*, but not of *IFN-α1*, was also slightly induced by CM Dox + CD38i compared to CM NT and CM Dox, albeit only for *CCL5* the difference was statistically significant. C) THP-1 and NKL cells were treated for 48h with cGAMP; the expression of surface CD38 on living cells was then measured by flow cytometry. The results indicate that, at least in some types of cells, CD38 protein expression can be increased, directly or indirectly, by cGAMP. The same phenomenon was not observed on MM cell lines SKO-007(J3), ARK and RPMI-8226 (only the latter is shown). The results shown are representative of two independent experiments. D) Negative feedback loop model of the relationship between cGAMP and CD38 levels.
Statistical significance was calculated using two-tailed paired Student's T test; * $p \leq 0.05$, ** $p \leq 0.01$

5.8 - cGAMP can bind to human recombinant CD38 causing a conformational change of the protein that involves tryptophan residues

To corroborate the hypothesis of a direct interaction between CD38 and cGAMP, fluorescence quenching measurements of recombinant human CD38 (rhCD38) in the presence of increasing concentrations of cGAMP were performed in collaboration with Dr. Annamaria Sandomenico and colleagues (Institute of Biostructure and Bioimaging, National Research Council, Napoli, Italy). This technique is based on the measurement of intrinsic fluorescence of tryptophan at around 350 nm following excitation at 295 nm. Containing 8 tryptophan residues, CD38 is suited for this method, which indeed has been previously employed in literature for the study of CD38-ligand interactions and for the determination of dissociation constants[175,176].

As expected, the spectrum of rhCD38 intrinsic fluorescence revealed a peak of emission around 345 nm due to tryptophan residues. When increasing concentrations of cGAMP were added to the mix, a pronounced dose-dependent decrease in fluorescence intensity (quenching) was observed (**Figure 7A**). Excitation of cGAMP alone at 295 nm resulted in a fluorescence spectrum whose intensity is very low in the interval 300-500 nm, making its interference with the assay negligible (not

shown). The value of the dissociation constant (K_D, the concentration of the ligand at which half of the protein sites are occupied) for the interaction CD38-cGAMP was calculated by applying a non-linear regression analysis on the fluorescence quenching data, assuming the presence of a single binding site; the estimated K_D was 33.23 ± 5.45 μM with a R^2 of 0.956 (**Figure 7C**).

The commercially available ENPP1-resistant analog 2'3'-cG^{s}A^{s}MP, which was synthesized by Li and collagues with the goal of creating a more stable version of cGAMP[69], also induced quenching of CD38, albeit to a lesser extent (**Figure 7B**). Accordingly, the estimated K_D was 46.32 ± 9.76 μM, indicating a lower affinity for the enzyme compared to the natural cGAMP, despite the two compounds have almost identical structure (**Figure 7C**).

The technique was validated using NAD, the best-known substrate of CD38, as previously reported in literature[175]. NAD similarly induced fluorescence quenching, as expected, exhibiting a K_D of 15.4 ± 1.5 μM (R^2 of 0.94) (**Figure 7D and E**). This value is comparable with K_D values reported in literature and, more importantly, is of the same order of magnitude of cGAMP.

Collectively, these observations suggest that cGAMP can physically interact with CD38 and the interaction involves tryptophan residues.

5.9 - Measurement of fluorescence recovery over time suggests that CD38 can actively transform cGAMP into a different chemical species

Following the dynamic changes in fluorescence intensity (F.I.) of the enzyme in the presence of a ligand at fixed wavelength can provide evidence of the occurrence of enzymatic reactions when proper controls are included in the experiment[175,176]. Therefore, time-course measurements of F.I. at 345 nm of rhCD38 in the presence of the natural cGAMP or the ENPP1-resistant analog 2'3'-cG^{s}A^{s}MP were performed following excitation at 295 nm and at the constant temperature of 25 °C.

Incubation of rhCD38 with cGAMP (both at fixed concentrations, 1 μM and 10 μM, respectively) resulted in a rapid decrease in F.I. within the first minute, confirming the rapid interaction of the enzyme with cGAMP. The presence of 2'3'-cG^{s}A^{s}MP induced a less pronounced decrease in F.I., in accordance with the fluorescence quenching experiments. Importantly, however, only in the presence of the natural cGAMP the decrease was followed by a recovery phase that brought back

the F.I. to the initial level within approximately 12 minutes, whereas for 2'3'-cG^{s}A^{s}MP the signal remained around its lowest point after the quenching phase (**Figure 7F**).

These results can be interpreted postulating the capacity of CD38 to transform cGAMP. According to this hypothesis, the quenching phase should correspond to the binding event; as the time passes, an increasing number of cGAMP molecules are transformed into other chemical species, which have lower affinity for CD38 catalytic site, and therefore induce progressively lower quenching, until all (or most) cGAMP molecules are consumed.

The different kinetics observed for 2'3'-cG^{s}A^{s}MP can be interpreted as the inefficacy of CD38 in transforming this molecule. However, it is possible that at longer times the curve of cG^{s}A^{s}MP would eventually return to the initial point; this is because the molecule is only more resistant to ENPP1 hydrolysis, but other enzymatic reactions may presumably occur.

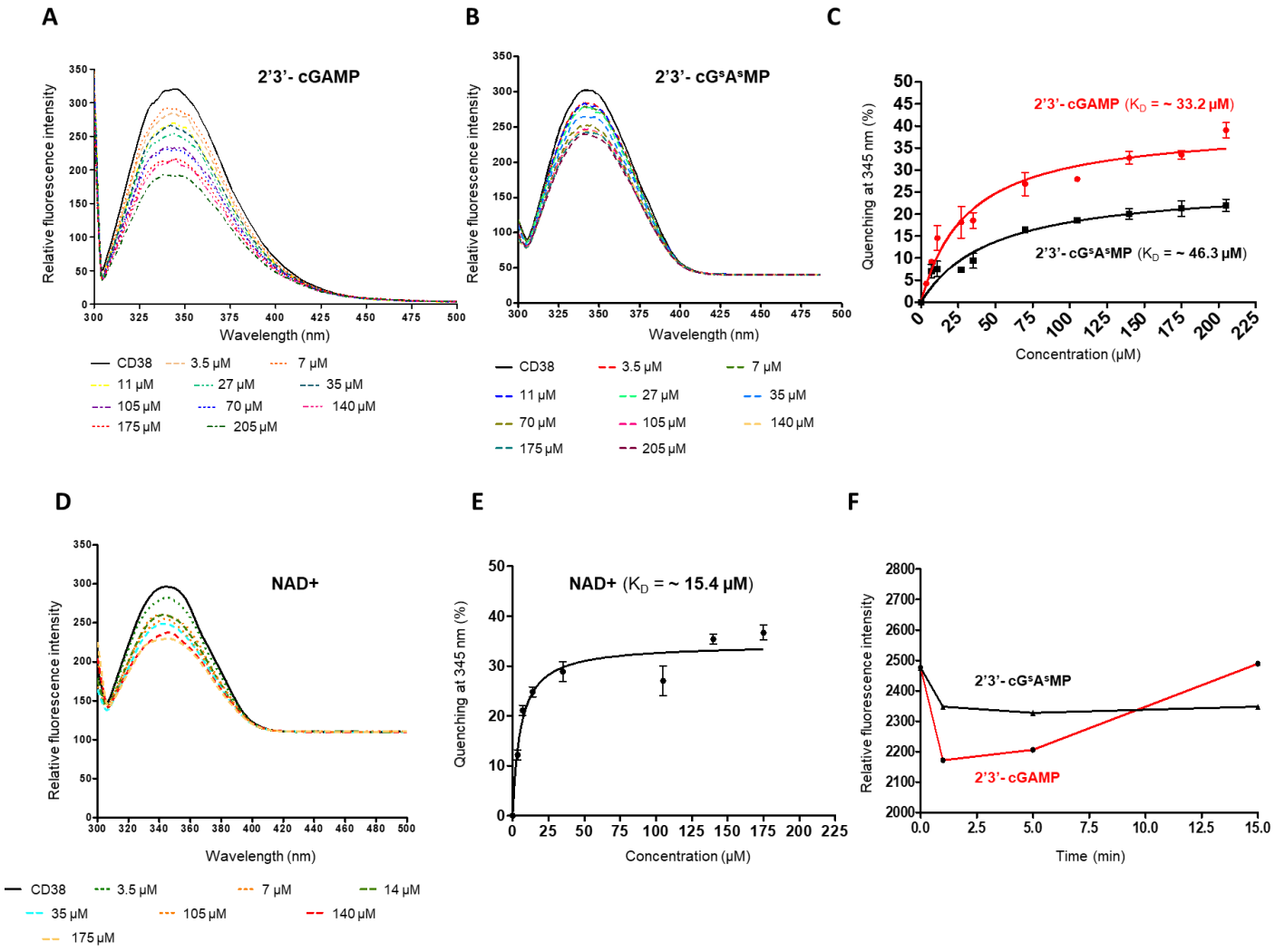


Figure 7: cGAMP can bind to CD38, causing a conformational change of the enzyme, and can be converted into a different chemical species. A) Intrinsic fluorescence spectrum of rhCD38 alone (1 µM) and with increasing concentrations of cGAMP. The progressive reduction of F.I. at 345 nm is mainly due to quenching of tryptophan fluorescence, indicating a conformational change of the protein that involves tryptophan residues. B) The ENPP1-resistant 2'3'-cG^{s}A^{s}MP also induces quenching at 345 nm, albeit at lower level compared to natural cGAMP. C) Saturation curves of cGAMP and 2'3'-cG^{s}A^{s}MP with rhCD38 (1 µM) describing the relationship between concentration (µM) and percentage of quenching at 345 nm. This type of graph allows to estimate the dissociation constant (K_D) of the interaction between CD38 and the putative ligand. D) and E) To validate the technique, the same experiment was performed with NAD, the best-known substrate of CD38. The resulting K_D is comparable with the values reported in literature. F) Measurements of fluorescence recovery over time at 345 nm of CD38 (1 µM) with cGAMP or 2'3'-cG^{s}A^{s}MP suggest the capacity of CD38 to efficiently transform cGAMP but not its analog.

5.10 – Reverse phase HPLC and ESI-TOF mass spectrometry show the degradation of cGAMP and the appearance of hydrolysis products after incubation with CD38

To study in further detail the mechanism of cGAMP degradation by CD38, an experiment of reverse phase high-performance liquid chromatography (RP-HPLC) coupled with UV absorption at 260 nm was performed. The chromatographic analysis of the cGAMP solution (used as reference) showed that the molecule eluted as a single peak with a retention time of 15.43 minutes. When rhCD38 was added to the same reaction mix containing cGAMP, a prominent shift in retention time (14.88 min) was observed after 5 minutes at 37°C. The peak further shifted at RT 14.58 and 14.67 minutes after 30 and 60 minutes, respectively (**Figure 8A**).

In contrast, no difference in retention time was observed for 2'3'-cG^{s}A^{s}MP incubated with rhCD38 under the same conditions, indicating that the enzyme is unable to transform this molecule. Notably, since the molecule 2'3'-cG^{s}A^{s}MP is a mix of diasteroisomers (as reported in the quality control sheet provided by the manifacturer), two distinct peaks were recorded at 19.05 and 19.55 RT (**Figure 8A**). This experiment confirms that cGAMP, but not the analog 2'3'-cG^{s}A^{s}MP, is rapidly transformed into a different chemical species when incubated with rhCD38. The observed peak shift between 30 e 60 minutes may imply that additional molecular rearrangements have occurred during that time.

Finally, in order to identify the breakdown products of the putative enzymatic reaction, electrospray ionization-time of flight (ESI-TOF) mass spectrometry analysis was performed in negative ion mode. cGAMP alone and the enzymatic reaction mixture with rhCD38 (5 minutes of incubation at 37°C) were prepared in water and directly infused into the mass spectrometer. The recorded mass spectra for water (black), intact cGAMP (red) and reaction mix cGAMP-rhCD38 (green) are reported in **Figure 8B**. The mass spectrum of water was acquired to exclude the presence of solvent related ions (background). As expected, the signals of a singularly deprotonated ($[M-H]^{-}$, m/z 673.07) and doubly deprotonated molecular ion ($[M-2H]^{-2}$, m/z 336.03), corresponding to intact cGAMP, were found only in the spectrum of cGAMP alone (**Figure 8C and D**). Instead, in the mass spectrum of the enzymatic reaction mixture four main molecular ions of m/z 217.04, 295.04, 377.07 and 455.09 were detected, while the two peaks corresponding to cGAMP disappeared completely (**Figure 8E to H**).

Assuming that CD38 catalyzes the same reaction of the phosphodiesterase ENPP1, the expected main molecular ions should be:

- the GAMP linear dinucleotide as product of hydrolysis of a single phosphodiester bond with addition of one molecule of H_2O (m/z 691.09 $[M-H]^-$).
- The two mononucleotides (AMP and GMP) derived from the cleavage of both phosphodiester bonds.

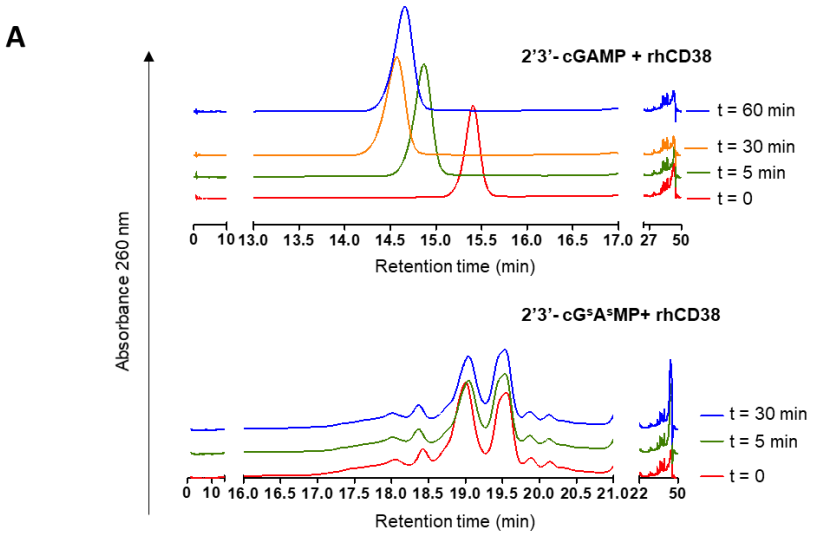
The nomenclature, chemical formula and expected m/z ratio of the mono-deprotonated ion of these expected products are reported below:

Adenosine monophosphate, AMP	$C_{10}H_{12}N_5O_5P$	$[M-H]^-$	m/z 346.05
Adenosine diphosphate, ADP	$C_{10}H_{15}N_5O_{10}P_2$	$[M-H]^-$	m/z 426.02
Guanosine monophosphate, GMP	$C_{10}H_{14}N_5O_8P$	$[M-H]^-$	m/z 362.06
Guanosine diphosphate, GDP	$C_{10}H_{15}N_5O_{11}P_2$	$[M-H]^-$	m/z 442.01

Since these products were not observed in the mass spectrum is very likely that under the experimental conditions of ionization in the electrospray source, the molecular ions undergo subsequent fragmentations and rearrangements, causing an additional breakage at level of the methylene group (CH_2) of ribose. According to this hypothesis, it can be speculated that the molecular ions $[M-H]^-$ of m/z 217.04 and m/z 455.09 (**Figure 8E and H**) correspond to the products adenosine dehydrated and guanosine methylen-diphosphate, respectively, whereas the molecular ions $[M-H]^-$ of m/z 295.04 and m/z 377.07 (**Figure 8F and G**) correspond to adenosine monophosphate dehydrated and guanosine methylen-monophosphate.

To elucidate the exact nature of these ions further MS^n mass spectrometry experiments (tandem MS) are needed. However, these preliminary data strongly indicate that cGAMP is processed by CD38, producing species at lower molecular weight, and HPLC analyses confirm this observation, further corroborating the results obtained in cellular models. The event of a non-enzymatic degradation of cGAMP (due to contaminants in rhCD38 preparation) can be excluded considering that after only 5 minutes the intact molecule became undetectable; it is unlikely that non-enzymatic degradation could occur so rapidly.

It is important to note that the catalytic mechanism of cGAMP breakdown by CD38 and ENPP1 could differ substantially; indeed, hydrolysis of NAD into ADPR by CD38 does not involve the cleavage of a phosphodiester bond.



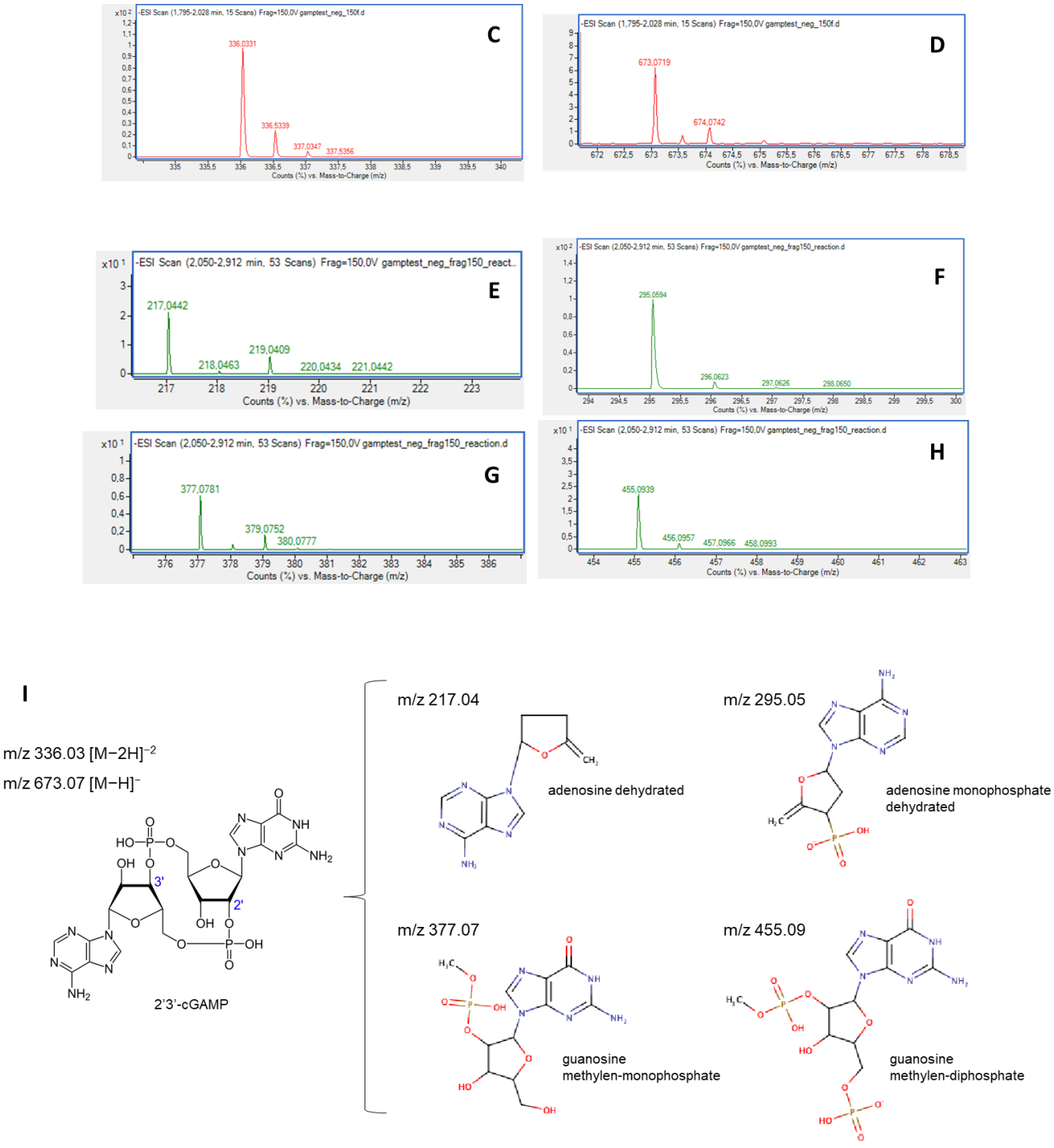


Figure 8: RP-HPLC and ESI-TOF mass spectrometry confirm the ability of CD38 of degrading cGAMP.

A) RP-HPLC time course analysis of cGAMP or 2'3'-cG^{s}A^{s}MP (100 µM) incubated with rhCD38 (1 µM) at different time points. The pronounced shift in retention time of cGAMP after incubation with rhCD38 suggests a catalytic transformation of the molecule, which does not occur for 2'3'-cG^{s}A^{s}MP. The double peak of the analog was expected since the commercial product is a mixture of diastereoisomers. B) ESI-TOF Mass spectrum in the m/z interval 120-1000 of cGAMP alone (red), cGAMP + rhCD38 (green) and water (black). The disappearance of the peak at m/z 336.03 (corresponding to doubly

deprotonated cGAMP) following incubation with rhCD38 is evident. C) and D) Zoom on the peaks corresponding to doubly and singularly protonated cGAMP. E) to H) Peaks corresponding to the products of hydrolysis and subsequent in-source fragmentation of cGAMP upon incubation with rhCD38. These peaks are absent in the spectrum of cGAMP alone and in water, suggesting the occurrence of an enzymatic reaction followed by further in-source fragmentations. I) Nomenclature and proposed molecular structures of the four putative products in their neutral form.

6. Discussion

In this study, I showed the ability of human multiple myeloma cells to secrete the immune-stimulating second messenger cGAMP in the extracellular environment upon treatment with the genotoxic drug doxorubicin. In addition, I provided evidence for a possible role for the ectoenzyme CD38 in regulating the concentration of extracellular cGAMP.

The concept that doxorubicin-induced immune response can mimic those of viral infection, with a typical type I IFN-signature, was already proposed by Sistigu and colleagues[177], but was not correlated with the activation of cytoplasmic DNA-sensing pathways. More recently, however, it was found that doxorubicin triggers the production of type I IFNs through the activation of ATM and cGAS-STING pathways in cells infected with Ebola virus, thus circumventing the block of IFN signalling caused by viral antagonist proteins[178].

A drug screening to identify, among different topoisomerase II inhibitors, including doxorubicin, the most powerful molecule in inducing immunogenic cell death (ICD) and T cell activation, showed that the drug teniposide can trigger ICD through DNA damage and STING activation in mouse melanoma and colon cancer cell lines[179]. Curiously, doxorubicin was excluded from the screening by the investigators because it did not induce the features of ICD associated with STING pathway activation.

Doxorubicin is thought to exert its antineoplastic effect mainly by inducing DNA fragmentation in rapidly dividing, repair-defective cancer cells; however, disruption of mitochondrial homeostasis is probably another important mechanism underlying its broad-spectrum citotoxicity[180]. Therefore, it would be of interest to understand whether the activation of the cGAS-STING pathway in the aforementioned models (and in the present work) is caused by the accumulation of genomic or rather mitochondrial DNA in the cytoplasm. Still, it is worthy of note that cytoplasmic DNA has not been observed yet upon doxorubicin administration. The existence of an unknown mechanism by which the drug activates the cGAS-cGAMP-STING axis should not be excluded.

According to recent works, the release of cGAMP by cancer cells may represent a general phenomenon[86,87,89]. Nonetheless, its biological significance is still debated. Some reports indicate that cGAMP released by malignant cells can trigger a potent IFN-mediated immune response, in which type I IFN-activated NK cells are the final effectors of tumor clearance[86]. According to this view, the active production and secretion of cGAMP by tumor cells should be detrimental for the tumor itself, unless it represents a mechanism to avoid self-STING activation, which might be hypothetically even more detrimental. On the contrary, it is also possible that cGAS activity is necessary for cancerous cells, since the axis cGAS-cGAMP-STING activates the NF-κB transcriptional program, which is often crucial for tumor survival[181]. Accordingly, cGAMP release in the extracellular environment might be, for cancerous cells, a "necessary evil" that can be exploited by the immune system to mount a protective response against the tumor. On the other hand, the channel proteins responsible for cGAMP export, such as LRRC8A/E VRACs, might be indispensable for tumor cell survival by regulating cell's osmolarity and favouring drug resistance[182].

Another fascinating hypothesis, supported by the work of Wu and colleagues[81], is that cancer cells secrete cGAMP in the surrounding microenviroment as a mean to provoke the apoptosis of infiltrating T cells, for which STING overstimulation is highly toxic, as previously discussed[88]. If this is the case, then the change of concentration of cGAMP in the TME could represent a "switch" from an anti-tumor, immune-stimulatory environment, fostered at low concentrations, to a cytotoxic one towards infiltrating lymphocytes, when the concentration of extracellular cGAMP exceeds a certain threshold. More *in vivo* experiments will be required to verify this hypothesis.

Finally, the recent observation that cGAMP can be quickly converted into adenosine through the sequential action of ENPP1 and CD73[89] suggests yet another explanation for how liberating cGAMP in the extracellular environment could be advantageous for cancer cells.

To date, there are only few reports that investigate the role of the cGAS-STING pathway in the immunopathology of multiple myeloma. In spite of this, MM is arguably a good model disease to study the involvement of cGAS-STING pathway, for several reasons. First, myeloma cell lines express cGAS at the highest levels compared to other cancer cell lines from different sources (**Results, Figure 1E**); secondly, myeloma is characterized by an elevated degree of genomic

instability[6], which positively correlates with the activation status of the pathway[90]; finally, different cell populations in the bone marrow express a set of enzymes potentially able to generate adenosine starting from cGAMP, in particular ENPP1, which hydrolyzes cGAMP into AMP and GMP, and CD73, which breaks AMP into adenosine.

It is worthy of note that, in the present work, ENPP1 surface expression on primary PCs from MM patients (measured by flow cytometry) ranged from undetectable to very low. This finding is not fully in accordance with the work of Morandi and colleagues, which reported relatively high levels of ENPP1 on CD138+ cells from BM aspirates of MM and MGUS patients[163]. However, this discordance can be easily due to the significant difference in the staining protocol, antibody and gating strategy employed, in addition to the small dimension of the data sample.

Established the capacity of MM cells to secrete cGAMP, I investigated how these cells respond to cGAMP itself by administrating it exogenously to the cell line SKO-007(J3), chosen as model. The most striking effect observed was a substantial induction of apoptosis, measured by Annexin V and propidium iodide double staining, after 48 hours of stimulation with 20 μM cGAMP. This finding is in accordance with the results obtained by Tang et al., who demonstrated that mouse normal and neoplastic B cells (A20 B-cell lymphoma cells and 5TGM1 multiple myeloma cells) undergo STING-dependent mitochondria-mediated apoptosis when challenged with the same concentration of the analog 3'3'-cGAMP, showing that these cell types cannot efficiently degrade STING following activation[93]. Interestingly, although the natural cGAMP has much higher affinity to STING compared to 3'3'-cGAMP, at lower cGAMP concentrations (5 μM) SKO-007(J3) cells do not undergo apoptosis, but rather acquire elevated β-galactosidase activity, a marker of cellular senescence, after 4-5 days of stimulation. It was recently observed that cGAMP administration to THP-1 cells results in DNA damage response (DDR) activation, phosphorylation of histone H2AX and cell cycle arrest[172], characteristics that are also frequently associated with senescence. However, I did not detect any increase in H2AX phosphorylation in SKO-007(J3) cells following cGAMP stimulation. Clearly, more evidence will be needed to understand the signalling pathways involved in the toxicity of high dose cGAMP in human cells.

It is known that cGAMP paracrine signalling can trigger the production of cytokines in bystander cells, in particular type I IFNs, CXCL10, CCL5 and several others. These cytokines are activators of NK cell effector functions and potent chemoattractants. Thus, the activation of NK cells by tumor-derived cGAMP is thought to be mostly indirect, depending on the intervention of a cytokine-producing cell type in the TME. Nonetheless, NK cells are among the highest STING-expressing immune cells[169]. Moreover, they express higher levels of the channel subunits LRRC8A/E compared to other blood cells[183,184], suggesting that they may have the potential of responding to extracellular cGAMP directly. In this work, I observed that 16-hour treatment of NK cells with exogenous cGAMP can potently increase their cytotoxicity towards the MM cell line SKO-007(J3). NK cells reacted to cGAMP without previous permeabilization with digitonin-based buffers (which has been frequently employed in literature for treating cells with cGAMP), implying that they can uptake cGAMP from the extracellular environment through channel proteins.

Theoretically, there might be a number of mechanisms by which cGAMP increases the killing ability of NK cells. It is possible that cGAMP stimulates the production of type I IFNs or other cytokines, which in turn boost NK cell effector function in an autocrine manner. NK cells can be an important source of IFN-γ and TNF-α, but whether they are able to secrete relevant amounts of type I IFNs needs to be established. Blocking IFNAR1/2 receptor with mAbs during cGAMP stimulation could help to demonstrate the contribution of type I IFNs in the increased killing ability.

Another possibility is that activation of IRF3 or NF-κB by the cGAMP-STING-TBK1 axis results in higher expression of activating receptors and/or protein involved in NK cell cytotoxicity (granzymes, perforin, granulysin), or in diminished expression of inhibitory receptors. Accordingly, I observed a subtle but reproducible increase in perforin and granzyme B in $CD56^{dim}$ NK cells from healthy donors upon 16-hours cGAMP stimulation. As mentioned above, both proteins are target genes of NF-κB complex. However, considering the vast number of genes induced upon NF-κB and IRF3 activation, it is unlikely that the augment in cytotoxicity is only due to increased perforin and granzyme content. More experiments will be needed to dissect the molecular mechanism underlying this phenomenon.

Apart from being both hydrolases, CD38 and ENPP1 share an important similarity. In particular, the molecule ADP-ribose (ADPR) is respectively a product of NAD hydrolysis by CD38 and substrate of

ENPP1, which hydrolyzes it into AMP[161]. In general, these two ectoenzymes act on chemically and structurally related molecules. This fact suggested us a possible similarity between the catalytic sites of CD38 and ENPP1. Still, a crucial difference between the two ectoenzymes is that ENPP1 requires Ca^{2+} and Zn^{2+} ions for the catalysis, as discussed above, while CD38 does not.

The data presented in this work strongly suggest that ENPP1 and CD38 share the function of degrading cGAMP. However, to prove indubitably this novel function of CD38 and to study in detail the possible reaction mechanism, it would be extremely valuable to crystallize CD38 in complex with cGAMP. It is possible that the mechanism by which CD38 degrades cGAMP could be essentially different from that of the phosphodiesterase ENPP1.

CD38 is an evolutionarily ancient enzyme, with a high degree of similarity between human and the mollusk *Aplysia*[185]. It would be worth investigating whether the cGAMP-degrading function of CD38 is conserved across mammals, or even across different animal phyla. This would provide insight on the importance of cGAMP catabolism in the homeostasis of the immune system from an evolutionary perspective.

As previously stated, CD38 is virtually ubiquitous among the cells of the immune system. The concept that cGAMP can be degraded by CD38 could have vast implications, not just in tumor immunology and IFN-mediated autoimmune diseases but also (and perhaps especially) in the context of antiviral immunity. It is not known whether non-transformed cells can secrete cGAMP upon viral infection; if this phenomenon actually occurs *in vivo*, the breakdown of cGAMP by CD38 could represent a homeostatic mechanism to preserve tissue integrity. For instance, it can be speculated that, during the acute tissue inflammation generated by DNA viruses, cGAMP would gradually accumulate in the extracellular space as the infection spreads across neighbouring cells. In a similar scenario, cGAMP levels should be regulated in order to avert massive and uncontrolled cytokine release. The existence of a negative feedback loop could possibly solve this problem. Interestingly, there is evidence that type I IFNs are inducers of CD38, at least in some models[173]; hence, it is possible that cGAMP released by infected cells and uptaken by tissue-resident type I IFN-producing cells, like pDCs, may stimulate the production of type I IFNs, which in turn could induce the upregulation of CD38 on the surface of infiltrating immune cells, like macrophages, cDCs

and NK cells. Increased levels of membrane CD38 would eventually bring extracellular cGAMP concentration back to a “safety level”.

Unfortunately, measuring cGAMP concentration is particularly challenging in vivo, and indeed such experiment has not been performed so far. Therefore, in order to find evidence supporting this hypothesis, it would be pivotal to develop techniques that allow accurate measurements of extracellular cGAMP fluctuations over time *in vivo*. Nonetheless, independently of the existence of such feedback loop, it is largely accepted that inflammatory stimuli induce or upregulate the expression of CD38 on several immune cell types[149].

Analogous speculations can be extended to the field of tumor immunology, where the biological relevance of cGAMP secretion has already been established in *in vivo* models[86,89].

The results of the present work may be relevant for the therapy of multiple myeloma. Albeit CD38-targeted therapy in MM is routinely adopted with Daratumumab, the efficacy of this monoclonal antibody is believed not to be based on the modulation of CD38 enzymatic activity. Isatuximab, on the other hand, interferes with the catabolism of NAD of MM cells, and its efficacy is thought to be due at least in part on this mechanism. It is tempting to speculate that the abrogation of cGAMP breakdown may be another mechanism by which Isatuximab exert its anti-myeloma activity. Blocking of cGAMP degradation may indeed boost innate immune response against tumor cells, and possibly interfere with the metabolic pathway that generates adenosine from cGAMP, as discussed above.

Nevertheless, this work has several limitations. One is related to the scarcity of methods to accurately quantify low amounts of cGAMP. Here, a commercially available competitive ELISA was used for this purpose; however, this method is reliable only for relatively high concentrations of the molecule. Based on works published in the recent literature, it is likely that, *in vivo*, the single cancer cell produces small amounts of cGAMP, whose concentration becomes biologically relevant only in the context of the tumor microenvironment, where numerous cancer cells are packed at high density. Moreover, it is generally assumed that extracellular cGAMP exerts its function on short distance. Accordingly, I was not able to measure biologically relevant amounts of cGAMP in the highly diluted culture medium of primary myeloma cells from MM patients treated with doxorubicin, not even in the presence of CD38 inhibitor. On the other hand, these cells are difficult to maintain in a healthy state

for prolonged time under standard culture techniques, and a high proportion of vital cells is crucial for detecting secreted cGAMP. It is also possible that, for unknown reasons, primary myeloma cells are intrinsically unable to release cGAMP in the extracellular environment. Only a more sensitive method of detection would allow to assess this important issue.

Another limitation is the arbitrariness of the doses of exogenous cGAMP used for treating the different types of cells used in the experiments. Unfortunately, the range of concentrations at which extracellular cGAMP operates *in vivo* is still unknown; consequently, most investigators use arbitrary concentrations and/or unrealistic methods of membrane permeabilization when treating cells with exogenous cGAMP. Hopefully, in the next future more *in vivo* studies will help to clarify the quantitative aspects of cGAMP physiology.

Finally, a major caveat of the present work is the lack of a genetic model of CD38-deficient or CD38-mutated MM cells, that would in principle avoid the off-target effect of the chemical inhibitor. A future perspective will be of generating a MM cell line carrying specific substitutions in the catalytic site of CD38, in order to obtain a model with the ectoenzyme inactive towards cGAMP and, at the same time, to better understand the mechanism of reaction of cGAMP breakdown. The catalytically dead enzyme would also be extremely valuable as control for the experiments of fluorescence recovery over time and mass spectrometry, whose results, as stated above, present some ambiguities. Indeed, from the evidence collected so far, it is only possible to attribute to CD38 the function of degrading cGAMP, but the formation of specific hydrolysis products is an issue that needs to be further investigated. Additional experiments of liquid chromatography-tandem mass spectrometry (LC-MS/MS) with the inclusion of controls such as inactive CD38 (negative control) and recombinant ENPP1 (positive control) will help to identify unambiguously the products of CD38-catalyzed reaction.

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