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In memoriam - Francesco di Virgilio: P2X7 receptors in purinergic pathophysiology

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Francesco di Virgilio passed away September 2024. It was a remarkable death for a scientist because it happened just after he had taken part in a Symposium in Chengdu, China. It occurred suddenly, during a post-congress excursion to the holy Buddhist mountain of Emei in the province of Sichuan. Of course it is an awfully sad event both for his family (especially for his wife, Dorianna), and for all his friends around the world, but this left his features also untouched and vigorous in our remembrance. (see Figs. 1–6).

He was a great figure in the research community interested in ATP signaling, probably the third important one besides Geoff Burnstock and Alan Richard North, both of which stopped working recently. Geoff died peacefully 2020, aged 91, and Alan retired from his position at Manchester University several years ago. It is unnecessary to mention that Geoff was the "Father of Purinergic Signaling" and Alan cloned numerous P2X receptors (Rs), thereby giving the badly needed boost to ATP research in the 1990s of the last century. Francesco devoted his energy to clarifying the role of the P2X7R in immunology and cancer; this receptor is considered by many of us to be the most important and interesting one of its whole class [1].

P2XZ/P2X7 receptors

Francesco began his scientific career in Padova, where he published his very first article, which appeared in *Proceedings of National Academy of USA*, on ATP, as a forebode of his lifetime activities, although at this time the involvement of this molecule in mitochondrial electron transport was his subject [2]. Then, he gradually discovered his interest in immunological cell types such as neutrophils, T-lymphocytes, leukocytes, and macrophages, until a scholarship in the USA at the Columbia University (New York) has got him associated with Samuel Silverstein and Thomas Steinberg, who discovered the P2ZR in macrophages [3,4] and lymphocytes [5]. His, at that time favorite *in vitro* model, the J774 macrophage responded to ATP with three different increases in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$; there was a first rapid and transient peak, followed by a sustained second peak of $[Ca^{2+}]_i$. Peak I was due to the mobilization of Ca^{2+} from intracellular pools, while peak II was due to the influx of extracellular Ca^{2+} [3]. The sustained second peak composed of a low and high threshold response, the latter one was caused by the permeabilization of the cell membrane to Ca^{2+}. This response could be exclusively elicited by ATP, and no other nucleotide, and was induced by the free acidic form of ATP (rather than by the more abundant Mg^{2+}-ATP). Nowadays, we know that peak I was caused by a metabotropic P2YR stimulation, while peak II was caused by the combined effect of a high-sensitivity non-P2X7R and a low sensitivity P2X7R-mediated stimulation, called at this time a P2ZR effect.

The application of high concentrations of ATP onto microglial cells of the mouse (considered to be the resident macrophages of the CNS) resulted in massive influx of Ca^{2+}, but also in pronounced uptake of the fluorescent dyes lucifer yellow and ethidium bromide [6]. The sizes of these molecules exceeded the diameter of the resting orifices of the cationic P2X1-6R-channels, reaching about 900 Da (for explanation see later). Incidentally, at the same time ion currents through P2XZRs were directly recorded from microglia *in situ*, in corpus callosum slices [7].

From this time on, Francesco clarified many characteristics of the P2ZR, dealing with aspects such as its

Figure 1

A photo of Francesco di Virgilio in 2005. It is noteworthy that his hair turned grey already in young age. There was an interesting contrast between the grey hair and the young complexion.

Figure 2

Francesco and his most narrow colleagues and coworkers in the garden facing the Department of Medical Sciences, University of Ferrara, in the year 2022 before the launch of the PRESTO COST Action. From left to right: Maria Luiza Thorstenberg, Simonetta Falzoni, Anna Lisa Giuliani, Elena Adinolfi, Anna Pegoraro, Elena De Marchi, and Francesco Di Virgilio himself.

Figure 3

Francesco in the famous cafeteria Pedrocchi in Padova, during a visit of Yong Tang, Patrizia Rubini, and Peter Illes. His wife, Dorianna Sandona is sitting between Francesco and Peter Illes. The photo was taken by Patrizia Rubini, whose face can be seen to reflect in the middle window in behind.

prominent cytotoxicity in immune cells [8,9]. The ability to kill foreign, neoplastic, and viral-infected cells is a primary function of cytotoxic T cells, natural killer cells, and macrophages. A number of soluble mediators of T-cells causing lysis have been proposed: Perforins are probably the best known ones, but soon ATP also joined this collection of cytotoxic substances. Moreover, it is now universally acknowledged that ATP is the archetypal and most ancient "danger-associated molecular pattern" (DAMP), a universal indicator of cell death [7].

Francesco noted that cytotoxic lymphocytes were insensitive to the lethal effects of ATP, and at first hypothesized that ATP-degrading enzymes were enriched on the external plasma membrane of these cells. Nonetheless, eventually he favored an alternative explanation, namely the lack of P2ZRs, which are responsible for cellular changes culminating in cytolysis. Francesco also made the important observation that cell death occurred both by necrosis (loss of important cell ingredients by the permeabilization process) and by apoptosis triggered by the P2ZR and leading to DNA fragmentation [10]. He discovered also an irreversible inhibitor of the macrophage P2ZR, oxidized ATP (oxATP; [11]). Incubation with oxATP at 37 °C for at least 2 h prevented ATP-dependent cell swelling, vacuolization, and lysis. It is noteworthy that oxATP was for a considerable time the only antagonist known to exhibit relative selectivity for the P2ZR.

Over the years Francesco discovered that peripheral human blood monocytes showed responses to P2YRs

Figure 4

Francesco with his beloved dog Isa before a mountain shelter in South Tyrol.

(intracellular Ca^{2+} release) but not membrane-permeabilizing P2ZRs. This low-sensitivity ATP receptor appeared only in the course of developmental changes of monocytes into macrophages [12]. Interferon-γ (IFN-γ) enhanced both membrane permeabilization and cytotoxic ATP effects, which were

Figure 5

Francesco before a peak of the Asiago Plateau, during a visit to Veneto.

Figure 6

Francesco with his birthday cake. He loved "meringata" and "tiramisu" cakes. He became just before his demise 70 years old.

completely antagonized by oxATP, and also inhibited multicellular giant cell generation stimulated by IFN-γ. Further, he reported that macrophage clones expressing high levels of P2ZRs spontaneously fuse to form such multinucleated giant cells [13].

Gradually, Francesco recognized most of the characteristics of the P2ZR. At that time, the valid terminology suggested that there are four types of P2Rs: (1) The P2YR, sensitive to ATP/ADP; (2) the P2UR, sensitive to UTP/UDP; (3) The P2XR sensitive to low concentrations of ATP; (4) and the permeabilizing/necrotic/apoptotic P2ZR, sensitive only to high concentrations of ATP. However, in 1996 Ann-Marie Surprenant and Alan North succeeded in cloning the P2X7R which had a considerable structural identity with the other P2XRs [14]. Although the general structural traits of the P2X7R resembled that of P2X1-6, there were also distinguishing features, such as the much longer intracellular C-terminus. Surprenant and North also reported that this receptor exhibits all previously described functional characteristics of the P2XZR, especially the astounding ability to increase its conductance on long-lasting or

repetitive contact with ATP, or the more potent agonist dibenzoyl-ATP. Eventually, it was demonstrated that J774 macrophage cells generated P2X7R-like currents, and P2X7R-mRNA was strongly expressed in these macrophages.

P2X7 receptors and interleukin-1β

A beautiful summary of the synergistic action of the P2X7R with the toll-like receptor-4 (TLR4) to release interleukin-1β (IL-1β) was published in one of Francesco's clear and concise reviews in 2007 [15]. The multifaceted and fascinating intracellular network promoting IL-1β release was apparently discovered by the combined efforts of many scientists, but the contribution of Francesco was decisive. ATP has many properties to serve as a so called DAMP [15]. It is available in high concentrations within the cytoplasm of every cell, a quick release occurs following tissue damage, and ready inactivation by powerful ubiquitous ecto-ATPases follows. Therefore it is only self-evident that ATP has been assumed and confirmed to be a stimulus to release pro-inflammatory cytokines e.g. from mouse microglia [16] or human macrophages [17] *via* P2X7R activation. The best-characterized stimulus of IL-β release is, however, a bacterial endotoxin, lipopolysaccharide (LPS), termed a "pathogen associated molecular pattern" (PAMP) [15]. LPS was described already in 1985 to cause *in vitro* rapid and large intracellular accumulation of its precursor pro-IL-1β, followed by slow intracellular liberation of the mature form [18]. Hence, it was reported that LPS-dependent IL-1β release involves activation of the purinergic P2X7R, and is inhibited by its selective blocker, oxATP [10]. The initiation of inflammasome (see next paragraph) activation is apparently due to the massive outflow of intracellular K^+ through the open P2X7R pore and the subsequent depletion of K^+ besides the inflammasome components [19]. This is an important step of activation, since IL-1β processing and release are inefficient processes; only about 10 % of the total pro-IL1β is converted by caspase-1 to its mature product and externalized by activated monocytes [20]. Hence, the "two signal model" for IL-1β predicts that the first signal is stimulation of the TLRs leading to accumulation of cytoplasmic pro-IL-1β, and the second one an ATP-dependent stimulation of the P2X7R, promoting inflammasome-mediated caspase-1 activation [17,21,22].

Hence, a last and important step in combining the TLR4 and P2X7R pathways is the nod-like receptor (NLR) family pyrin domain containing 3 (NLRP3) inflammasome, a central scaffold molecule of immunocytes, driving activation of the caspase cascade, which catalyzes the activity of caspase-1 also called IL-1β converting enzyme [15]. The inflammasome is a protein complex containing among others NLR and the adaptor protein ASC, which then recruits and proteolytically splits the inactive form of caspase-1, pro-caspase-1. Macrophages isolated from mice genetically depleted of ASC or NLRP3 are unable to mature and secrete IL-1β in response to ATP [15].

Then, the above observations were extended to mouse dendritic cells, known to present antigens to T cells [23,24]. During co-culture with T helper lymphocytes, P2X7R-containing dendritic cells synthesized large amounts of IL-1β, although their counterparts, which did not contain functional P2X7Rs, failed to do so. Human primary fibroblasts also possess P2X7Rs, coupled to ion fluxes, microvesicle formation and in this case IL-6 release [25]. The microvesicles apparently served as an exit pathway for interleukin liberation.

P2X7 receptors and inflammatory/infectious diseases

Based on their anti-inflammatory properties, small molecular (drug-like) P2X7R antagonists were strongly hoped to efficiently treat chronic inflammatory diseases. However, these hopes did not verify in more than 30 clinical studies, performed to test the efficacy of P2X7R blockade in osteoarthritis, rheumatoid arthritis, and chronic pulmonary disease; somewhat more encouraging results were obtained in Crohn's disease [26,27]. In host−microbe interaction, P2X7R activation was reported to mediate apoptosis of human monocytes/macrophages infected with *Mycobacterium tuberculosis*, reducing the intracellular bacterial viability [28]. Unfortunately, the therapeutic relevance of these findings was controversial, because the pro-inflammatory and cytotoxic activities were reported to aggravate the disease severity and the associated tissue damage [29]. Nonetheless, recently developed P2X7R partial agonistic substances, such as clemastine, may have a more favorable profile for the treatment of tuberculosis.

Two diagnostic approaches were crowned with more success. (1) P2X7Rs were valid targets for positron emission tomography (PET) by developing carbon- and fluorine labelled tracers, which became valid means in the diagnosis of neuroinflammation [26,27]. This procedure could evade the questionable identification of the translocator protein 18 kDa, a protein localized to the outer mitochondrial membrane, and instead recognized the pro-inflammatory phenotype of microglia. (2) The shed/soluble (s)P2X7R was found in the blood serum and plasma of healthy individuals and patients with acute infection; the levels of sP2X7R were considerably higher in the diseased patients and showed positive correlation with the C-reactive protein level [30]. sP2X7R was also elevated in the early phases of COVID-19 and predicted an adverse clinical outcome [31]. Hence, in spite of moderate results obtained in relation to therapeutic applicability of P2X7R ligands, the diagnostic field appeared to be more promising.

Measurement of extracellular ATP *in vivo* and P2X7R-mediated signaling in cancer

In recent years, Francesco pioneered studies on the role of extracellular ATP in pathology thanks to the invention of a luciferase-based probe, able to measure ATP on the outer leaflet of the plasma membrane [32]. One of his famous statements was: "For a messenger molecule to truly be considered as such, it must be measurable at the site where it exerts its effect." In fact, this probe allowed for live measurement of extracellular (e)ATP in mice and was central in many cancer related articles, allowing to demonstrate that eATP concentration is very high (in the millimolar range) in the tumor microenvironment [33], and that various anticancer treatments can alter eATP concentration in solid and liquid tumors thus affecting tumor eradicatinonal responses. The same probe was also useful in several other animal models to measure the release of eATP as a danger signal in inflammation [34].

Francesco's group was also the first to associate tissue proliferation with P2X7R activation [35]. Together with Elena Adinolfi he demonstrated that the P2X7R can play an *in vivo* growth promoting role in cancer cells affecting several pathways, of which vascular endothelial growth factor (VEGF) secretion and neovascularization are especially important [36]. A similar activity could also be attributed to a human splice variant of the wild-type receptor P2X7A, termed P2X7B, which has lost its pore forming activity but retained the capacity of facilitating cell growth [37]. These preliminary studies were followed by other study groups demonstrating the significance of the P2X7B isoform in anticancer therapy resistance and metastasis.

The role of the P2X7R in cancer can be considered somewhat controversial; it plays a role also in tumor eradicating immune responses, and in consequence, if the host loses P2X7R expression, tumor growth can be favored [38,39]. However, by contrast, if the tumor cells express high levels of P2X7Rs, anti-cancer drugs become more effective in reducing tumor growth/spreading, as the lack of P2X7Rs causes compensatory effects on the expression of other players of purinergic signaling, such as the ectonucleotidase CD73 [40], that are known to stimulate immunosuppression and thereby tumor growth.

Current work

In the last few years, Francesco also intensified his work on the analysis of vesicular release from the cell membrane activated by P2XRs. Indeed, his group was among the first to demonstrate that activation of this receptor triggered the release of vesicles containing cytokines or tissue factors from dendritic cells and macrophages [41,42]. Recently, Francesco reported most interesting findings on the role of the P2X7R as a master regulator of intercellular organelle and mitochondria trafficking in immune cells [43]. Activation of P2X7Rs promoted in mouse microglial cell lines the release of mitochondria to recipient cells, increasing their energy level and conferring a pro-inflammatory phenotype.

Scientific and organizational activities of Francesco

Francesco was not only an outstanding figure of the purinergic community, but also a highly important representative of biological/medical science worldwide. He had more than 370 publications enlisted on PubMed, had more than 35,000 citations in Scopus, and an H-index of over 100. At the University of Ferrara, where he has spent most of his career, he has held several important positions. He was from 1994 to 2006 Full Professor of General Pathology and from 2006 to 2024 Full Professor of Clinical Pathology, Dean for Education of the Medical School, Director of the Department of Experimental and Diagnostic Medicine, Deputy Rector for Research and Technology Transfer, Chairman of the PhD Program in Molecular Medicine and Pharmacology, and Head of a Research Program in Inflammation and Autoimmunity at the Laboratory of Clinical Pathology at Ferrara University Hospital.

He worked continuously with a large group of academic co-workers of about 10–15, of whom Simonetta Falzoni, Juana Sanz, Anna Lisa Giuliani, Elena Adinolfi, and Davide Ferrari, are probably the most prominent. He was lucky to have as co-operation partners and friends, the pharmacologist Pier Andrea Borea and the medicinal chemist Pier Giovanni Baraldi, in Ferrara. He was most active both in the Italian and the European (International) Purine Clubs; he organized in 1998, 2006, and 2024 multinational Congresses with a large number of attendants for these Societies [44]. In 2001 he hosted in Ferrara a symposium on "Nucleotides and their Receptors in the Immune System" together with Peter Illes and Pier Andrea Borea, sponsored by the VW-Foundation [45]. He was one of the driving forces behind the PRESTO COST ACTION of the European Community entitled "P2X Receptors as Therapeutic Opportunity". Francesco was also currently president of the Italian Purine Club.

He was a passionate scientist who always enjoyed talking about research and pushed his collaborators towards creative thinking and rigorous planning and analysis of scientific data. He enjoyed the numerous convivial occasions with his closer colleagues and students but also with the many scientists with whom he collaborated worldwide. He was an inspiring figure for all those who had the fortune of knowing him and will be much missed by all of us.

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