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Purinergic signaling in giant cell formation

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1. ABSTRACT

Cell fusion into multinucleated giant cells (MGC) is an essential process that contributes to many important biological mechanisms in mammalians. In the bone and immune system, macrophages are endowed with a remarkable potential for cell fusion events as evidenced by their propensity to fuse with other cells and between themselves during both normal processes and disease. Macrophage fusion is critical for the normal development of multinucleated osteoclasts, the cells responsible for bone resorption. Macrophages from various tissue compartments also undergo fusion into MGC, a hallmark of granulomatous inflammation. To date, the mechanisms underlying macrophage fusion remain poorly understood. Receptor-ligand interactions are thought to mediate this process and several lines of evidence implicate purinergic receptors in both osteoclast and MGC formation. Notably, the P2X7 receptor for extracellular ATP is expressed in osteoclasts and in many types of granulomas associated with infection, foreign body response and sterile inflammation. Through their ability to sense extracellular cues and ATP, a messenger of intercellular communication, purinergic receptors likely contribute to cell-cell interactions that result in macrophage fusion.

2. INTRODUCTION

Herein, we highlight the importance of macrophage fusion in bone and inflammation and the implication of purinergic signaling as a critical component of this process. In particular, a role for P2X7 in MGC formation is emphasized and evidence that may provide insights into the mechanisms underlying P2X7 action is presented. The relationships of P2X7 to known molecular mediators of cell fusion and its potential contribution to membrane destabilization and reorganization are discussed. We also address the putative role of the nuclear factor of activated T cells (NFAT) as an important purinergic-driven signaling pathway in osteoclast and macrophage fusion.

3. OSTEOCLASTS AS PHYSIOLOGICAL MULTINUCLEATED GIANT CELLS

Osteoclasts are multinucleated giant cells that are found in bone of healthy individuals; they are cells of the monocyte-macrophage lineage deriving from hematopoietic progenitors. The basic physiological role of osteoclasts is extracellular resorption of mineral and organic bone matrix components. Indeed, despite its static appearance, bone is a dynamic tissue that continuously undergoes a process

involving breakdown (resorption) and buildup (formation), which are mediated by osteoclasts and osteoblasts respectively. Resorption is necessary for remodeling during skeletal growth and tooth eruption but also to guarantee the continous replacement of fatigued bone with new one and to regulate blood calcium levels (1). Skeletal bone is the main calcium source in the body since a constant extracellular Ca^{2+} concentration is guaranteed by osteoclast-mediated bone disruption. To maintain a constant bone mass, a negative feedback is activated by the high calcium concentrations (40 mM) reached in bone lacunae during bone resorption which cause apoptosis of osteoclasts. At the same time, osteoblast proliferation and differentiation are increased resulting in bone maintenance (2). Perturbations of the balance of osteoclast/osteoblast activities can result in skeletal abnormalities, such as osteopetrosis, osteoporosis and bone loss linked to rheumatoid arthritis or cancer (3). Osteopetrosis is due to increased bone density and is mainly associated with rare hereditary disorders. By contrast, osteoporosis is a disease affecting millions of people in the world, especially post-menopausal women and elderly of both genders, but also immobilized patients and even astronauts exposed for a long time to zero gravity. Osteoporotic patients present reduced bone density and frequently undergo invalidating fractures. Bone loss is also a secondary effect of several pathologies such as rheumatoid arthritis and hematological neoplasias, like multiple myeloma (4). The detrimental effects of excessive osteoclast activity render the study of these cells really important. In particular, osteoclasts are the only cells known to behave as polykarions in physiological conditions.

Differentiation of osteoclasts from monocyte/macrophage lineage precursors requires the presence of the cytokine receptor activator of NF-kappa B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) that are produced by osteoblasts and stromal cells exposed to PTH or other bone resorbing hormones. Membrane-bound or soluble RANKL binds to its receptor RANK on osteoclasts, giving rise to a series of signals responsible for osteoclast differentiation and fusion. Among the signaling pathways activated are those of the transcription factors NF-kappa B, AP1 and NFAT (3). Osteoclasts to become active, after cytokine-mediated differentiation, go through a three stage process: 1) monocyte/macrophage precursors become preosteoclasts expressing characteristic proteins such as TRAP and calcitonin receptor; 2) mononucleate preosteoclasts fuse together to become non functional multinucleated osteoclasts that are polykarions lacking ruffled borders and thus cannot resorbe bone; 3) non functional polykarions are activated into resorbing osteoclasts by various factors such as RANKL, TNF-alpha and LPS. These fully activated, mature osteclasts have the capacity to resorbe bone and die after the resorption process is completed (1). A main requisite for the osteoclasts to resorb is to fuse forming multinucleated giant cells. Among multinucleated giant cells, osteoclasts are morphologically similar to foreign body giant cells (FBGC) with their nuclei randomly diffused into the cytoplasm. Nevertheless, osteoclasts, in humans, have a considerably reduced number of nuclei when compared to FBGC (5). Various evidence indicate a direct relashionship between the number of nuclei of the active osteoclast and its resorbing activity. For example osteoclasts of patients with Paget's disease that involves excessive bone resorption, present a greatly increased number of nuclei. Similarly, in birds, which require the ability of a fast mobilization of calcium to allow for egg formation, the osteoclasts show a greater number of nuclei than those of mammals (1). As for the other polykarions, the mechanisms underlying osteoclast multinucleation is far from being elucidated.

4. MULTINUCLEATED GIANT CELLS , A FEATURE OF CHRONIC INFLAMMATORY DISORDERS

Myeloid precursor cells in the bone marrow differentiate directly or via circulating monocytes, into tissue macrophages that harbour specific characteristics dictated by their respective microenvironment. Resident macrophages are present constitutively in all tissues in absence of overt inflammation where they perform trophic and homeostatic roles. They are at the interface of innate and adaptive immunity due to their ability on one hand, to engulf invading microbes or cell debris and remove apoptotic cells, and on the other hand, to secrete a wide array of immunomodulatory cytokines, present antigens to T cells and act as accessory cells in lymphocyte activation (6). Consequently, macrophages are characterized by a high degree of heterogeneity and adapt their phenotype to suit the microenvironment in which they reside. In an attempt to classify/distinguish macrophage populations, a nomenclature based on the different behaviour of macrophages has been introduced (7). It is generally thought that M1 macrophages function as bactericidal, antigen presenting cells and promote the differentiation of naïve CD4+ T cells into Th1 effector lymphocytes. M1 macrophages are induced by lipopolysaccharide, the archetype of bacterial antigen, and interferon-gamma (8), and are defined as highly activated pro-inflammatory macrophages. In contrast, M2 macrophages promote CD4+ Th2 lymphocyte and regulatory T cell (Treg) differentiation (9), and are activated by the type 2 cytokines IL-4, IL-10 and IL-13. M2 macrophages are referred to as alternatively activated macrophages and have been associated with repair processes and attenuation of excessive inflammation.

Macrophage plasticity is further illustrated by their singular capacity to fuse into MGC (10), in response to either physiologic stimuli to form osteoclasts or inflammatory agents such as microorganisms or non digestible foreign materials. During this process, macrophages switch their pluripotent activity to more specialized functions aimed at resorbing tissue or eliminating invading agents. As a continuum of macrophage plasticity, MGC also harbour various morphologic phenotypes depending on the local environment and the chemical and physical nature of the agent to which the MGC and the monocyte-macrophage precursors are responding. The most described morphological variants of MGC include Langhans Giant Cell (LGC) with nuclei located at the cell periphery in a

Table 1. Typical features of immune response and MGC in different pathological conditions

Granuloma related pathologies	Immune response	Pattern of cytokine	Giant Cell type
Mycobacterium tuberculosis	Th1	IFN-gamma, TNF-alpha	Small LGC with bactericidal functions
High virulent *M. Tuberculosis*	Th1 shift to Th2	IL-4, IL-10, IL-13	antigen presenting LGC with low phagocytic properties
Mycobacterium leprae: Tubercoloidleprosy	CD4+Th1	IFN-gamma, IL-12, IL-15	LGC
Lepromatous leprosy	CD4+,CD8+ Th2	IL-10, IL-13	LGC
Schistosomiasis: Eggs induced granuloma	CD4+ Th2	IL-4, IL-2, IL-13	Large LGC (> 50 nuclei in IL-12 KO) absence of Th1 response
Sarcoidosis	CD4+Th1	IFN-gamma, IL-2, IL-6, TNF-alpha	LGC and rarely FBGC
Giant cells artheritis	CD4+ Th1	IFN-gamma, IL-1beta, IL-6	FBGC associated with calcification of internal elastic lamina and rarely LGC
Foreign body reaction to biomaterials: implants	Th2	GM-CSF, IL-4, IL-13	FBGC
Silicosis, Asbestosis (silica particles, asbestos fibers).	Th1	IL-1beta, TNF-alpha, IL-6	LGL and FBGC

horseshoe fashion, and Foreign Body Giant Cells (FBGC) characterized by a large cytoplasm with nuclei randomly distributed inside the cells (11). It is not clear whether these morphological variants arise through the fusion of macrophages polarized to the M1 or M2 state (12-13). Alternatively, they may represent different stages of macrophage fusion with LGC, which contain a smaller number of nuclei, preceding that of FBGC (14).

Although considered an uncommon event, homotypic fusion of macrophages into MGC is a hallmark of chronic inflammatory granulomatous reactions (15), and has been observed in many pathological conditions, including infection, immune reactions to foreign materials and various diseases of unknown etiology (16). This is summarized in Table 1.

4.1. Infective inflammation

In infective granulomas**,** including tuberculosis and leprosy, the MGC derived from infection by intracellular bacteria are referred to as 'immune MGC' and morphologically, are typical of LGC (17). In tuberculosis, macrophages are the principal host cells for the intracellular replication of *M. tuberculosis.* At the same time, they act as antigen-presenting cells (APCs) and play an important role in the killing of mycobacteria (18). Protective immunity to *M. tuberculosis* relies on cell-mediated immune response, driven mainly by CD4+ and CD8+ T cells with a Th1 cytokine profile and consequent release of IFN-gamma, IL-2 and TNF- alpha. Overall, acquired resistance against *M. tuberculosis* appears to require the generation of a T cell-mediated immune response, the activation of infected macrophages and the formation of granuloma that prevents dissemination of the mycobacteria and acute tuberculosis (19). The majority of cells in the granuloma are epithelioid macrophages with abundant cytoplasm and Langhans giant cells. In spite of macrophage activation by Th1-derived IFN-gamma that allows acidification of phagosome and production of nitrogen species, some bacteria escape macrophage killing (20). This in turn induces a chronic response by the host with the development of a granulomatous response promoted by Th2 cells with release of IL-4, IL-13 and IL-10 (21-22). LGC become enlarged and are incapable of phagocytosis but still retain a strong antigen presentation capability (23). For many intracellular bacteria, clearly an imbalance of Th1/ Th2 response is thought to contribute to the dissemination of infection and fibroproliferative disorder (24).

Recently Li and co-workers (25) have shown that a loss-of-function P2X7 polymorphism contributes to the variability in susceptibility to mycobacterial infection through abolition of P2X7-mediated macrophage apoptosis. In addition, J774 macrophages with defective P2X7 function as well as macrophages from individuals with polymorphism of the P2X7 gene infected *in vitro* with *Bacillus Calmette Guerin*, become resistant to apoptosis and loose the ability to fuse and form MGC (Falzoni and Chiozzi, unpublished observations). These observations suggest a link between P2X7 and steps that are fundamental for killing *mycobacterium* and limiting its dissemination (26-27).

Granuloma is also a feature of reactions to helminth and in particular, to *Schistosoma mansoni.* Schistosomiasis is a disease characterized by hepatic and intestinal granuloma formation around deposited parasite eggs and tissue fibrosis. It is known that deposited eggs secrete soluble egg antigens into surrounding tissue that evoke a T-cell mediated granuloma(28). The granuloma consists of eosinophils, monocytes, lymphocytes, epitheloid cells and some MGC that surround eggs together with fibroblasts and various amounts of collagen fibres. In murine schistosomiasis, pathogenesis is induced by CD4+Th2 cells and IL-4 and IL-13 drive the response against soluble egg antigens and induce fibrosis (29), while IL-10, IFN-gamma and a subset of regulatory T-cells act to limit schistosome-induced pathology (30).

4.2. Inflammation of unknown etiology

The formation of MGC is also observed in many different disorders classified as inflammatory autoimmune diseases of unknown etiology including Crohn's disease, sarcoidosis, giant cell arteritis, atherosclerosis, rheumatic arthritis, Langerhans cell histiocytosis and multinucleated giant cells in bone, lymph node and skin lesions (31-33). As mentioned above, those are disorders of the T-lymphocyte-mediated inflammatory response to unknown antigenic stimuli, and most of them share a typical Th1 response.

In giant cells arteritis, cellular infiltrates in inflamed adventitia of artery walls are composed of Th1 lymphocytes that secrete a large amount of IFN-gamma able to activate macrophages and induce MGC formation (34). Activated macrophages in the adventitia produce significant levels of IL-1beta and IL-6, and resemble the highly activated monocytes circulating in the blood of patients. Moreover, activated macrophages release matrix metalloproteinases (MMP) that are able to digest arterial wall components, causing tissue destruction (35) . Antigens involved in the pathogenesis are not well defined but analysis of giant cells arteritis lesions indicated the presence of FBGC with LGL in some cases, suggesting the possibility that infection may also contribute to the pathogenesis of this disease (36).

Recent investigations in immunology and molecular biology have provided evidence for a link between infectious agents such as *mycobacterium* and propionibacterial, and the development of sarcoidosis. Although the nature of the antigenic stimuli is not known (37-38), it is generally recognized that T lymphocytes in sarcoidal lesions are CD4+ that release Th1 cytokines such as IFN-gamma and IL-2 (39). Among the immunocompetent cells that constitute sarcoidal lesions, alveolar macrophages release a large amount of TNF-alpha, IL-1beta and IL-6 while macrophages isolated from granulomatous lymph nodes express high levels of IFN-gamma, TNF-alpha and IL-12 (40). In addition, alveolar macrophages isolated from patients express high levels of intercellular adhesion molecule-1 and high density of class II indicating that antigen-presenting activity is enhanced (41). Quite interestingly, Mizuno and co-workers have shown that monocytes from sarcoidosis patients express high levels of P2X7 receptor (42). Moreover, MGC formation in these patients was inhibited by irreversible blockade of P2X7 (43).

4.3. Sterile inflammation

Foreign body reactions to biomaterials is well documented and have implications in tissue engineering and regenerative medicine. It is thought to be responsible for the failure of orthopaedic implants and many animals models have demonstrated the presence of MGC in response to different types of biomaterials (44). These reactions are predominantly driven by a Th2 response and are characterized by the presence of FBGC.

Pulmonary response to inhaled toxic foreign particles and dusts is also characterized by macrophage fusion in MGC. The presence of MGC has been observed in lungs of patients with pneumoconiosis and irreversible fibrosis, notably following exposure to silica particles and asbestos fibers (45). Animal models of these diseases are characterized by chronic inflammatory reactions and the presence of both types of morphological variants in bronhoalveolar lavage and pulmonary interstitium (46-48).

The basic cellular machinery that drives macrophage fusion and MGC formation is not well understood. This process is likely to encompass a series of well ordered sequential steps including cell aggregation or clustering, adhesion, membrane merging and formation of intercellular bridges that ultimately lead to numerous nuclei contained within a single continuous plasma membrane. The formation of FBGC has been the most studied and several mediators including transcription factors, transmembrane proteins and cell surface molecules involved in the fusion process have now been identified. This has been the subject of recent reviews (33, 44, 49) and their detailed description is beyond the scope of this work. Our discussion will be limited to those molecules that might be relevant to purinergic receptor-mediated macrophage fusion.

5. P2X7 RECEPTOR-DEPENDENT SIGNALING AND MGC FORMATION

Most of the evidence implicating purinergic signaling in the formation of multinucleated giant cells relate to the P2X7 receptor for extracellular ATP. Following the original observation by Falzoni *et al.* (50) that P2X7 is expressed during human monocyte/macrophage differentiation into multinucleated macrophages, both direct and indirect evidence suggest a crucial role for this receptor in macrophage fusion: 1) P2X7 function/expression has been documented in relation to all three types of inflammatory granulomas as described above, notably in tuberculosis (51), sarcoidosis (42-43) and foreign body responses (52); 2) P2X7 is present on a wide range of macrophages and a number of *in vitro* studies using macrophage preparations from various tissue compartments including human peripheral blood-derived macrophages (50), rat lung alveolar macrophages (53) and murine peritoneal macrophages (Lemaire, unpublished observations), have demonstrated the participation of P2X7 in this process; 3) pharmacological blockade of P2X7 inhibits macrophage fusion (27,50) whereas P2X7 stimulation increases this process both in HEK293 cells transfected with full length P2X7 and in macrophages (54); 4) heterologous cells transfected with a truncated inactive P2X7 (54), as well as macrophages devoid of P2X7 function (27) and macrophages from mice lacking the P2X7 gene (Lemaire, unpublished observations) all display defective macrophage fusion.

The observations that macrophages from mice lacking the P2X7 retained the ability to form osteoclasts (55) and MGC (Lemaire, unpublished observations) have brought into question the role of P2X7 in this process. However it should be emphasized that these macrophages display a lower capacity than their wild-type counterparts to form MGC. The basal fusion index observed in these knockout macrophages may be related to compensatory mechanisms, or the contribution of other P2 receptor(s). In connection with this, prolonged exposure of osteoclast precursors from P2X7-null mice to ATP was found to inhibit osteoclast fusion (56). Quite interestingly, macrophages lacking P2X7 fail to respond to stimuli or inflammatory cues suggesting that P2X7 is required for efficient promotion of MGC (Lemaire, unpublished observations). This would be consistent with the important role of P2X7 in inflammation, a reaction associated with MGC formation by macrophages *in vivo*. To date the implication of P2X7 in the cellular events resulting in

macrophage fusion is not understood. It is not clear what mediators trigger P2X7 activation, which P2X7 function(s) are required and what stage(s) of the fusion process are mediated by P2X7. Herein we highlight existing evidence that may provide some insights with respect to the mechanisms underlying P2X7 action. We also discuss the potential implication of some P2X7-related pathways in this process.

5.1. What turns on P2X7 activation during macrophage fusion and MGC formation?

A wealth of evidence have linked P2X7 activation to high millimolar concentrations of ATP and cytotoxic lethal effects, and this characteristic has been used to distinguish P2X7 from other members of the P2X receptor family. However such well established paradigm represents only one facet of this intriguing receptor and is challenged by observations that high millimolar levels of extracellular ATP are unlikely to be available within the cell microenvironment given that intracellular ATP concentration is only 3-5 mM, and that most cells express significant ecto-ATPases activities. Even during chronic inflammation both ATP release and ATP metabolism are increased with the net balance likely resulting in low ATP levels present in the vicinity of P2X7. Furthermore, MGC formation is a regulated process involving many functional steps that require cell survival, as evidenced by the presence of long-lived macrophages within granulomas. Yet, there is no direct evidence that extracellular ATP, the physiological ligand of P2X7 can by itself increase MGC formation. Punctual *in vitro* addition of ATP to bulk extracellular milieu is unlikely to mimic the basal dynamic concentration of ATP available to P2X7 receptor located on plasma membranes of closely juxtaposed cells. As postulated for the reported trophic effects of P2X7 (57-59), an autocrine purinergic loop triggered by the release of endogenous ATP in the P2X7 microenvironment may be responsible for P2X7 activation during MGC formation. Consistent with this, P2X7 transfected cells display higher fusion index and MGC formation than empty vector transfectants in the absence of exogenous stimuli (54). P2X7 has been identified as a non-lytic pathway for ATP secretion (60) and evidence for autocrine activation of P2X7 via the release of endogenous ATP have been demonstrated in macrophages (60), human monocytes (61) and T cells (62). This process may occur following ATP accumulation within diffusion-restricted microdomains of the cell membrane that are not readily accessible to the extracellular compartment. Quite interestingly, P2X7 which is uniformly distributed on the plasma membrane of cells, concentrates in membranes clusters at the site of cell-cell interaction during the fusion of macrophages and transfected cells (54, 63), a condition that may favor P2X7-dependent ATP release, increased juxtamembrane ATP concentration and autocrine activation of P2X7 at discrete membrane sites.

In this scheme, activation of P2X7 may be dependent on the critical balance between nucleotide metabolism and nucleoside generation. Such assumption is supported by a number of studies. Thus P2X7 reportedly desensitizes following continuous leakage of ATP from cells, and treatment with apyrase, a functional analog of CD39/NTPDase, re-established sensitivity to tonic stimulation by ATP resulting in trophic effect (64). More direct evidence for a putative role of CD39 in the regulation of P2X7 activation has been provided by the observations that cell-surface expression of CD39 was inversely correlated with P2X7 stimulation (65) while absence of CD39 was linked to unimpeded activation of P2X7 (66). Notably, overexpression of CD39 in macrophages was found to reduce ATP release by 71%, but did not totally suppress it with some residual ATP presumed to be leakage or release from macrophages themselves (65). This in turn, was correlated with diminished P2X7 stimulation. All together, these studies suggest a novel paradigm by which CD39 expressed on macrophage surface autoregulates the macrophage ambient nucleotide concentration thereby controlling P2X7 activation. In this regard, it is tempting to speculate that CD39/P2X7 interplay at juxtaposed membranes may control repeated episodes of brief ATP activation of P2X7, a condition known to result in membrane and cytoskeletal disruptions that are harmless and fully reversible (67).

Low threshold activation of P2X7 by extracellular ATP may be achieved through positive allosteric modulation via conformational changes of P2X7. Such mode of regulation requires the participation of accessory molecules and appears to be carried out through sutble membrane perturbations including changes in membrane constituents during cellular responses. Among these, arachidonic acid, which regulates a range of biological effects including cell growth, differentiation and viability, increases the potency of ATP acting at P2X7 (68). Other membrane components, notably lysolipids, also increase the effects of sub-maximal ATP concentrations at P2X7 (69-70), and relatively high concentrations of lysolipids in close proximity to the P2X7 receptor can be generated by phospholipase A_2 activation. An important feature of active lipids is the presence of a single long acyl chain, suggesting that disruption of the ordered structure of the lipid bilayer following their membrane insertion is required for their effects (70). Of particular interest is the observation that lysophosphatidylserine increased agonist potency without apparent cell lysis. Given that P2X7 activation causes externalization of phosphatidylserine (71), as suggested (70), this may favour lysophosphatidylserine production via extracellular phospholipase A_2 thus providing a mean by which P2X7 could autoregulate its activation by low ambient ATP concentrations. There is evidence that some antimicrobial peptides, notably the peptide antibiotic polymyxin B (PMB) (72-73) and human cathelicidin LL-37 (74), potentiate the effects of low ATP concentrations through allosteric modulation of P2X7. Their effects are thought to be related to their amphipathic conformations and their ability to insert within hydrophobic/hydrophilic interface of membranes and bind to phosphatidylcholine/cholesterol bilayers. The findings that PMB promotes MGC formation by HEK cells tranfected with full length P2X7 and macrophages (54) suggest that a lipid-P2X7 loop may be involved during the process of macrophage fusion. Consistent with this, GM-CSF which stimulates

macrophage fusion (75-76) reportedly induces phospholipid metabolism (77). Another potential mechanism for positive allosteric action at the P2X7 may involve extracellular nicotinamide adenine nucleotide acting via a GPI-anchored ADP-ribosyltransferase (ART2.1). This ectoenzyme catalyses ADP-ribosylation of arginine residues on the P2X7 ectodomain, a modification sufficient to decrease the threshold for gating of P2X7 in response to ATP binding (78). Therefore, NAD which is released during the early stage of inflammatory response can act synergistically with ATP to regulate P2X7 signaling in macrophages during the fusion process.

5.2. Pore-forming activity: a critical component of P2X7-driven macrophage fusion?

Pore formation is an important step in a variety of fusion events notably fusion between target cell and viral membranes (79), cell-cell fusion triggered by viral proteins (80) and myoblast fusion during myotube formation in muscle (81). The hallmark of P2X7 activation is its ability to form a pore that allows influx of low molecular mass solutes into the cytoplasm. Therefore it is reasonable to assume that P2X7-induced macrophage fusion may be related to its capacity to undergo conformational changes through membrane lipids reorganization and to form a membrane pore. As yet, unequivocal evidence that the P2X7-dependent pore is a requisite for effective macrophage fusion is lacking and its putative role in the fusion process remains an unresolved issue. However, indirect/circumstantial evidence indicate that pore-forming activity may be an important attribute for inducing cell fusion. Thus pharmacological blockade of P2X7- pore-forming activity by oxidized ATP and the antagonist KN-62 also blocks macrophage fusion (27,50). Conversely, PMB which enhances pore activity (73) also promotes macrophage fusion (54). Additional evidence come from observations that cells transfected with full length P2X7 display an higher fusion index whereas such pro-fusing phenotype is lost in cells expressing a P2X7 lacking the C-terminal domain and unable to form the pore (54). Similarly, macrophage clones selected for ATP resistance and loss of pore forming activity fail to form MGC (27). In line with this, an independent study reported that osteoclast formation in RAW macrophage-like cells is prevented by down-regulation of P2X7 in cells made resistant to ATP and transiently devoid of pore formation, but resumes upon recovery of P2X7-dependent pore-forming activity (82).

It can be argued that P2X7-dependent pore formation has been linked to cytolytic effects of the receptor. How this paradigm could be reconciled with potential implication of such pore in macrophage fusion, a regulated process that requires healthy cells? Some insight may be provided by the observations that brief ATP activation of P2X7 is coupled to pore-forming activity that is fully reversible and non-lethal (67). In addition, a recent study using a P2X7 point mutant with ablated pore forming capacity demonstrates a trophic role for P2X7 pore in driving microglial cell proliferation (83). Indirect support for the existence of such P2X7 trophic pore comes from other observations that human cathelicidin LL-37, which stimulates fibroblast growth, also enhances P2X7-dependent pore formation (74). Quite intriguingly, LL-37 leads to pore-forming activity in cells expressing a truncated P2X7 unable to generate the non-selective pore typical of the full length receptor. This coupled to the finding that such effect is not abrogated by inhibition of Pannexin-1 (Panx-1) raise the interesting possibility that induction of the trophic pore may be triggered through distinct mechanisms.

Assuming that pore formation is critical for macrophage fusion, the question remains as to what could be the putative role of the pore in the process? Its implication as a conduit for ATP release that could contribute to autocrine stimulation or as an effector of membrane bridging between partner cells is at present unknown. These two possibilities however are not mutually exclusive. As summarized before, macrophages must undergo an ordered sequence of functional events in order to be able to fuse with partner cells. It is noteworthy that a series of parallel and consecutive podosomes expressing high levels of P2X7 at sites of cell contact is seen during interaction between partner macrophages (63). This may be related to the formation of a network of small pores within apposed membranes which, following membrane reorganization, generate larger areas of junctional cytoplasm that eventually result in complete mixing of cytoplasm from the two partners involved. This is clearly speculative but is reminiscent of the proposed model for anchor cell fusion in *C.elegans* which suggests that multiple pores form, enlarge and coalesce (84).

5.3. P2X7- downstream effectors that might mediate macrophage fusion

The final cellular steps identified as critical for cell fusion are membrane alignment and membrane pore and resolution (81). During fusion of target cell with virus, fusion is thought to proceed through a "hemifusion" intermediate in which the outer membrane leaflets undergo lipid mixing prior to fusion pore formation, enlargement and completion of fusion (79). By analogy with this type of fusion event, macrophages may undergo similar cellular changes many of which could be potentially mediated through P2X7.

In particular, brief activation of P2X7 triggers rapid (within seconds) exposure of phosphatidylserine (PS) on the outer membrane (71) in a non apoptotic fashion (85). This in turn, as discussed previously, may favor other lipids-P2X7 interactions with consequent increase of P2X7 gating in response to low ambient ATP levels. Transient and reversible PS translocation has been associated with a number of physiological cell responses (86) and within the context of cell fusion, may represent a pre-requisite step for regulated pore formation. Indeed, PS exposure on the cell surface has been associated with the fusion of cytotrophoblast (87), cardiomyocytes (88), myoblasts (89) and more recently, was found to be required for macrophage fusion (90).

It is known that PS distribution modulates rapidly the activities/distribution of several membrane proteins (85). One obvious candidate that may regulate pore

formation during macrophage fusion is Panx1 hemichannel protein. Panx1 has been shown to be part of the pore forming unit of the P2X7 receptor (91-92). The gating of Panx1 hemichannels by P2X7 appears to result on one hand, in pore forming activity and on the other hand, in the facilitation of ATP release for extracellular signaling that include both autocrine/paracrine actions (93). Additional evidence indicate that modulation of Panx1 hemichannel opening may be achieved by submillimolar levels of extracellular ATP that rapidly and reversibly suppress pore formation as assayed by ethidium bromide influx in *Xenopous* oocytes (94). This points to Panx1 as an important molecule involved in feedback inhibition of P2X7 activation through ATP release and suggest that fine tuning of Panx1 hemichannel opening may be critical for regulated pore formation. Such mechanism would be compatible with repeated cycles of brief pulses of P2X7 activation and regulated pore formation, a condition likely required for macrophage fusion. As yet, there is no evidence for a role of Panx1 in macrophage fusion, and the use of animals lacking Panx1 may help clarify this issue.

The formation of intercellular bridges allowing direct intercellular communication via diffusion of ions, metabolites and small soluble molecules during macrophage fusion may require the docking of hemichannels present on partner cells. Such process resulting in gap junction formation appears to be a selective property of connexins since the formation of intercellular channels by Panx1 is unlikely given the presence of a glycosylation site in the extracellular domain of this protein (95). Intriguingly, the expression of P2X7 on both partner cells is required for fusion of cells transfected with P2X7 (Lemaire, unpublished observations) and macrophages (63). Of direct relevance to this, P2X7 has been shown to interact with connexin 43 (Cx43). Thus P2X7 and Cx43 co-localize to the membrane of macrophages, and some evidence has been provided that the presence of P2X7 at the cell surface facilitates membrane insertion of Cx43 and the formation of gap-junction channels (96). Quite interestingly, human cytrophoblast treated with Cx43 antisense oligonucleotides were found to fuse poorly (97). Moreover, Cx43 expression as well as gap junctional communication have been observed between macrophages and FBGC and between FBGC after implantation of biomaterial in animals (98). All together these observations point to a role of connexins, particularly Cx43 in mediating P2X7-dependent macrophage fusion.

The macrophage cytoskeleton is important for numerous functions associated with macrophage fusion including changes in cell morphology, migration and membrane organization. The P2X7 has been shown to form a large macromolecular complex in the membrane with cytoskeletal proteins, notably with actin (99). Recent evidence indicate that P2X7 is involved in rapid assembly of actin at the plasma membrane (100), an event likely required for membrane organization. Also, a close interaction between P2X7 and nonmuscle myosin, another component of the cytoskeleton, has been demonstrated (101). Quite interestingly, ATP was found to dissociate nonmuscle myosin from the P2X7 complex, an event that leads to increased P2X7 pore formation. It has been proposed that this may be a mechanism by which P2X7 undergoes transition from channel to pore, and that dissociation of P2X7 from its cytoskeletal attachments may allow pore formation possibly through interaction with pore-forming proteins such as Panx1.

Overall, without excluding the potential implication of P2X7 at multiple steps of the fusion process, its attributes which include interactions with membrane lipids, hemichannel proteins, and cytoskeletal actin and myosin speak for a more prominent role of P2X7 in the final stages of membrane fusion.

5.4. Potential link between P2X7 and P1 receptors for adenosine in the fusion process

The observations that apyrase potentiates MGC formation by macrophages (63) and HEK293 cells tranfected with P2X7 (Lemaire, unpublished observations) raise interesting questions with respect to P2X7 implication in MGC formation. As discussed previously, CD39 may play a role in regulating basal low ATP stimulation of P2X7 (65). Even in the presence of apyrase, a functional analog of CD39/NTPdase, suboptimal concentrations of ATP sufficient to cause P2X7 activation may be present in the vicinity of the receptor. On the other hand, considering that P2X7 is a conduit for ATP release, ATP metabolism through the serial actions of ecto-apyrase and ecto-5' nucleotidase would result in the formation of adenosine which has been shown to promote both MGC (102) and osteoclast formation (103). Consistent with this, lung inflammation resulting from elevated adenosine in adenosine deaminase-deficient mice is characterized by accumulation of MGC (104). Therefore, P2X7-dependent ATP release may provide a functional link between P2X7 and P1 receptors for adenosine which may be important in terminating or otherwise, modulating the fusion signals.

6. PUTATIVE ROLE OF PURINERGIC SIGNALING IN OSTEOCLAST PHYSIOLOGY AND FUSION

The function of purinergic receptors in bone formation is extensively covered by other authors in this issue of Frontiers in Bioscience. In this section we will focus on some potential, non conventional links, between purinergic signaling and osteoclast physiology and fusion. In particular we will address the putative function of purinergic receptor-activated calcium-NFAT signaling.

Osteoclasts express a wide range of purinergic receptors comprising A1, A2A, A2B, A3 (105) P2X2, P2X4, P2X7, P2Y1, P2Y2, P2Y6, P2Y13 (106,107). Upon activation, all purinergic receptors cause an increase in intracellular calcium either through G protein coupled ER release (Adenosine and P2Y receptors) or directly acting as calcium channels (P2X receptors). Due to the importance of bone as the main body's calcium reservoir it is easy to imagine how calcium signaling plays a central role in osteoclast physiology. Indeed, calcium waves both depending on ER release and extracellular calcium entry are fundamental for osteoclastogenesis. Different proteins including IP3R2 on the ER and TRP calcium channels on

plasma membrane have been shown to play a role in osteoclast calcium waves but none of them has been definitively proved to be the only responsible for the oscillations (2). In this regard, purinergic receptors could be important players in calcium entry and modulation. Among P2X receptors, there is clear evidence of ATP-mediated extracellular calcium entry only through P2X7 (108). Despite their expression (109), P2X4 appears to activate a current but lacks the calcium permeating ability (110), while P2X2 is non functional (106).

When activated by pharmacological ATP concentrations (i.e 1-3 mM), P2X7 receptor mediates the opening of a big unselective pore that causes a massive and lethal entry of calcium into the cytosol (111) and mitochondria (57). At these agonist concentrations an activity as cytotoxic receptor has been proved for P2X7 also in osteoclasts (112), suggesting a role of the receptor in terminating the resorption when lacunae calcium is too high. Moreover, at physiological extracellular ATP concentrations, P2X7 receptor also exerts a trophic activity in several cell types (57,58,64,83,113). This growth-promoting potential has been shown to be dependent on P2X7- mediated calcium increase in different cell compartments, comprising cytosol (114), mitochondria (57) and ER (58). P2X7- induced increase of basal levels of mitochondrial calcium was also linked to augmented ATP production and mitochondrial activity (57) that might be relevant in osteoclastogenesis. Indeed, mitochondrial respiration and glucose-pyruvate consumption are augmented during multinucleated osteoclast formation (115) while inhibition of oxidative chain also blocks osteoclast differentiation and fusion (116). An effect on mitochondrial calcium increase has been recently shown also for metabotropic P2Y2 receptors in submandibular gland cells (117). In this respect osteoclast P2X7 and $P2Y_2$ could exert a facilitating function in multikarion formation and bone resorption.

Osteoporosis is a frequent secondary effect in post-transplant patients treated with immunosuppressants inhibiting calcineurin-NFAT axis such as cyclosporin or FK506 (2). Interestingly NFAT signaling pathway is activated through calcium influx from P2X7 and several other purinergic receptors (118-121). This transcription factor that was originally identified in the context of T cell activation, has also been involved in cardiovascular and muscular systems differentiation and in the regulation of osteoclastogenesis (122). Several studies have shown that P2 receptors could exert an action on T cell receptor signaling (121,123), chemokine expression (124) or cell growth (58) through NFAT family members. Here we suggest that a similar function could also explain, at least in part, the importance of purinergic signaling in bone homeostasis.

Among the NFAT protein family members the one that is mainly involved in bone formation is complex 1 (NFATc1), which over-expression has been associated with different P2X7 isoforms (58-59). RANK/M-CSF mediated NFATc1 pathway seems to be more relevant for osteoclast differentiation than NF-kappa B and AP1, which are not sufficient by themselves to drive osteoclastogenesis (125). In support of this hypothesis, NFATc1 is able to induce osteoclast fusion even in the absence of RANKL stimulation (125). Moreover, osteoclast conditional NFATc1-deficient mice exhibit osteopetrosis (126). Indeed, calcineurin-NFATc1 signaling axis has been shown to be central in the genesis of osteoclasts as the application of cyclosporine, FK506 and intracellular calcium chelants (i.e. BAPTA) all stop the osteoclast maturation (125,127). Once activated by calcium, NFATc1 exerts a transcriptional activity on a series of osteoclastogenesis pivotal genes such as TRAP, calcitonin receptor (125), cathepsin K (125) and beta 3 integrin (128). A recent paper by Kim *et al.* has also demonstrated a direct involvement of NFATc1 in regulating osteoclast fusion as its overexpression caused an increase in multikarion formation and upregulated two proteins shown to be central to the fusion process: DC-STAMP and Atp6v0d2 (129). In this respect, the ability of different isoforms of P2X7 receptor to double levels of active NFATc1(58-59), could be relevant in the regulation of osteoclast physiology and also in MGC formation. The decrease of active NFATc1 could also explain why, either reduced expression or inhibition, by means of a blocking antibody or receptor antagonists, of macrophage and osteoclast P2X7 receptor causes a decrease in multikarion formation (27,54,82,111,130). Nonetheless, if *in vitro* data on osteoclasts all seem to converge to a pro-fusion role for P2X7 thus suggesting the receptor as a possible osteoporotic inducer, the *in vivo* data are controversial. As a matter of fact, different P2X7 KO strains show diverse bone phenotypes ranging from increased to decreased bone mass (107). Moreover, the incidence of osteoporosis related fractures is augmented in postmenopausal women carrying a loss of function polymorphism in the C terminal tail of P2X7 (131). These apparently contradicting data could be reconciled supposing that P2X7 loss could cause a reduction of NFATc1 levels that in turn will decrease osteoblast proliferation, as it does in other cell models (58). Moreover, in the KO model, compensation either by other P2Xs able to induce NFAT activation (121) or by alternative splice variants of the same P2X7 (132) should be taken into account. In the case of post-menopausal patients, if the NFATc1 hypothesis was proved to be true, one should also consider the presence of the C- terminally truncated isoform of P2X7 (P2X7B) that would be able to activate the NFAT pathway (59). In our hands P2X7B isoform is expressed by osteoclasts but lacking in osteoblasts (Adinolfi, Falzoni and Jørgensen unpublished data).

Other putative activators of the NFATc1 signaling pathways are P2Y receptors expressed by osteoclasts. When activated by their ligands P2Y1, P2Y2 and P2Y6 all cause an intracellular calcium spike in osteoclasts but jointly P2Y6 receptor strongly activates NF-kappa B pathway (133). Nonetheless, the proliferative advantage caused by PY agonist can be only partially ascribed to NF-kappa B, thus suggesting that NFAT-mediated cell growth could be contemporary or alternatively activated. Indeed, there is evidence for the ability of all three osteoclast P2Ys to be able to activate NFAT in other cell models (134-136).

Besides its effects on NFAT, P2Y2 receptor could exert a regulatory effect on osteoclastogenesis through its plasma membrane association with alpha(v) beta 3 integrins (137). These molecules are important in mediating the first phases of osteoclast interaction and there is evidence for their direct interaction with P2Y2. Membrane bound P2Y2-alpha(v)beta 3 integrin complex is able to regulate focal adhesion kinase signaling pathway that is also central in osteoclast activation (138). In a similar fashion P2X7 could also affect osteoclastogenesis through other proteins that have been shown to be part of the membrane complex of the receptor (99). For example one of the P2X7 interacting proteins is integrin beta 2 that as well as being calcium-regulated is also part of a family of proteins linked to fusion of both MGCs and osteoclasts. Moreover, inhibition of Hsp90, that is part of the P2X7 membrane complex, has been shown to upregulate receptor expression and activity (139). This could be interesting in terms of osteoclastogenesis as Hsp90 inhibitors have been proposed as antitumoral drugs but show as side effects potentiation of bone metastasis (140-141). We suggest that this could be due to P2X7 overexpression causing neoplasic osteoclast proliferation.

We strongly believe that an in depth investigation in purinergic driven calcium-NFAT signaling would prove useful to elucidate the important role of extracellular purines receptors in bone patho-physiology and in granuloma formation.

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Abbreviations: MGC: multinucleated giant cells; NFAT: nuclear factor of activated T cells; RANK: receptor activator of NF-kappa B; RANKL: RANK ligand; M-CSF: macrophage colony-stimulating factor; Treg: regulatory T cell; LGC: Langhans giant cells; FBGC: foreing body giant cells, APCs: Antigen presenting cells; MMP: matrix metalloproteinases; PMB: polymyyxin B; PS: Phosphatidylserine; Panx1: Pannexin 1; Cx43: connexin 43; NFATc1: NFAT complex1.

Key Words: Macrophage, Osteoclast, Fusion, Polykarion, P2X7 Receptor, Inflammation, NFAT, Review

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