Common signalling pathways in macrophage and osteoclast multinucleation

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Abstract

Macrophage cell fusion and multinucleation are fundamental processes in the formation of multinucleated giant cells (MGCs) in chronic inflammatory disease and osteoclasts in the regulation of bone mass. However, this basic cell phenomenon is poorly understood despite its pathophysiological relevance. Granulomas containing multinucleated giant cells are seen in a wide variety of complex inflammatory disorders, as well as in infectious diseases. Dysregulation of osteoclastic bone resorption underlies the pathogenesis of osteoporosis and malignant osteolytic bone disease. Recent reports have shown that the formation of multinucleated giant cells and osteoclast fusion display a common molecular signature, suggesting shared genetic determinants. In this Review, we describe the background of cell-cell fusion and the similar origin of macrophages and osteoclasts. We specifically focus on the common pathways involved in osteoclast and MGC fusion. We also highlight potential approaches that could help to unravel the core mechanisms underlying bone and granulomatous disorders in humans.
Introduction

The fusion of plasma membranes is an active, frequent and universal phenomenon in living cells. Here, two forms of fusion can be distinguished as they lead to functionally diverse cell activities. First, the fusion between intracellular membrane-bound vesicles and the inner leaflet of the plasma membrane is crucial for a broad range of cellular activities, such as protein maturation, neurotransmission, hormone secretion and receptor trafficking. By contrast, the merging of outer plasma membranes is a hallmark of cell-cell and virus-cell fusion. The fusion of an enveloped virus with the host plasma membrane involves viral fusion proteins, known as fusogens, which have been the subject of many structural and functional studies owing to their relevance for infection (Harrison, 2008). Surprisingly, the molecular processes governing cell-cell fusion remained less explored and are thus only poorly understood.

The study of cell–cell fusion has emerged as a new field of research to understand the molecular mechanisms and consequences of membrane fusion (Harris and Watkins, 1965, Harris et al., 1969, Ephrussi et al., 1969, Blau et al., 1983, Blau et al., 1985). Under certain developmental and homeostatic conditions, mammalian cells fuse and give rise to cells with specialised functions. The ability of two or more cells to fuse and form a new syncytial cell is evolutionary conserved among eukaryotes (Ogle et al., 2005). At the cellular level, cell–cell fusion in mammals is essential for fertilisation (fusion of sperm and egg) to combine haploid genomes. At the organ level, it is required for formation of skeletal muscle (i.e. the fusion of myoblasts) to increase myofibre size for improved contractile strength, and it can occur during formation of the placenta (fusion of trophoblast cells) (Ogle et al., 2005).

Among the cells that can undergo cell–cell fusion, macrophages appear to have a particularly pronounced potential to fuse among themselves, both under normal or pathological conditions. In bone, the fusion between cells of the monocyte/macrophage lineage leads to the formation of osteoclasts, which become efficient bone-resorbing cells. Mutations in key genes affecting osteoclast fusion and function can result in rare diseases of the skeleton (e.g. Osteopetrosis, Paget’s disease) (Mellis et al., 2011). Under pathological conditions, such as in granulomatous diseases, macrophages can fuse into multinucleated giant cells (MGCs). However, although MGCs have been associated with inflammatory disorders, their exact role is unknown.

Despite their different environmental milieu, osteoclasts and MGCs share common mechanisms that govern cell fusion and multinucleation. In this Review, we describe the common origin of osteoclasts and MGCs and provide evidence that macrophage fusion is most likely driven by molecular mechanisms that are common to MGC and osteoclast
formation. We focus specifically on the signalling molecules and molecular pathways that are shared in osteoclasts and MGCs, and describe these within the context of cell fusion and multinucleation.

**Monocyte lineage: a common origin for osteoclasts and giant cells**

*Macrophages: immune cells with a fusogenic potential*

Macrophages have been originally identified in 1882 by Elie Metchnikoff based on their phagocytic properties as mononucleated cells that belong to the myeloid lineage (Gordon, 2008). They are ubiquitously present in tissues, in which they adapt to their local tissue environment and physiology to maintain homeostasis and repair. The ontogeny of mononuclear macrophages in a tissue has received widespread interest and has been reviewed thoroughly elsewhere (Perdigueró and Geissmann, 2015, Wynn et al., 2013, Ginhoux and Jung, 2014). As part of the innate immune response, macrophages have a unique potential to fuse with themselves and other cells to form multinucleated cells, also known as polykaryons. An early observation of macrophage multinucleated cells was already reported by Langhans in 1868 in granulomata that were associated with tuberculosis (TB) (Langhans, 1868). In contrast to most other fusion-capable cell types, which undergo fusion as a required part of their developmental program, the majority of macrophages fuse only infrequently and reside in tissues as mononuclear cells.

Macrophage-cell fusion and multinucleation are fundamental processes that occur in specific instances and give rise to MGCs that can be classified into several morphological subtypes, depending on the arrangement and composition of their organelles and their functional characteristics; these include Langhans giant cells and foreign-body giant cells (FBGCs) (Anderson, 2000). In general, MGCs arise in response to an infection and are considered a hallmark of granulomatous diseases, where they are also referred to as Langhans giant cells (Mariano and Spector, 1974, van Maarsseveen et al., 2009). MGCs can also be found in Giant-cell arteritis, an inflammatory disease of blood vessels. While the exact cause of this pathology is unknown, the disease process initiates when dendritic cells in the vessel wall recruit T cells and macrophages to fuse and form granulomatous infiltrates (Salvarani et al., 2012). Similarly, T cell-macrophage fusion results in MGC formation allowing human immunodeficiency virus (HIV) spreading (Bracq et al., 2017). MGCs can also form in response to large foreign materials, such as implanted biomedical devices and are then designated as FBGCs (Anderson, 2000). A less well characterised type of MGCs is the Touton giant cell, which is frequently found in lesions containing cholesterol and lipid deposits that are formed by fusion of macrophage-derived foam cells (Dayan et al., 1989).
FBGCs are thought to have a role in the uptake of large particles when large and/or poorly degradable material is present (Anderson et al., 2008). Their presence is associated with fibrotic encapsulation of engrafted biomaterials through VEGF release and neovascularisation (Dondossola et al., 2016). However, the general function of MGCs in disease is obscure, and it remains unclear whether their presence is beneficial or detrimental to the disease outcome. *In vitro*, interleukin-4 (IL-4)-induced MGCs have been reported to phagocytose large and complement-opsonized materials more effectively than their unfused macrophage precursors (Milde et al., 2015). In addition, following infection with mycobacterium, macrophages release anti-bacterial nitric oxide (NO), which drives the transformation into MGCs but, paradoxically, rendering the latter permissive for mycobacterial persistence (Gharun et al., 2017). Thus, it cannot be excluded that fused macrophages exhibit different properties, depending on specific stimuli and disease contexts.

It is noteworthy that in their morphology and origin, MGCs resemble another product of fusion between monocytes and/or macrophages: osteoclasts, which are discussed in the next section.

**The monocyte/macrophage origin of osteoclasts**

The bone contains another well-defined and characterised multinucleated cell, the osteoclast, which is derived from monocytes and is responsible for the resorption of mineralised bone (Box 1) (Parfitt, 2002). Mononuclear osteoclast progenitor cells use vascular routes to migrate to specific locations in the skeleton, where they fuse with each other to become mature osteoclasts (Baron and Kneissel, 2013, Teitelbaum, 2000).

Since their identification in 1873 (Kolliker, 1873), the origin of osteoclasts has been controversial; however, in the 1970s, their haematopoietic origin was confirmed by Walker's pioneering experiments, as transplantation of bone marrow and spleen cells from wild-type mice restored bone resorption in osteoclast-deficient osteopetrotic mice, demonstrating that hematopoietic tissues produce migratory cells (osteoclast progenitors) that are essential for the resorption of hard tissue (Walker, 1975a, Walker, 1975b). In 1986, Scheven and colleagues showed for the first time that osteoclasts could be generated from a subset of cells that is highly enriched in haematopoietic precursors (Scheven et al., 1986). Despite many attempts aimed to identify a specific precursor population of osteoclasts, studies hitherto failed to do so, possibly because of the lack of appropriate cell surface markers that are able to distinguish between precursors and osteoclasts.
The monocyte/macrophage origin of osteoclasts was subsequently confirmed in 1990 by Udagawa and colleagues when they showed that within the bone marrow, haematopoietic stem cells give rise to the monocyte-macrophage lineage upon stimulation by macrophage colony-stimulating factor (M-CSF) (Udagawa et al., 1990). Osteoclasts can be generated from immature cells from the monocyte-macrophage lineage, and also from mature tissue macrophages (Udagawa et al., 1990). Subsequently, different bone marrow cell populations were fractionated with the use of CD31 (a glycoprotein found on the surface of platelets, monocytes, neutrophils, and some types of T-cells) and Ly-6C (a class of antigens differentially expressed on monocytes/macrophages and endothelial cells) as markers (Nikolic et al., 2003, de Bruijn et al., 1994). In addition, a common myeloid progenitor that gives rise to monocytes, macrophages and dendritic cells (DCs), termed myeloid blasts (CD31+/Ly-6C+), has been proposed (de Vries et al., 2009). This progenitor was found to be the myeloid cell most capable to rapidly differentiate into functional osteoclasts following stimulation with receptor activator of nuclear factor kappa-B ligand (RANKL) (de Vries et al., 2009), although other progenitors have also been shown to be able to differentiate into osteoclasts (de Bruijn et al., 1994) (Fig. 1). Moreover, differentiated macrophages stimulated with M-CSF and RANKL have the potential to fuse and form osteoclasts (Udagawa et al., 1990). Immature dendritic cells can differentiate into conventional DCs, but can also form osteoclasts under the influence of M-CSF and RANKL (Speziani et al., 2007, Miyamoto et al., 2001).

In human peripheral blood, three monocyte subpopulations have been identified and classified based on their surface expression of CD14 and CD16: the classical, the intermediate and the non-classical monocytes (Ziegler-Heitbrock et al., 2010). It has been proposed that classical monocytes are the main source of osteoclasts (80–90%), whereas intermediate monocytes generate osteoclasts with high resorption potential in inflammatory conditions, and non-classical monocytes become non-resorbing osteoclasts that may be FBGCs (Sprangers et al., 2016). This suggests that monocyte subpopulations determine the functional properties of osteoclasts and further studies are required to further elucidate the link between monocyte/macrophage ontogeny and osteoclast function.

Taken together, it is now well established that MGCs and osteoclasts are multinucleated macrophages derived from common progenitors that exert specialised functions in their respective tissue micro-environments. Thus, despite their distinct location and function, MGCs and osteoclasts are closely related, as evident from the molecular pathways involved in macrophage fusion, which suggest a core fusion program existing in both cell types as discussed below.
Common mediators involved in MGC and osteoclast fusion and multinucleation

Cell fusion resulting in multinucleation in MGCs and osteoclasts is a complex and multi-step process that is spatiotemporally regulated by common genetic determinants. The fusion events that occur during multinucleation can be categorised into four main cellular phases: (1) pre-fusion programming of the cells, (2) chemotaxis, cellular adhesion and cytoskeletal rearrangements, (3) membrane fusion and multinucleation, and (4) post-fusion macrophage reprogramming. Below, we describe the four cellular phases focusing on the common determinants of fusion in macrophages and osteoclasts. We also briefly mention the major downstream pathways of the fusion mediators that are distinct between an osteoclast and a MGC.

Pre-fusion programming of macrophages

To become multinucleated, macrophages have to become fusion-competent. This fusion competence is acquired through the enhanced transcription of essential fusogens, which is governed by the action of both exogenous stimuli and endogenous signalling pathways. Signals that contribute to the pre-fusion programming of macrophages have been studied in osteoclast and MGC precursors. Based on recent evidence, a complex picture of pre-fusion mechanisms is emerging with both similarities and differences between osteoclasts and MGCs. The common pre-fusion determinants in osteoclasts and MGCs involve soluble factors that are crucial for macrophage differentiation (e.g. M-CSF) (Box 2) and membrane receptors with their ligands (Fig, 2).

Before we describe common fusion mediators, two essential cell-type-specific fusion mediators that initiate cell fusion are worth mentioning. RANKL is the key cytokine that differentiates osteoclast precursors into mature multinucleated osteoclasts and its role has been widely described and reviewed elsewhere (Feng and Teitelbaum, 2013). Similarly, the cytokines IL-4 and IL-13 specifically induce macrophage fusion to form MGCs in vitro (DeFife et al., 1997, McInnes and Rennick, 1988) and in vivo (Chensue et al., 1992, Kao et al., 1995).

As for the common mediators of macrophage fusion, the signalling adaptor DNAX-activating protein 12 (DAP12) is the co-stimulatory immunoreceptor tyrosine-based activation motif (ITAM)-bearing factor that is essential for macrophage fusion. It was shown that Dap12<sup>−/−</sup> mice exhibited a slight increase in bone mass (Zou and Teitelbaum, 2015) and defective
formation of MGCs (Helming et al., 2008), which was associated with a significant reduction in macrophage fusion (Helming et al., 2008). Furthermore, the triggering receptor expressed on myeloid cells 2 (TREM2) was found to be essential for macrophage multinucleation, which is mediated through its interaction with the co-stimulatory DAP12 (Helming et al., 2008). It has been shown that TREM2 is essential in macrophage fusion (Helming et al., 2008), activation (Turnbull et al., 2006), and osteoclast formation (Cella et al., 2003, Humphrey et al., 2004, Otero et al., 2012). Moreover, TREM2 was recently identified as a trans-acting genetic regulator of a macrophage multinucleation gene co-expression network in the rat (Kang et al., 2014). With regard to signalling mechanism, binding of TREM2 to DAP12 leads to the recruitment and activation of spleen tyrosine kinase (Syk), which, in turn, results in activation of signalling phosphoinositide 3-kinases (PI3K) in both osteoclasts (Peng et al., 2010) and MGCs (Fig. 2) (Helming et al., 2008). However, beyond these common mechanisms, osteoclasts and MGCs use distinct downstream pathways to induce fusion-competency (Fig. 2). In osteoclasts, RANKL signalling and ITAM cooperate to induce expression of nuclear factor of activated T cells c1 (NFATc1), the master transcription factor for osteoclastogenesis (Negishi-Koga and Takayanagi, 2009), whereas in MGCs, IL4 and ITAM induce expression of the transcription factor STAT6 to acquire a fusion-competent status (Moreno et al., 2007).

During an inflammatory reaction, signals delivered by extracellular ATP and adenosine are detected and transduced by purinergic P2 and P1 receptors, respectively, making purinergic signalling a general hallmark of immunity and inflammation (see (Di Virgilio and Vuerich, 2015) for review). Among the P2 receptors, a common mediator of both osteoclast and MGCs pre-fusion is the purinergic receptor P2X7 (P2RX7). ATP released into the extracellular space of inflamed site activates P2RX7, acting like a danger signal (Di Virgilio and Vuerich, 2015). Both macrophages and osteoclasts express P2RX7, which is involved in inflammasome activation and cytokine release (Di Virgilio, 2007, Ferrari et al., 1997). P2RX7 signalling also has a role in multinucleation as macrophages expressing high levels of P2RX7 were unusually prone to form MGCs (Chiozzi et al., 1997, Falzoni et al., 1995). Indeed, the transfection of cells with P2RX7 caused spontaneous fusion (Chiozzi et al., 1997), while an anti-P2RX7 antibody inhibited multinucleation in MGCs (Lemaire et al., 2006). In osteoclasts, extracellular ATP is not a fusogen per se, but its degradation product adenosine strongly potentiates osteoclast fusion through the A1/A2A receptors (Pellegatti et al., 2011), leading to the hypothesis that P2RX7 is not a fusogen that promotes cell-to cell interaction, but instead performs a regulatory role in the ATP-adenosine axis to prime cells for fusion, possibly by fine tuning cell-fusion signalling pathways. Although a role of adenosine was proposed in giant cell formation in rheumatoid nodules (Merrill et al., 1997),
further studies are required to understand the role of adenosine in fusion. Little is known about the downstream effectors of P2RX7 in either osteoclasts or MGCs. Nevertheless, it has been shown that NFATc1 is triggered by P2RX7 activation in microglial cells (Kataoka et al., 2009), suggesting that it could act downstream of P2RX7 signalling in osteoclasts.

Tumour necrosis factor (TNF) is involved in pathological bone resorption (osteolysis) associated with rheumatoid arthritis (RA), periodontitis, as well as in granulomatous diseases, such as TB and Crohn’s disease (CD) (Zhao et al., 2012, Van Deventer, 1997, Lukacs et al., 1994). TNF has been implicated in multinucleation in both osteoclasts and MGCs (Lampiasi et al., 2016). Although the disease-driving role of TNF is established in osteolysis, its implication in granulomatous disease outcome remains to be fully elucidated. TNF can act directly on osteoclast precursors, in synergy with RANKL, to promote NFATc1-dependent osteoclast and macrophage fusion and multinucleation through activation of c-Jun N-terminal kinase (JNK) (Lam et al., 2000, Yarilina et al., 2011). Accordingly, NFATc1 expression was found to be elevated in synovial macrophages from patients with TNF-driven inflammatory arthritis (Yarilina et al., 2011), reinforcing its pathological role in osteoclast activity. Furthermore, granuloma macrophages have been shown to fuse to form MGCs, suggesting that fusion and multinucleation are a consequence of bacterial infection (McCLean and Tobin, 2016). TNF is essential for the formation and maintenance of granulomas in vitro and in vivo (Roach et al., 2002, Algood et al., 2004, Chensue et al., 1994, Flynn et al., 1995, Kindler et al., 1989) and anti-TNF agents interfere with granuloma integrity in bovine tuberculosis (Kindler et al., 1989) and sarcoidosis (Broos et al., 2013). These studies suggest that, in general, TNF has a beneficial effect in inducing granuloma formation, and, accordingly, TNF-deficient mice show an increased susceptibility to TB and contain many bacteria in poorly formed granulomas (Flynn et al., 1995). The exact mechanisms by which TNF regulates granuloma formation remain to be determined.

Modulation of Ca\textsuperscript{2+} is a crucial regulator of RANKL signalling and therefore multinucleation, and several Ca\textsuperscript{2+} regulators have been found to mediate osteoclast fusion (Yeon et al., 2015, Kim et al., 2017); however, their role in multinucleation of MGCs has been less investigated. The potassium calcium-activated channel subfamily N member 4 (KCNN4, also known as KCa3.1) is thus far the only Ca\textsuperscript{2+} regulator that has been identified in both osteoclasts and MGCs (Grossinger et al., 2018, Kang et al., 2014). Through modulation of Ca\textsuperscript{2+} signalling and its oscillations, KCNN4 can regulate the translocation of NFATc1 and subsequent Akt activation, in turn controlling osteoclast and macrophage multinucleation (Kang et al., 2014, Grossinger et al., 2018). Beyond its effect on multinucleation of MGCs and osteoclasts, pharmacological blockage of KCNN4 was associated with a reduction of
glomerular MGCs following the induction of glomerulonephritis. In keeping with this, $Kcn4^{-/-}$ mice showed significantly reduced bone erosion following collagen-antibody induced arthritis (Kang et al., 2014).

While M-CSF, DAP12/TREM2, P2RX7, TNF and KCNN4 are common pre-fusion mediators in the osteoclast and MGC precursor cell, their downstream signalling mechanisms that induce fusion-competency differ between osteoclasts and MGCs, giving rise to two distinct cell types (Fig. 2). Indeed, in osteoclasts, most studies show that RANK-mediated signalling pathways and/or transcription factors converge towards NFATc1 as the ultimate master regulator of osteoclast multinucleation (Kim and Kim, 2014). In MGCs, the downstream mechanisms are less well-understood. The signal transducer and activator of transcription 6 (STAT6) has a pivotal role in transducing the signals from IL-4 and IL-13, which both induce a fusion-competent state in precursors of MGCs and FBGCs (McInnes and Rennick, 1988, Miyamoto et al., 2012a). While there it is not fully clear whether all factors involved in fusion of MGCs and/or FBGCs follow the same downstream signalling, there is some evidence pointing to convergence on a common STAT6-IL-4-induced MGC activation pathway (Fig. 2).

**Macrophage chemotaxis, adhesion and cytoskeletal rearrangements**

The migration and positioning of macrophages (chemotaxis) is an important element preceding cell fusion. The chemotactic properties of osteoclasts as part of their function in bone resorption were shown decades ago, as osteoclasts require the activity of the chemoattractant sphingosine 1 phosphate receptors (S1PRs) (Mundy et al., 1978). A common chemoattractant for osteoclasts and MGCs is the chemokine (C-C) ligand-2 (CCL2) and its receptor CCR2, and both were recently shown to be important for the formation of osteoclasts and FBGCs (Sul et al., 2012, Kyriakides et al., 2004). Indeed, fewer osteoclasts and FBGC were observed when isolated from the bone marrow of mice lacking either CCL2 or CCR2 (Khan et al., 2016). In addition, there was a significant reduction in the number of nuclei and the size of these cells as macrophages need to be brought close together to fuse.

For cellular fusion, cells must adhere to each other and bring their membranes into close contact. Integrins as well as cadherins, which are involved in homotypic cell–cell contacts, have been implicated as mediators of fusion in multiple cell types (Van den Bossche et al., 2009). The specific integrins required for adhesion of MGCs and osteoclasts differ. The macrophage-1 antigen, a β2 integrin, is involved in adhesion of MGC precursors to the fusogenic surface (McNally and Anderson, 2002), whereas integrin αvβ3 is important for
osteoclast fusion and the organisation of its cytoskeleton (McHugh et al., 2000). Consequently, deletion of this integrin impairs cytoskeletal organisation and the resorbing function of osteoclasts (Faccio et al., 2005, McHugh et al., 2000). In addition, E-cadherin also mediates cell–cell adhesion in osteoclasts and MGCs (Moreno et al., 2007, Fiorino and Harrison, 2016).

The cytoskeleton forms a scaffold that determines cellular shape, organelle organisation and movement. Therefore, during cell fusion, cytoskeletal rearrangements are not only important for the movement of macrophages towards each other, but also to reorganise the resulting fused multinucleated cell. This integrin-mediated re-arrangement of the cytoskeleton involves phosphorylation of Syk and recruitment of the co-stimulatory ITAM protein DAP12, in both MGCs and osteoclasts (Helming and Gordon, 2009, Zou et al., 2007). Guanine nucleotide exchange factors that activate the small Rho GTPase RAC1 are also involved in rearranging the cytoskeleton (Pajcini et al., 2008), as well as in promoting vesicle transport, polarising microtubules, in both osteoclasts and MGCs. (Faccio et al., 2005, Jay et al., 2007). Furthermore, podosomes are specialised macrophage adhesion structures; they are conical, actin-rich structures found on the outer surface of the cell membrane (McNally and Anderson, 2005). Podosomes exhibit two distinct features: an actin core and an adhesive ring complex, containing integrins and integrin-associated proteins, and both important for cellular motility and invasion (Pfaff and Jurdic, 2001). Podosomes are striking features of osteoclasts, and their arrangement is highly interconnected through a dense, network of filamentous actin (F-actin) (Luxenburg et al., 2007, DeFife et al., 1999a). Furthermore, in osteoclasts, the formation of actin rings by podosomes (called the “sealing zones”) maintains local acidification and accumulation of matrix-degrading enzymes between the cell and bone surface (Luxenburg et al., 2006). This sealing zone becomes the site of osteoclast attachment to the bone matrix and allows bone resorption. Remarkably, FBGCs are also characterised by actin rings and sealing zones (ten Harkel et al., 2015). It has been shown that F-actin is necessary for osteoclast and FBGC formation, as its disruption was shown to inhibit human macrophage fusion (DeFife et al., 1999b).

**Multinucleation as a result of membrane fusion**

To date, the genes described to have a direct role in macrophage membrane fusion are scarce. Even though several factors have been implicated, including the putative seven transmembrane receptor DC-STAMP (Yagi et al., 2005), CD44 (Cui et al., 2006, Sterling et al., 1998), CD47 and the tetraspanins CD9 and CD81 (Takeda et al., 2003), the underlying mechanisms are not clear (Fig. 3).
DC-STAMP is essential for cell–cell fusion in both osteoclasts and MGCs, and directly regulates fusion (Yagi et al., 2006). It is often used as a cell-fusion marker in fusing MGCs and osteoclasts (Eleveld-Trancikova et al., 2005). Moreover, a genetic variant of the DCSTAMP gene was found to simulate osteoclastogenesis in patients with Paget’s disease of bone (Laurier et al., 2017). Its downstream signalling in osteoclasts and FBGCs appears to be via the RANKL-NFATc1 and IL-4-STAT6-NF-κB axes, respectively (Yagi et al., 2005). Paradoxically, despite the established role of DC-STAMP in both osteoclast and MGC fusion, its ligand is still unknown, and the exact mechanism by which DC-STAMP promotes cellular fusion in osteoclasts and MGCs thus remains unclear. The identification of osteoclast stimulatory transmembrane protein (OC-STAMP), which modulates fusion in osteoclasts and FBGCs independently of DC-STAMP, suggests the existence of other essential fusogens in these cells (Miyamoto et al., 2012b, Khan et al., 2013).

Tetraspanins are a superfamily of membrane proteins, and among them, CD9 and CD81 are known to control cell–cell fusion as they negatively regulate fusion of osteoclasts and MGCs (Takeda et al., 2003, Parthasarathy et al., 2009). Indeed, CD9 and CD81 double-null mice spontaneously develop MGCs in the lung and show enhanced osteoclastogenesis in the bone (Takeda et al., 2003). CD44 is another cell-surface adhesion molecule known to mediate macrophage adhesion and fusion, and to mediate the formation of MGCs both in vitro and in vivo (Cui et al., 2006, Sterling et al., 1998). CD44 is also involved in osteoclast fusion, as CD44 deficiency significantly inhibited osteoclast activity and function through the downregulation of the NF-κB/NFATc1 pathway (Li et al., 2015).

Matrix metalloproteinases (MMPs) are endopeptidases required to process or degrade extracellular matrix components. Membrane type 1 MMP (MT1-MMP; also known as MMP14) is a membrane-anchored collagenase with important roles in pathophysiological settings, including the development of the skeleton (Holmbeck et al., 1999) (Gonzalo et al., 2010). MT1-MMP has been recently identified to be part of the macrophage cell fusion machinery through its interaction with the fusogen CD44 (Chellaiah and Ma, 2013). MT1-MMP is also required for Rac1 signalling (Gonzalo et al., 2010). In addition, a physical interaction between tetraspanins and MT1-MMP has been reported but it requires further characterisation in multinucleating macrophages (Lafleur et al., 2009, Gonzalo and Arroyo, 2010).

**Post-fusion macrophage reprogramming**

Once they have fused and thus are multinucleated, both osteoclasts and MGCs are terminally differentiated and exert tissue-specific functions (Fig. 3). It is worth noting that macrophages fuse not only with mononuclear cells, but also with other multinucleated cells
(Jansen et al., 2012), suggesting that fusion and multinucleation are not simply one-off events. There are also reports suggesting that osteoclasts can lose nuclei through fission, giving rise to a novel functional osteoclast (Jansen et al., 2012). In osteoclasts, the role of multinucleation is well established as it is tightly linked to bone resorption. A mononucleated osteoclast can resorb bone, but the osteoclast resorption activity has been shown to correlate with the number of nuclei per cell (Boissy et al., 2002). Bone resorption is essential to ensure optimal bone mass and bone mineral homeostasis as it is coupled to bone formation and increased or diminished bone resorption leads to pathological conditions, such as osteoporosis or osteopetrosis. While there is a cause-effect relationship linking osteoclast multinucleation to function in physiopathology, MGC multinucleation and activity is not well-documented in disease. Interestingly, FBGCs have the capacity to dissolve the mineral phase of the bone, without digesting its matrix fraction (ten Harkel et al., 2015). As FBGCs are often found under conditions where large and/or poorly degradable material is present, they might facilitate the uptake of large particles though phagocytosis (Anderson et al., 2008, Milde et al., 2015), but rigorous quantitative studies are needed to substantiate this hypothesis.

The reprogramming of cells after fusion has allowed for a number of widely used cellular markers of macrophage multinucleation. The terminal differentiation of multinucleated osteoclasts is characterised by the acquisition of mature phenotypic markers, such as cathepsin K (CTSK) and MMP-9 (Pagani et al., 2005). CTSK is a protease involved in bone and cartilage breakdown, owing to its ability to catabolise elastin, collagen, and gelatin (Chiu et al., 2004). MMP9 is required for osteoclast migration and has an important role in skeletal remodelling (Reponen et al., 1994). In addition, osteoclasts are positive for tartrate-resistant acid phosphatase (TRAP), which has been extensively used as a marker of differentiated osteoclasts (Burstone, 1959), as TRAP-staining intensity increases with multinucleation of osteoclasts. However, TRAP stains both mononucleated and multinucleated osteoclasts, and is also expressed in certain activated and pathological macrophages and dendritic cells (Hayman, 2008). In contrast to osteoclasts, MGC-specific markers are less-well defined. Under pathological conditions, MGCs express effector molecules that are also present in osteoclasts, such as CTSK and MMP9 (da Costa et al., 2005). CTSK and MMP9 transcripts have also been found to be part of a macrophage multinucleation network in the rat (Kang et al., 2014). In addition, MGCs have also been shown to express TRAP (Park et al., 2012), suggesting that the existence of some phenotypic markers of cell multinucleation that are shared by MGCs and osteoclasts.

*Osteoclasts and MGCs: a lack of side-by-side comparison*
Above, we have discussed the so far described common mediators of fusion between osteoclasts and MGCs, but there are other determinants of fusion that have been found in only one of these cell types. This could be because studies that use osteoclasts as a multinucleation model might not necessarily investigate the functional conservation in MGCs, or *vice versa*. As an example, the scavenger receptor CD36 is known to be required for fusion of MGCs (Helming et al., 2009), but its exact role in osteoclastogenesis has not been yet established. Conversely, several factors, such as for instance Atp6v0d2 or CD200, have been shown to be involved in osteoclastogenesis, but their role in MGCs has not been investigated (Lee et al., 2006, Cui et al., 2007). Therefore, there is a clear need to study osteoclasts and MGCs side-by-side in order to broaden our understanding of the shared fusion machinery in these cells.

Some hints might come from the study of human Mendelian diseases with genes involved in macrophage multinucleation. It was shown that deletions or loss-of-function mutations in either *DAP12* or *TREM2* genes, which are involved in both MGC and osteoclast fusion, are causally associated with Nasu-Hakola disease, a dementia associated with bone cystic lesions (Paloneva et al., 2000, Paloneva et al., 2002). Mutations in these two genes induced defective multinucleation in osteoclasts resulting in impaired bone resorption (Humphrey et al., 2004, Humphrey et al., 2006). Moreover, both MGCs and osteoclasts have been reported to play a role in the pathogenesis of osteoarthritis and rheumatoid arthritis (Weinberg et al., 1993, McInnes and Schett, 2011). Furthermore MGCs and osteoclasts have been shown to be an HIV-1 target, suggesting that macrophage multinucleation could contribute to the spreading of the virus (Bracq et al., 2017) and osteoclast-specific bone loss (Raynaud-Messina et al., 2018). Thus macrophage multinucleation could be considered as a basic phenomenon perturbed in bone, inflammatory and infectious disorders (Box 3).

**Conclusions and perspectives**

Osteoclasts and MGCs have been reported to be involved in a wide range of pathologies. The specific role of MGCs in disease outcome has not been studied in detail, whereas the contribution of osteoclast multinucleation in health and diseases, such as in osteopetrosis (Sobacchi et al., 2013) and Paget’s disease (Albagha, 2015), is already well characterised and documented (Helfrich et al., 2007). For each disease, it will be important to determine the precise triggers and signalling pathways involved in precursor fusion, as well as the phenotypes and functions of the resulting osteoclasts and MGCs. If one considers macrophage multinucleation as an intermediate phenotype for the bone, inflammatory and
infectious disorders discussed above, it is plausible to hypothesise that key genetic determinants of macrophage multinucleation, common in MGCs and osteoclasts, might orchestrate the fundamental signalling pathways that regulate bone resorption and are perturbed in bone and granulomatous disorders. Comprehensive molecular profiling of multinucleating macrophages could unravel such factors and pathways and thus provide the starting point to assess their role in both bone homeostasis and inflammatory disease (Box 3) (Kang et al., 2014).

However, there are many outstanding questions: given the relative low numbers of multinucleated cells in a given organism, what prevents cells in general from fusing and what differs in these multinucleated cells? Osteoclasts are known to resorb the bone but is the resorptive activity a direct reflection of cell fusion? What is the advantage of being multinucleated for resorbing the bone? What is the tissue and disease-specific function of an MGC?

The vast majority of cells are capable of cell division. However, only a selected group of cell types undergo the opposite process: fusion between cells. It will be therefore interesting to compare the fusion of macrophages to the fusion of other cells. Indeed, ‘fusion proteins’ structurally similar to viral proteins have been identified in sperm cells, myoblasts and trophoblasts (Vignery, 2000). To date, few mediators of fusion (RAC1 and tetraspanins) shared between macrophages and other fusing cells have been identified (Helming and Gordon, 2009, Chen et al., 2007). While most of the fusion molecules are fusogens per se, the tetraspanins are the only inhibitors of fusion that have been identified until now. Because their downregulation is known to induce membrane fusion (Aguilar et al., 2013), it would be of interest to investigate to what extent tetraspanins are expressed in non-fusing cells and whether they can prevent cells from fusing with their siblings. In addition, it is important to note that multinucleation can occur in the absence of membrane fusion. For instance, incomplete cytokinesis can contribute to multinucleation (Takegahara et al., 2016) and should be considered as an alternative mechanism to multinucleation of osteoclasts and MGCs.

The fact that osteoclasts are fusing cells suggests that multinucleation does confer some advantages in resorbing the bone. While it is accepted that there is a positive correlation between the number of nuclei per osteoclast and their resorptive activity, it has been shown that a mononucleated osteoclast can also resorb bone (Boissy et al., 2002). In contrast, MGCs are often very large in size but do not resorb bone. The specific species has also an effect on osteoclast multinucleation and/or size as mouse osteoclasts are often small and can be mononuclear at times (Boissy et al., 2002), whereas feline (Mundy, 1995) and
chicken (Collin-Osdoby and Osdoby, 2012) osteoclasts have been previously described to be rich in nuclei. In that sense, the question of a ‘mechanism of brake’ for multinucleation is a valid one. A recently identified mutation in SNX10 has caused uninhibited fusion of precursors \textit{in vitro} and resulted, paradoxically, in osteopetrosis, as the cells showed low resorption potential (Stattin et al., 2017). In Paget's disease, where there is excessive fusion, the opposite is seen with an excessive resorption by very large osteoclasts, suggesting that fusion \textit{per se} is not an indicator of functionality in osteoclasts (Anderson, 2001). The different triggers of osteoclasts fusion and multinucleation resulting in such an array of pathological conditions remain to be identified.

In conclusion, macrophage and osteoclast fusion and multinucleation share common signalling pathways and a core gene regulation programme that requires in depth analysis. Identifying the genetic determinants of this core fusion programme will help understanding the pathologies related to osteoclast and MGC dysfunction. The current technological advances in single cell RNA-sequencing (scRNA-seq) will help to unravel the shared fusion mediators in their tissue environment. When applied during early stages of macrophage multinucleation, scRNA-seq offers the advantage of classifying the precursor cells based on their pre-fusion transcriptome profiling. Beyond the transcriptome, the shared metabolic and epigenetic signatures between osteoclasts and MGCs will further deepen the characterisation of the core fusion programme and allow understanding of macrophage multinucleation in health and disease.

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Figure legends

Fig.1: Differentiation of osteoclasts from the monocyte/macrophage lineage.

Haematopoietic stem cells give rise to myeloid and lymphoid precursors. Myeloid precursors further differentiate into macrophage/dendritic cell precursors that differentiate into monocytes, macrophages and dendritic cells (DCs). Monocytes (Ly-6C−) stimulated by macrophage colony-stimulating factor (M-CSF) mature into macrophages, but addition of receptor activator of nuclear factor kappa-B ligand (RANKL) drives monocytes into osteoclast commitment. Early stage Ly-6C+ monocytes show a strong osteoclast commitment potential upon stimulation with M-CSF and RANKL, while retaining the ability to become Ly6C− monocytes. Fully differentiated macrophages stimulated with M-CSF and RANKL can fuse to form osteoclasts. Under pathological conditions (shown here in red), upon stimulation with macrophage colony-stimulating factor (M-CSF) or granulocyte macrophage colony stimulating factor (GM-CSF) and interleukins (IL-4,IL-13), macrophages can form multinucleated giant cells (MGCs) that resemble osteoclasts.

Fig. 2: Common pre-fusion mediators in osteoclasts and multinucleated giant cells.

Summarised here are the shared major signalling pathways between multinucleated giant cells (MGCs) and osteoclasts that have been described until now. Within the bone marrow, osteoclast or MGC progenitors differentiate into a precursor cell upon stimulation by macrophage colony-stimulating factor (M-CSF), and various factors are then required to induce fusion-competency. In both osteoclasts and MGCs, triggering receptor expressed on myeloid cells 2 (TREM-2) recognises its unknown ligand, associates with DNAX activating protein 12 (DAP12) and leads to signalling through spleen tyrosine kinase (SYK). In addition, Purinergic Receptor P2X7 (P2RX7) has a regulatory role and acts through the ATP-adenosine axis, while tumor necrosis factor (TNF) binds to the TNF receptor to activate c-Jun N-terminal kinase (JNK) downstream. Moreover, the potassium Calcium-activated channel subfamily N member 4 (KCNN4), contributes to the sustained Ca²⁺ signalling and downstream activation of protein kinase B (AKT). These pre-fusion mediators are common in osteoclasts and MGCs, but their signalling mechanisms vary. In osteoclasts, binding of RANKL to its receptor induces a fusion-competent status through activation of the transcription factor nuclear factor of activated T-Cells 1 (NFATc1) (shown in red). In macrophages, interleukins IL-4 and IL-13 bind to their common IL-4 receptor and induce a fusion-competent status through the activation of the transcription factor signal transducer and activator of transcription 6 (STAT6) (shown in green), giving rise to MGCs.

Fig. 3: Common molecular mechanisms and steps in cell fusion leading to osteoclasts and MGCs.

Cell fusion comprises several distinctive steps. First, programming of macrophages and osteoclasts results in a fusion-competent status (Top; for details see Fig. 2). Then chemotaxis of macrophages brings them towards each other, which is mediated by binding of C-C motif chemokine ligand 2 (CCL2) to its receptor. Thereafter, cell–cell adhesion is partly mediated by E-cadherin and integrins, and subsequent cytoskeletal rearrangements are regulated by RAC1 which is regulated by membrane type 1-matrix metalloprotease (MT1-MMP) and results in actin cytoskeleton organisation within podosomes. Finally,
membrane fusion, and thus the formation of osteoclasts and multinucleated giant cells (MGCs) by cell-cell fusion, is mediated by dendrocyte expressed seven transmembrane protein (DC-STAMP), the master regulator of fusion in both cell types. In addition, the downregulation of the tetraspanins CD9 and CD81 is necessary for membrane fusion. An interaction between tetraspanins and MT1-MMPs has been identified but requires further investigation. CD44 can also mediate membrane fusion facilitated by its interaction with MT1-MMP. Once they have become multinucleated, the resulting osteoclasts and MGCs produce tartrate-resistant acid phosphatase (TRAP), matrix metallopeptidase 9 (MMP9) and Cathepsin K (CTSK).

Boxes

Box 1: Osteoclast differentiation

The differentiation of osteoclast progenitors into osteoclasts is promoted by soluble factors such as receptor activator of nuclear factor kappa B (RANK) ligand (RANKL, also known as TNSF11), which stimulates osteoclastogenesis by binding to receptor for activation of RANK (Yasuda et al., 1998) in presence of macrophage colony-stimulating factor (M-CSF). Osteoclast differentiation and function is under a tight regulation of stromal cells of the bone, such as osteoblasts and osteocytes, with the latter being the terminally differentiated form of osteoblasts (Bonewald and Johnson, 2008). Osteocytes are capable of orchestrating biochemical signals to maintain bone homeostasis by locally secreting osteoclastogenic factors such as RANKL, which affects osteoclast precursors in the vicinity (Xiong et al., 2015). The coupling between osteoclasts and osteoblasts is called bone remodelling and requires a balance between the processes of bone resorption and formation, thus preventing accumulation of microdamage (Baron and Kneissel, 2013, Cohen, 2006).

Box 2: Macrophage Colony-Stimulating Factor, a crucial cytokine for macrophage differentiation

Both osteoclasts and macrophages undergo differentiation from haematopoietic stem cells, which is mediated by M-CSF, a cytokine that stimulates the proliferation, differentiation and survival of macrophage and osteoclast precursors (Tanaka et al., 1993, Wiktor-Jedrzejczak et al., 1990). M-CSF primarily promotes proliferation and survival of macrophages by binding to and activating its cognate receptor, c-Fms (Sherr, 1990). In c-Fms-deficient mice, the number of blood monocytes is dramatically reduced (Dai et al., 2002, Cecchini et al., 1994), emphasising the role of M-CSF in controlling monocyte development. The essential role of M-CSF in osteoclastogenesis was revealed by studies of rodents that expressed non-functional M-CSF (due to a point mutation in the Csf1 gene encoding M-CSF), which lacked
osteoclasts and developed a severe osteopetrotic phenotype (Wiktor-Jedrzejczak et al., 1990, Marks et al., 1992). Although M-CSF is essential for the proliferation of osteoclast and MGC precursors, additional stimuli are required in order to achieve cell multinucleation.

**Box 3: Systems genetics and epigenetic approaches for macrophage multinucleation**

Integration of gene expression with DNA sequence variation in macrophages (i.e., expression quantitative trait locus (eQTL) analysis) provided a first step towards the mapping of genetic determinants that affect both osteoclast and macrophage function, and allowed to investigate their role in bone and inflammatory disorders. Taking advantage of an inbred rat strain (Wistar Kyoto, WKY) that displays spontaneous MGC formation during macrophage differentiation, the genetic control of macrophage multinucleation transcriptome was previously investigated (Kang et al., 2014). Multinucleated macrophage mRNA levels have been used as a quantitative trait to carry out genome-wide linkage analysis using a panel of single nucleotide polymorphisms (SNPs) throughout the rat genome. This led to the identification of a co-expression network that regulates macrophage multinucleation (i.e. ‘Macrophage Multinucleation Network’ or MMnet), containing 143 genes that are regulated in trans by Trem2 (Kang et al., 2014). MMnet was identified using an eQTL approach in spontaneously fusing bone marrow-derived macrophages (without addition of RANKL) and was found to be enriched for osteoclast genes (Ctsk, Dcstamp, Mmp9, Cd9, P2rx7). Within this macrophage multinucleation network, KCNN4 has been identified as a regulator of both MGC and osteoclast multinucleation (Kang et al., 2014), suggesting that MMnet contains novel genes involved in macrophage multinucleation. In addition, epigenetic and metabolic mechanisms underlying macrophage/osteoclast multinucleation have been the focus of recent studies (Rotival et al., 2015, Nishikawa et al., 2015, Park-Min et al., 2014). To date, the epigenetic mechanisms of multinucleation were only investigated in osteoclasts, and the study of conservation of these in MGCs will help understanding the shared fusion machinery between the two cell types.

**References**


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Lymphoid/Myeloid Precursor

Granulocyte
TRAP negative

Myeloid Precursor

Erythroid
TRAP negative

Macrophage/Dendritic cell Precursor

Ly-6C- monocyte
CD11b+, F4/80+, cFMS+, RANK+

Ly-6C+ myeloid blast
CD31-, cFMS+, RANK+

M-CSF

MGC
M-CSF/GM-CSF
IL-4
IL-13

Common multinucleation/fusion mediators

Osteoclast
M-CSF RANK-L

Dendritic cell

?
Osteoclast/ MGC Progenitor

M-CSF →

Osteoclast/ MGC Precursor

NFATc1 /RANKL-induced osteoclast activation

NFATc1 /RANKL

RANKL

RANK

TNF receptor

KCNN4

ATP

Adenosine

P2RX7

SYK

JNK

Ca²⁺

AKT

IL-4/IL-13

IL-4/IL-13 receptor

TREM 2 ligand

TREM 2

DAP12

STAT6

STAT6 /IL-4-induced MGC activation

IL-4/IL-13

Fusion-competent osteoclast/ MGC

IL-4/IL-13