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# Inhibition of RhoA and mTORC2/Rictor by Fingolimod (FTY720) induces p21-activated kinase 1, PAK-1 and amplifies podosomes in mouse peritoneal macrophages

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ABSTRACT

Macrophage functions in the immune response depend on their ability to infiltrate tissues and organs. The penetration between and within the tissues requires degradation of extracellular matrix (ECM), a function performed by the specialized, endopeptidase- and actin filament- rich organelles located at the ventral surface of macrophage, called the podosomes. Podosome formation requires local inhibition of small GTPase RhoA activity, and depends on Rac 1/Rho guanine nucleotide exchange factor 7, β-PIX and its binding partner the p21-activated kinase (PAK-1). The activity of RhoA and Rac 1 is in turn regulated by mTOR/mTORC2 pathway. Here we showed that a fungus metabolite Fingolimod (FTY720, Gilenya), which is clinically approved for the treatment of multiple sclerosis, down-regulates Rictor, which is a signature molecule of mTORC2 and dictates its substrate (actin cytoskeleton) specificity, down-regulates RhoA, up-regulates PAK-1, and causes amplification of podosomes in mouse peritoneal macrophages.

## 1. Introduction

Macrophages play protective and destructive roles in innate and adaptive immunity, inflammation, chronic rejection of transplanted organs, and cancer development (Bonnardel and Guilliams, 2017; Liu et al., 2016a; Kloc and Ghobrial, 2014; Kloc et al., 2014). In order to perform their functions macrophages have to be mobile and be able to infiltrate various organs, navigate and penetrate between and within the tissues, and digest (degrade) extracellular matrix (ECM). To do so, macrophages (as well as other motile and invasive cells, such as dendritic cells, microglia, osteoclasts, myocytes and cancer cells) are equipped with the specialized extracellular matrix degrading organelles called the podosomes (or the invadosomes in the invasive cells) located on the ventral surface of the cell. To degrade ECM the podosomes use the endopeptidase enzymes – the matrix metalloproteinases (MMPs). Interestingly, in human macrophages the podosomal membrane type-1 matrix metalloproteinase (MT1-MMP) has an additional and unexpected function far beyond its proteolytic activity. It imprints spatial memory of podosome location, which enables for faster reassembly of podosomes at the specific, predetermined sites within the cell (El Azzouzi et al., 2016; Gucciardo et al., 2016). Matrix degradation depends also on the activity of p21-activated kinase 1 (PAK-1) (Pignatelli et al., 2012). Podosome consists of plasma membrane protrusion with the actin filament column/core surrounded by vinculin and other focal adhesion proteins (Linder and Wiesner, 2015; Veillat et al., 2015). It has been shown that, at least in endothelial cells, the podosome formation also involves G-protein coupled receptor (GPCR) kinase-interacting protein 1(GIT-1); (Wang et al., 2009). Podosomes are extremely dynamic and constantly assemble and disassemble. Recent studies indicate that podosome formation involves local inhibition of small GTPase RhoA activity, which induces actin filament assembly/disassembly, and activation of GTPase Rac-1 (Iwatake et al., 2017; Weaver et al., 2003; van Helden et al., 2008). Studies on dendritic cells showed that activation of RhoA and deactivation of Rac-1 leads to podosome disassembly (van Helden et al., 2008). The formation of podosomes also depends on PAK-1, which is the effector of the Rac-1 and Cdc42, and plays a role in the scaffolding/reorganization of actin cytoskeleton (Webb et al., 2005; Ke et al., 2013) and on the Rho guanine nucleotide exchange factor 7 β-PIX, which activates Rac-1 (ten Klooster et al., 2006; Md Hashim et al., 2013). It has been shown that PAK-1 and β-PIX

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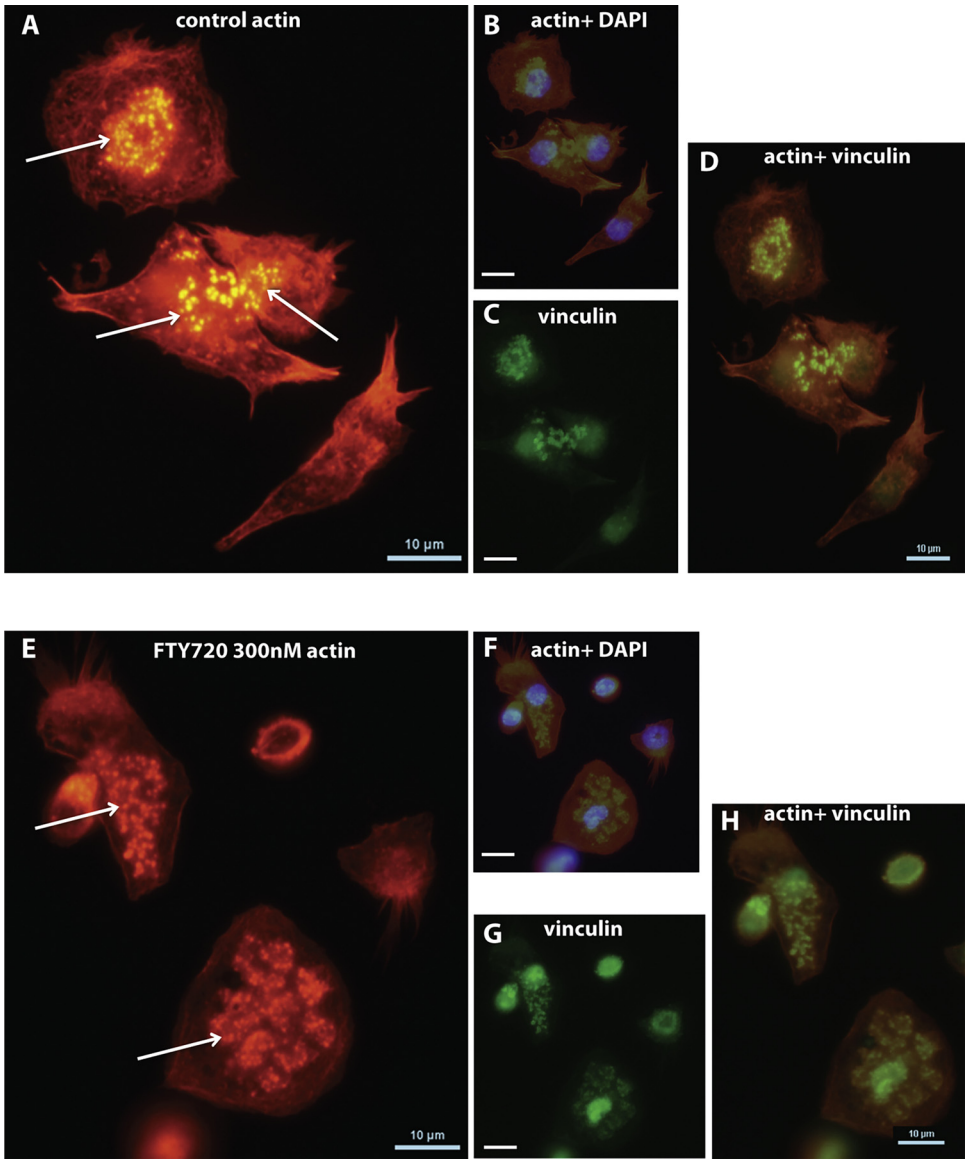


**Fig. 1.** FTY720 amplifies podosomes in mouse peritoneal macrophages.
Control or incubated with 300 nM FTY macrophages were fixed and stained with Rhodamine-phalloidin to visualize actin (red) and immunostained with anti-vniculin antibody and FITC-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). A) Control macrophages stained for actin show actin-rich podosmes (arrows). B) Merged image of actin and DAPI stained macrophages. C) Podosmes contain vinculin. D) Merged image of actin and vinculin staining showing colocalization of actin and vinculin in the podosomes. E) FTY720 treatment causes amplification of podosomes (arrows), which occupy nearly entire surface of the macrophage. F) Merged image of FTY720 treated macrophages; actin and DAPI staining. G) Amplified podosomes contain vinculin. H) Merged image of actin and vinculin staining showing colocalization of actin and vinculin in the amplified podosomes. Bar is equal to 10 μm. (For interpretation of the colour in this figure legend the reader is referred to the web version of this article).

form a signaling module that reciprocally activates Rac-1 (Mayhew et al., 2007). In addition, it has been shown that, at least in human hepatoma cells, the PAK1 is regulated by mTOR pathway (Ishida et al., 2007). We showed previously that macrophage specific deletion of RhoA, inhibition of ROCK kinase, which is a downstream effector of RhoA, by Y27632 inhibitor or inhibition of the guanine exchange factors (GEFs), which are upstream regulators of RhoA, cause reorganization of macrophage actin cytoskeleton and disrupt actin-dependent organelles such as Golgi complex and receptor recycling pathway. All these changes prevent macrophage movement and abrogate chronic rejection of cardiac allografts in rodent models (Chen et al., 2017; Liu et al., 2017, 2016b; Liu et al., 2016c; Zhang et al., 2012). It has been shown that RhoA pathway is reciprocally regulated by Rac-1 and mTOR pathway (Gordon et al., 2014; Gulhati et al., 2011; Jeruschke et al., 2013; Liu et al., 2010; Zhang et al., 2013). The mTOR serine-threonine kinase is a catalytic subunit of two distinct multiprotein complexes with different functions and different substrate specificity, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Guertin and Sabatini, 2009). The mTORC1, which regulates cell metabolism, growth and proliferation, has five components: mTOR, regulatory-associated protein of mTOR (Raptor); mammalian lethal with Sec13 protein 8 (GβL, also known as mLST8); proline-rich AKT substrate 40 kDa (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor) (Guertin and Sabatini, 2009). The mTORC2, which, via RhoA and Rac-1, regulates actin cytoskeleton, has six different proteins, mTOR; rapamycin-insensitive companion of mTOR (Rictor), which dictates substrate (actin cytoskeleton) specificity of mTOR; mammalian stress-activated protein kinase interacting protein (mSIN1); protein observed with Rictor-1 (Protor-1); GβL; and Deptor (Guertin and Sabatini, 2009). The fungus metabolite Fingolimod (FTY720, trade name Gilenya, Novartis) is clinically approved and used for the treatment of multiple sclerosis. It has been shown that FTY720 is a substrate for the sphingosine kinase and its phosphorylated form binds to sphingosine 1-phosphate (S1P) receptors, and that FTY720 and phospho-FTY720 activate the Smad signaling pathway in mesangial cells, and upregulate the expression of collagen (Xin et al., 2006). FTY720 has also immunomodulatory activity. It is phosphorylated *in vivo* by sphingosine kinase 2 (SphK2) to FTY720-phosphate (FTY720-P), which is an agonist of the S1P receptors (S1P1) preventing immune cells trafficking between lymphoid organs and blood. It also affects endothelial cells and endothelial barrier function by enhancing formation of adherens junctions (Brinkmann et al., 2004). Recently, (Yang et al. (2015)) showed that in liver injury mouse model, the S1P/S1PR2/3 system mediates motility of bone marrow derived macrophages