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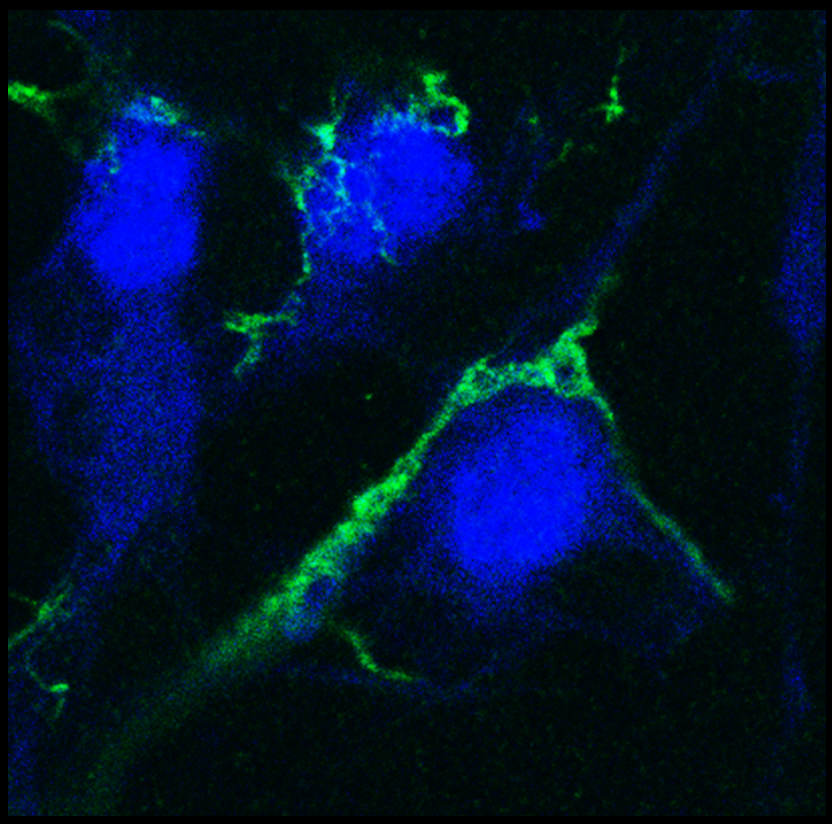

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A tutti quelli che hanno creduto in me

Contents

Chapter 1

1. NICOTINAMIDE PHOSPHORIBOSYLTRANSFERASE (NAMPT): A PLEIOTROPIC ENZYME

Nicotinamide phosphoribosyltransferase (NAMPT) is a pleiotropic protein that exists in two distinct forms: (i) an intracellular form (iNAMPT) and (ii) an extracellular form (eNAMPT) [1].

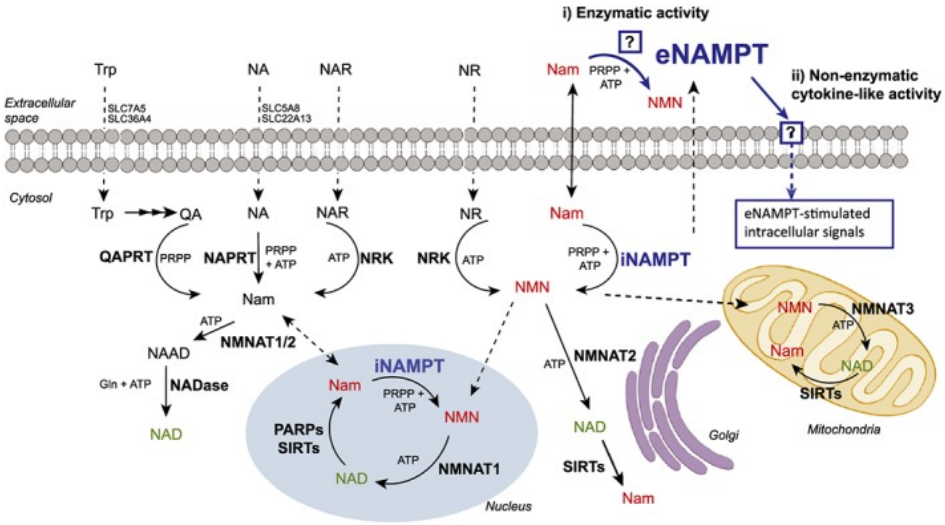


Figure 1. The role of sub-cellularly localized NAMPT in NAD synthesis. QAPRT, quinolinic acid phosphoribosyltransferase; NAPRT, nicotinic acid phosphoribosyltransferase; NRK, nicotinamide riboside kinase; NMNAT, NMN adenylyltransferase. NAD metabolites or substrates: Trp, tryptophan; NA, nicotinic acid; NAR, nicotinic acid riboside; NR, nicotinamide riboside; Nam, nicotinamide; QA, quinolinic acid[2].

1.1 iNAMPT: role in NAD metabolism

NAD is one of the most important cofactors in cellular metabolism and its main role is in redox reactions, transferring electrons from a reaction to another. The NAD synthesis starts from different precursors: L-triptophan (Trp), nicotinamide (NAM, vitamin B3), nicotinic acid (NA, vitamin B3), nicotinamide riboside (NR) and the recently described nicotinic acid riboside (NAR) (Figure 1). While lower eukaryotes and prokaryotes use the nicotinic acid pathway as a major source of NAD synthesis, in humans the main source

of cellular NAD is the uptake of NA, NAM, and NR from the diet or the reuse of NAM released intracellularly after consumption by NAD-utilizing enzymes, in the so-called NAD salvage pathway.

The intracellular form of NAMPT is a homodimeric class type II phosphoribosyltransferase, which represents the rate-limiting enzyme in this pathway. In detail, iNAMPT catalyzes the production of nicotinamide mononucleotide (NMN) through the condensation of nicotinamide (NAM) with 5-phophoribosyl-1-pirophosphate (PRPP) and ATP; afterwards, NMN is converted to NAD by nicotinamide mononucleotide adenylyltransferase (NMNAT).

Since NAD covers an important role in cells and iNAMPT regulates the production of NAD, it is clear that iNAMPT is a key enzyme in cellular energetics and cellular survival. Moreover, iNAMPT, affecting the intracellular levels of NAD, indirectly modulates the action of many NAD-dependent enzymes, such as Poly (ADP-ribose) polymerases (PARPs) and sirtuins (SIRTs), which explicate some of their functions in DNA damage and in the regulation of gene transcription[3, 4]. It is therefore not surprising that iNAMPT has been shown to be up-regulated in several disorders and has been considered a druggable target.

Structurally, NAMPT is a protein consisting of 491 amino acids with a molecular weight of 55 kDa. From the crystallographic structure, iNAMPT results in a dimeric form, and the interface of the two monomers serves as a pocket that accommodates NMN[5]. Different selective inhibitors have been designed, such as FK866 and CHS828, which compete with NMN for the catalytic site. These inhibitors entered into phase I/II clinical trials for non-solid and solid tumours; unfortunately, up to date there is no information about their efficacy *in vivo*. Recently, preclinical characterization of second-generation inhibitors have unmasked retinopathy and cardio-toxicity as potential side effects of these class of compounds[6].

1.2 eNAMPT: role as cytokine

NAMPT is not only an enzyme involved in metabolism, but it has been shown that this protein may be secreted through a non-classical pathway. For simplicity, I will refer to this form as extracellular NAMPT (eNAMPT) throughout my thesis.

Samal *et al*. described for the first time eNAMPT as an active cytokine in the extracellular space in 1994[7]. They reported its secretion from pre-B cells and its ability to synergize with stem cell factor and IL-7 to promote colony formation[7]. At the time, given that the identity with iNAMPT had not been recognized yet, eNAMPT was referred to as **pre-B-cell colony-enhancing factor 1 (PBEF1).**

This paved the way to its classification as a cytokine. Since then, a number of reports have strengthened its biological potential as a paracrine and autocrine factor. In 2005, once again its name was modified to "*visfatin*" because it was thought to be secreted preferentially by visceral adipose tissue in obese patients[8, 9]. Now, it is clear that eNAMPT is not only produced by pre-B cells and adipocytes but also readily detectable in conditioned media from cultures of most cell types (Table 1)[2]. Immune cells, such as macrophages and leucocytes, neurons and glia cells, cardiomyocytes, melanocytes and fibroblasts have been demonstrated to release eNAMPT in the extracellular space under basal conditions or under stimulation.

Few studies have focused the attention on the type of stimuli that might induce eNAMPT release from cells. First, it is clear that eNAMPT release occurs in the absence of cell death and therefore appears to be a true and specific phenomenon. Stress conditions, including ischaemia[10] and oxygen–glucose deprivation (OGD), strongly increase eNAMPT release in neurons and glial cells[11], and hypoxia has been shown to be a triggering stimulus in melanoma cells[12].

All these stress conditions appear to be highly relevant in cancer as nutrient deprivation, hypoxia and oxidative stress are all features of the tumour micro-environment.

	Cell type	Modulation of eNAMPT release	Reference
Adipocytes	• 3T3-L1 adipocytes	Increased by Ox-LDL, CTRP3, glucose, rosiglitazone, adipocyte differentiation	(Fukuhara *et al.*, 2005) (Tanaka *et al.*, 2007)
	• SGBS adipocytes		(Chen *et al.*, 2013) (Derdemezis *et al.*, 2011)
	• Adipocytes derived from healthy donors	Decreased by insulin, PI3K and AKT inhibitors, quercetin	(Li *et al.*, 2014a) (Haider *et al.*, 2006a, b)
	• HIB-1B adipocytes		(Haider *et al.*, 2006b) (Revollo *et al.*, 2007)
Immune cells	• LPS-activated monocytes	Increased by ATP, LPS	(Schilling and Hauschildt, 2012)
	• Macrophages in visceral adipose tissue		(Curat *et al.*, 2006)
	• Leucocytes		(Friebe *et al.*, 2011)
	• Peripheral blood lymphocytes		(Samal *et al.*, 1994)
Brain cells	• PC12 cells	Increased by $CoCl_2$, ischaemia, OGD	(Kang *et al.*, 2011)
	• Primary neurons		(Jing *et al.*, 2014)
	• Primary glial cells		(Zhao *et al.*, 2014)
Cancer cells	• Hepatoma cells (HepG2, Huh-7)	Increased by anti-CD38 and differentiation in CCL, oxidative stress (H_2O_2), hypoxia	(Samal *et al.*, 1994) (Garten *et al.*, 2010) (Soncini *et al.*, 2014)
	• Colorectal cancer cells (HCT-116, LS180)		(Ghaemmaghami *et al.*, 2013)
	• Breast cancer cells (MCF10A, MCF7, T47D, MDA-MB-231, BT549, MDA-MB-468)		(Audrito *et al.*, 2015)
	• Melanoma cells (B16, MeWo, HMCB, SkMel28, LB24)		(Lin *et al.*, 2015)
	• Neuroblastoma and glioma cells (SH-SY5Y, SK-N-Be, U87)		
	• Mesothelioma (MSTO)		
	• Prostate cancer cells (DU-145)		(Grolla *et al.*, 2015)
	• Cervical cancer cells (HeLa)		
	• Chronic lymphocytic leukemia lymphocytes		
Other cells	• Fibroblast (COS-7, PA317, CHO)	Increased by LPS, TNF-α, IL-1β, starvation and oxidative stress (H_2O_2), differentiation, glucose, C-peptide	(Samal *et al.*, 1994; Jia *et al.*, 2004)
	• Amniotic epithelial cells		(Ognjanovic *et al.*, 2005)
	• Inflamed HUVECs		(Romacho *et al.*, 2013)
	• Neonatal rat cardiomyocytes		(Pillai *et al.*, 2013)
	• Pancreatic beta cells		(Revollo *et al.*, 2007)
	• Isolated human islets		(Kover *et al.*, 2013)
	• Sebocytes		(Kovacs *et al.*, 2016)
	• Melanocytes		(Grolla *et al.*, 2015)

Table 1. Summary of cell types that release eNAMPT[2].

Only recently, it has been demonstrated that eNAMPT can be released by most if not all cancer cell lines. Interestingly, conditioned media from tumoural cells appears to contain substantially more eNAMPT when compared with non-tumoural cultures. However, this parallels with the over-expression of intracellular NAMPT reported in the majority of cancer cell lines and tumour tissues[13, 14].

Nutritional changes have also been shown to promote eNAMPT release. Indeed, other than the first evidence which linked increased eNAMPT levels to obesity-linked diabetes[8], secretion of eNAMPT promoted by glucose or high insulin has been reproduced by others[15, 16]. Last, eNAMPT, like many other cytokines, is also secreted in response to inflammatory stimuli, such as LPS and ATP in monocytes[17].

Overall, a wide range of stimuli has been used to modulate eNAMPT release and this may indicate that eNAMPT secretion is recruited to function under specific circumstances.

2. eNAMPT and disease: autocrine and paracrine effects

The secretion of eNAMPT by different cell types suggests that this cytokine has pleiotropic roles in physiology and pathology and it is not surprising that circulating eNAMPT levels are frequently increased/deregulated in patients affected by different metabolic and inflammatory disorders, including cancer.

In Figure 2 the main findings on the roles of eNAMPT in cells are summarized.

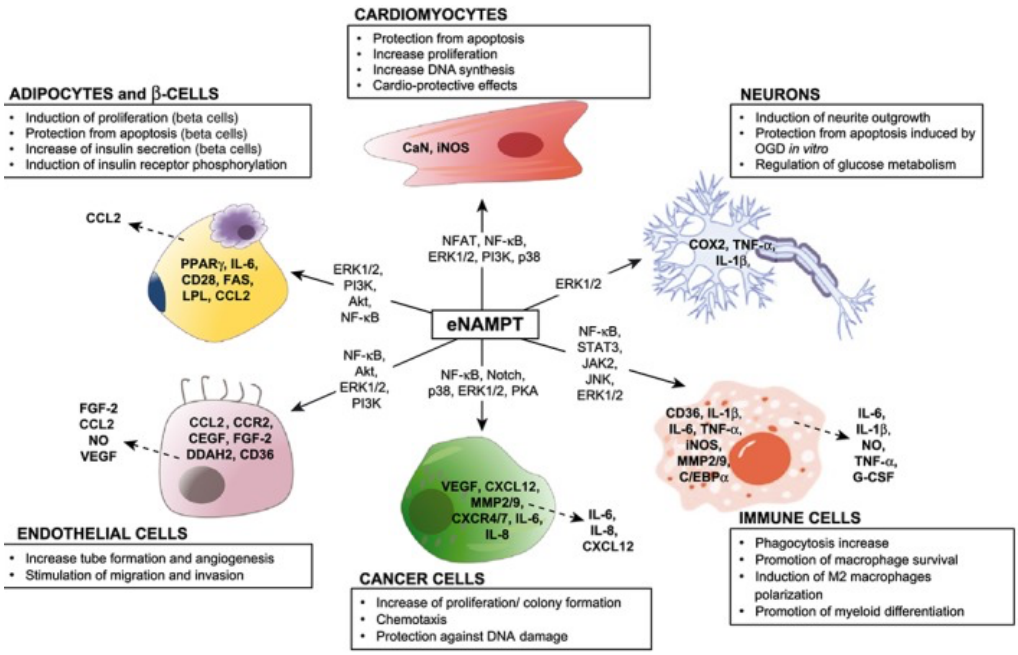


Figure 2. Autocrine and paracrine effects of eNAMPT[2].

2.1 eNAMPT: metabolic disorders

As described previously, Fukuhara *et al.* described for the first time that eNAMPT has an insulin-mimetic activity. Indeed, they demonstrated that eNAMPT exerted insulin-mimetic effects in cultured cells and lowered plasma glucose levels in mice. Furthermore, the administration of recombinant eNAMPT on adipocytes and β cells induces their proliferation and protects them from apoptosis[8, 18]. They suggested that these effects are mediated by the binding of eNAMPT to the insulin receptor, this article was retracted, since other groups were unable to demonstrate the binding of eNAMPT to the insulin receptor [19].

Since then, numerous reports have analysed the correlation between plasma eNAMPT levels and metabolic disorders. For example, Revollo *et al.* demonstrated that eNAMPT, partially thought NAD biosynthesis, plays a critical role in the regulation of glucose-stimulated insulin secretion (GSIS) in pancreatic β cells, but it does not possess insulin-like activity[19]. Indeed, they demonstrated that NAMPT through NAD biosynthesis regulates insulin secretion, but they failed to reproduce the insulin-mimetic effects using different recombinant eNAMPT obtained from different species (prokaryotic and eukaryotic).

On the contrary, Xie H *et al.*, reported in osteoblasts an insulin-like action of eNAMPT[20], indeed after eNAMPT treatment they observe an increased glucose uptake, proliferation and type I collagenase production. Similarly, Song HK *et al.* showed that eNAMPT treatment of kidney mesangial cells increases the uptake of glucose, GLUT-1 protein expression, and synthesis of pro-fibrotic molecules. In addition, they observed that eNAMPT is able to reduce the plasma glucose levels in mice[21].

Importantly, correlates to human diseases have also been found. Many research groups observed a possible correlation with circulating eNAMPT levels and the anthropometric and metabolic parameters in patients affected by **diabetes**

type II. Moreover, Berndt *et al.*, found a correlation between circulating eNAMPT levels and **obesity**, BMI (Body Mass Index) and body fat, but not with the size of the circumference of the waistline and with the WHR (Waist-hip Ratio)[22]. On the contrary, other researcher groups obtained opposite results regarding the correlation of plasma eNAMPT levels and metabolic disorders. These conflicting results might be explained by the variability in the commercial immunoassays available for the detection of eNAMPT. For example, Körner *et al.* identified an unknown protein (with high molecular weight of eNAMPT) that compromised the measurement of eNAMPT in one of the commercial immunoassay kits[24].

2.2 eNAMPT: inflammatory diseases

Many evidences have been provided for an involvement of eNAMPT in both the innate and adaptive immunity. First, eNAMPT is secreted upon inflammatory stimuli (listed in Table 1). For example, Jia *et al.* demonstrated that the amount of eNAMPT is increased in the presence of different inflammatory stimuli such as LPS, IL-1β and TNF-α in monocytes and neutrophils *in vitro*[25].

Not only, but the stimulation of innate and adaptive immune cells with eNAMPT is able *per se* to induce an inflammatory response, inducing a positive loop. Indeed, it has been reported that eNAMPT induces M2 polarization, increases phagocytosis and promotes macrophage survival and myeloid differentiation[26]. Moreover, eNAMPT treatment induces the activation of T cells and B cells. In this direction, different studies reported that eNAMPT treatment increased the mRNA levels and the release of different cytokine such as IL-1β, IL-1Rα, IL-10, IL-6, IL8 and TNF-alpha through the activation of p38, ERk1/2, AKT, JNK, NF-κB or the JAK/STAT3 pathways[2, 27-29]. Moreover, eNAMPT induces the expression of iNOS (inducible NO-synthase), which leads to the formation of peroxynitrite in macrophages[30].

It has been demonstrated also that the injection of eNAMPT in peritoneal mice stimulates the production of pro-inflammatory cytokines from mononuclear blood cells (such as IL-8 and IL-16) and increases mRNA expression and plasma levels of IL-6[31].

All these data support the evidence that eNAMPT plays a crucial role in inflammation. Likewise, eNAMPT circulating levels are up-regulated in different acute and chronic inflammatory conditions, such as lung injury, atherosclerosis, ulcerative colitis, rheumatoid arthritis, psoriasis and sepsis.

Rheumatoid arthritis. High levels of eNAMPT in plasma are associated with the severity of joint damage, prompting cartilage erosion[32]. Moreover, eNAMPT regulates the production of PGE2, MMP-3 and ADAMTS synthesis in chondrocytes, postulated as a degradation marker in patients[33, 34].

Acute respiratory distress syndrome (ARDS) and acute lung injury (ALI). It has been reported that eNAMPT is one of the proteins overexpressed in ALI and ARDS, determining increased levels of the cytokines such as IL-1β, TNF-α and IL-8. Moreover, in heterozygous $NAMPT^{+/-}$ mice, operating with a deletion of a single allele, there is a modest protection from lung injury[35].

Inflammatory bowel disease. Several studies have reported the correlation between high plasma levels of eNAMPT and ulcerative colitis and Chron's disease and there is a correlation with the stage of the disease. Due to the anatomic proximity of bowel and visceral fat, the bowel inflammation could activate adipocytes, which release several cytokines and adipokines, among which eNAMPT. Interestingly, after three months of treatment, eNAMPT levels were significantly reduced[36].

2.3 eNAMPT: cancer

The role of iNAMPT in the cancer pathology is well documented [13, 14, 37]. Indeed, iNAMPT has been observed overexpressed in cancer cells, and the treatment of tumoural cells with FK866 leads to cellular death *in vitro*[13]. Also in tumour-bearing mice, the treatment with FK866/CHS828 leads to reduction

in tumour volume and metastasis formation compared to controls. As described above, FK866/CHS828 entered phase I/II clinical trials for non-solid and solid tumour (*www.clinicaltrials.gov*).

To date, it is known that also eNAMPT has a pivotal role in cancer. Indeed, as observed for other cell types, eNAMPT has a cytokine effect on tumour cells and controls several cancer hallmarks.

Indeed, the treatment of eNAMPT on different tumour cells, such as PC3 (prostate cancer) cells, melanoma cells and MCF7 (breast cancer) cells, results in a proliferative effect[28, 29]. Indeed, it has been demonstrated that the eNAMPT treatment on cancer cells increases mRNA levels of VEGF, MMP-2/9, CXCL12 and CXCR4/7 thought the activation of MAPK, NF-κB and notch pathways[38-41]. All these effects are then translated in an increase in proliferation, migration and colony formation.

Furthermore, eNAMPT seems to promote the epithelial-mesenchymal transition (EMT), a process in which epithelial cells lose their adhesion properties and become mesenchymal stem cells with migratory and invasive properties. Regarding this aspect, Cheng *et al.* and Soncini *et al.* have shown that eNAMPT, in a NF-κB- dependent manner, promotes osteosarcoma and breast cancer cell migration through the reduction of E-cadherin expression and the increase of N-cadherin, vimentin and ZEB1[42, 43].

The tumoural microenvironment is a heterogeneous environment in which tumoural cells cooperate with immune cells, bone marrow-derived inflammatory cells, endothelial cells and fibroblasts. It is evident that eNAMPT may affect cell types other that tumoural cells *in vivo*.

In endothelial cells, eNAMPT treatment induces the activation of MAPK, PI3K, AKT and NF-κB intracellular pathways, which results in the increase of the release of Fibroblast Growth Factor 2 (FGF-2), Vascular-Endothelial Growth Factor (VEGF) and nitric oxide (NO)[44]. Kim *et al.* and Adya *et al.* reported that eNAMPT treatment of human umbilical vein endothelial cells (HUVECs) promotes angiogenesis through the activation of ERK1/2. Indeed,

in vivo and *in vitro*, it has been found that eNAMPT stimulates the formation of new vessels, facilitating the migration and formation of veins in HUVECs. This action is attributable to both the up-regulation of VEGF and of the matrix metalloproteases (MMP) and the suppression of metallopeptidase inhibitor 1-2 (TIMP 1-2). Moreover, the inhibition of PI3K/Akt and ERK1/2 leads to a drastic decrease in the effect of eNAMPT on the activation of the gene expression of VEGF and MMP, and consequently leads to a reduction of endothelium proliferation and capillary formation[45-48]. *In vivo*, Kim *et al.* observed that eNAMPT in matrigel plug assays and in chorioallantoic membrane assay in mice promotes angiogenesis[29, 46]. The pro-angiogenetic effects of eNAMPT observed on HUVECs are also reflected on cancer cells.

Moreover, Audrito *et al.* have shown that eNAMPT is able to polarize resting monocytes obtained from chronic lymphocytic leukaemia towards M2-macrophages with tumour-supporting properties[26]. Indeed, eNAMPT treatment increased CD163, CD206 expression and the release of IL-6 and IL-8 tumour-promoting cytokine and IL-10 immunosuppressive cytokine.

Therefore, eNAMPT may modulate cancer cell proliferation and migration but also angiogenesis and tumour-related inflammation.

In support of these observations, eNAMPT circulating levels are increased in cancer patients compared to healthy subjects[49-51]. Furthermore, a positive correlation between circulating levels of eNAMPT and cancer progression has been described[15-17] (Table 2).

condition	level of investigation	main finding
Breast cancer	Tissutal protein	Associated with poor disease-free and overall survival
Oostmenopausal breast cancer (PBC)	Serum level	Elevated (associated with risk of PBC)
Gastric cancer	Tissutal protein and mRNA	Increased
	Serum level	Increased (positive correlation with stage progression)
	Tissutal protein	Increased (correlation with VEGF-A expression, negatively correlated with survival)
Lymphomas	Tissutal protein	Increased (highly expressed in Hodgkin's lymphoma)
Prostate cancer	Tissutal protein	Increased
Ovarian cancer	Tissutal protein	Increased
Colorectal cancer	Blood level	Increased (correlated with stage progression)
Malignant astrocytomas: anaplastic astrocytoma (AA, grade III) and glioblastoma (GBM, grade IV)	Serum level tissutal protein and mRNA	Increased (correlated with tumor grade, coexpression with p53 in GBM tissue was associated with poor survival)
Esophageal cancer	Serum levels, tissutal mRNA	Increased (independent factor of mortality)
Melanoma	Tissutal protein and mRNA	Increased (independent of BRAF mutations and Clark's levels)

Table 2. eNAMPT as a cancer biomarker

Despite these encouraging results, there are, however, still numerous aspects to be revealed about the role and the biological importance of eNAMPT: (i) there is no information, in literature, about the physiological levels and role of eNAMPT, (ii) circulating eNAMPT levels, as described above, have been observed elevated in different pathologies and not only in cancer patients, and therefore there is no specificity associated to this phenomenon; (iii) different cells types, such as inflammatory cells, endothelial cells and adipocytes release eNAMPT and it is difficult to understand the source of circulating eNAMPT. In this regard, our group, recently, demonstrated that part of circulating eNAMPT derives directly from tumoural cells[12].

2.4 eNAMPT: neuro- and cardio- protection

While in cancer and autoimmune disorders eNAMPT seems to have predominantly a negative role, increasing the aggressiveness of the tumour and inducing inflammatory responses, in brain and heart the picture appears different, with a cardio-protective and neuro-protective described.

Evidences demonstrate that eNAMPT protects from apoptosis, induces proliferation and DNA synthesis via the activation of PI3K, p38, ERK 1/2 and NF-κB in cardiomyocytes[52, 53]. By doing this, eNAMPT reduces post-reperfusion myocardial injury. In neurons, eNAMPT treatment increases

mRNA levels of COX-2, TNF and IL-1β via ERK 1/2, resulting in neurite outgrowth, protection from apoptosis induced by OGD (oxygen-glucose deprivation) and regulates glucose metabolism[11].

In conclusion, there is ample evidence that eNAMPT triggers numerous intracellular signalling pathways and it can also participate in many biological processes. The discrepancies observed may suffer from poor systematic investigation and may be translated in different roles of this cytokine dependent on the type of context in which it exerts its actions, both physiological and pathological.

3 eNAMPT EFFECTS: ENZYMATIC ACTIVITY OR BINDING TO A RECEPTOR?

The manner by which eNAMPT triggers the above phenomena is still largely unknown. Given that eNAMPT has the potential to remain enzymatically active upon release, these effects can be attributed to (i) its extracellular enzymatic activity and/or to (ii) the binding and activation of a cell surface receptor (Figure 1). Currently, there are evidences both for and against these possibilities, which are not mutually exclusive.

3.1 Enzymatic activity of eNAMPT

Revollo *et al.* demonstrated through size exclusion chromatography that secreted eNAMPT can exist as a dimer in conditioned media of adipocytes[19]. Given that dimerization is required for the creation of the catalytic site of the enzyme, this represents the possibility that eNAMPT might be active in the extracellular space. Obviously, the availability of substrates is crucial for this hypothesis, and the presence of sufficient concentrations of phosphoribosyl pyrophosphate (PRPP), nicotinamide or ATP has been disputed[54].

In this regard, a recent work of Li *et al.* has reported that eNAMPT protects macrophages from apoptosis induced by ER-stress through its enzymatic

activity[55]. It is true that if eNAMPT acts as an active enzyme, its product, NMN, should be able to mimic the effects of eNAMPT exposure. While a minority of experiments have confirmed this, many others have failed to demonstrate an effect of NMN[19, 55]. Indeed, some reports have shown no effect of NAMPT inhibitors on its function, demonstrating that at least part of its effects are independent of enzymatic activity[26, 55].

Indeed, the mutants of eNAMPT: H247E, R392A, R311A (that lead to a loss in enzymatic activity) and S200D (that prevents the dimerization of proteins), have the same ability compared to wild-type protein to protect macrophages from apoptosis induced by ER-stress, to induce the activation of STAT3 and to stimulate the release of IL-6. Only one report in the literature has shown that treatment with wild-type eNAMPT, but not with the H247A enzymatically inactive mutant, attenuates the detrimental effect of OGD on cell viability of neuronal and glial cells[11].

To date, it is still difficult to define if eNAMPT acts as an extracellular enzyme to induce intracellular pathway activations.

3.2 Putative eNAMPT receptors

Since the effects of eNAMPT are observable at ng/mL concentrations and the activation of specific intracellular pathways occurs in minutes, eNAMPT may bind to and activate a cell surface receptor. In 2007, the insulin receptor was the first propose by Fukuhara *et al.*, but the article was subsequently retracted because the data were not reproducible[8, 56]. Only recently, other two different receptors were proposed: CCR5 and TLR4 but, up to date, no other research group has replicated or disputed those receptors.

Van den Bergh *et al.* have reported that eNAMPT inhibits HIV infection of macrophages by R5 HIV and not by X4 HIV, moreover using the Surface Plasmon Resonance found an interaction between CCR5 and eNAMPT with a nanomolar range affinity[57]. Unfortunately, there are no information about the physiological role of this interaction. In 2015, Camp *et al*., also have reported

that eNAMPT binds and activates TLR4 inducing NF-κB activation in lung injury. The authors confirmed this interaction using Surface Plasmon Resonance, but without indicating either a K_D or an affinity range[58].

Despite these evidences, the real identity of the receptor still remains unclear, and CCR5 or TLR4 may be reputed good candidates at best, given the incomplete data available.

Most of my thesis has been focused on exploring the possibility that CCR5 is the eNAMPT receptor, and my observations will be submitted soon for publication. Given that the chapter on CCR5 is written in a manuscript-like format, with only minor information given about this receptor, I will expand this information below. On the contrary, given that only preliminary evidence have been provided by myself on TLR4 and this is too preliminary for publication, the corresponding chapter is not yet publication-ready and I have included TLR4 details there.

4 CHEMOKINE RECEPTOR TYPE 5 (CCR5)

CCR5 (also known as CD195) is the C-C chemokine receptor type 5, which binds selectively the chemokines of the CC-family. Chemokine receptors are members of the family of seven transmembrane-spanning receptors that signal through heterotrimeric G proteins upon ligand binding [59-61]. In general, the effects of CCR5 include inhibition of cAMP production, stimulation of calcium release, activation of PI3-kinase and MAP kinases, as well as other tyrosine kinase cascades. Through these pathways, CCR5 regulates trafficking and effector function of immune cells and serves as the main co-receptor for the entry of R5 strains of the human immunodeficiency virus (HIV-1, HIV 2)[60].

4.1 CCR5 signalling

Endogenous agonists:

A number of inflammatory CC-chemokines, including macrophage inflammatory protein 1 alpha (MIP-1α) and MIP-1β, chemokine (C-C motif) ligand 5 (CCL5 also known as RANTES), monocyte chemotactic protein 2 (MCP-2 also known as CCL8) and HCC-1, structurally related to MIP- 1α, have been described as endogenous ligands of CCR5.

Signal transduction of CCR5 induced by binding with the ligand, starts with the dissociation of G-protein into Gαi and Gβγ subunits and the consequent inhibition of adenylate cyclase and the activation of phospholipase Cβ isoforms (PLCB). These events lead to the hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol-1,4,5- trisphosphate (IP_3) and diacylglycerol (DAG). Then, the increase of IP_3 production causes calcium release from the endoplasmic reticulum and the activation of several calcium- dependent signalling. Like other Gi-coupled receptors, also CCR5 stimulates the opening of inward-rectifying K^+ channels and activates different tyrosine kinase cascades[62, 63].

CCR5 ligands stimulate the activation of several intracellular pathways. Some of them involve the MAP kinase family, such as ERK1/2, p38 and JNK, p56Lck, the phosphatases SHP1 and SHP2, leading to the phosphorylation of Src-related kinase and the cytoskeleton associated protein kinases paxillin[64, 65]. Another one involves JAK-STAT signalling. The activation of JAK by RANTES leads to the translocation of STAT1-3 to the nucleus and the regulation of gene transcription[66-68].

All these RANTES-mediated signalling are inhibited by pertussis toxin (PTx), suggesting a Gαi mechanism[69, 70], except for the JAK-STAT pathway[67].

Moreover, RANTES has also been shown to promote the activation of PI_3 kinase and Rho A, a member of the Rho family of small GTPases, in T cells[71].

Rho GTPases, including Rho A, coordinate the reorganization of the actin cytoskeleton and regulate cell polarity, adhesion and motility.

The modulation of CCR5 activity by its endogenous agonists includes also a physical conformational change, which results in the receptor internalization. Indeed, after ligand binding, the G-protein–coupled receptor kinases (GRK2 and GRK3) phosphorylate the receptor and, subsequently, the β-arrestin recognises the phosphorylated receptor and mediates the internalization through clathrin-coated vesicles[62, 63].

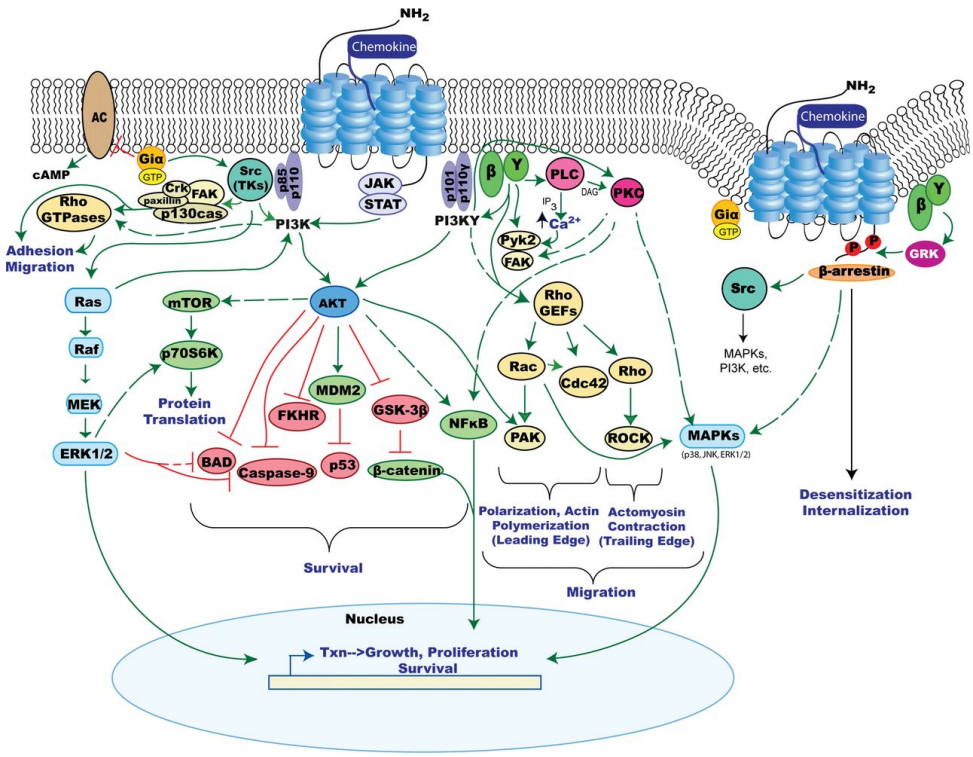


Figure 3. CCR5 intracellular signalling pathways[72].

Endogenous antagonists:

The chemokine Monocyte Chemotactic Protein-3 (MCP-3 also named CCL7) is a natural antagonist proposed for CCR5 receptor[73]. It competes with MIP-1β on CCR5 and reduces MIP-1β- dependent calcium signalling[73]. Blanpain *et al.,* demonstrated that MCP-3 is able to reduce the binding of the virus

membrane glycoprotein gp120 to CCR5 over-expressing CHO cells, but this is not translated into a reduction of the HIV infection[73].

Uguccioni *et al.,* reported that MCP-3 induces a migration response with bimodal concentration dependence, with different efficacy and curve trends. In detail, MCP-3 increased monocyte migration until the concentration of 100 nM, while higher concentrations inhibit the cell migration. They discovered that the effects of agonism obtained at low doses were mediated by CCR1, CCR2 and CCR3[74].

Synthetic antagonists:

The development of CCR5 inhibitors is mainly due to the need to find drugs that can reduce and prevent HIV R5 strain infection. Different small molecules have been synthetized such as maraviroc, cenicriviroc, aplaviroc and vicriviroc.

Maraviroc is a synthetic competitive antagonist of CCR5. It reduces both RANTES- mediated calcium signalling and HIV infection, without affecting the internalization rate of the receptor[75]. Maraviroc is defined as a slowly reversible small molecule antiretroviral drug, classified as an entry inhibitor, which impedes gp120 binding to CCR5 and the consequent fusion of the virus with the host[75, 76]. It is used in the treatment of HIV R5 strains positive patients and, recently, entered into phase II for HIV pre-exposure prophylaxis (PrEP) in woman and men[77, 78].

Maraviroc is the only drug of this class approved for HIV therapy, while the other compounds failed in primary efficacy endpoints (vicriviroc) or showed liver toxicity (aplaviroc) in clinical trials[79]. Clinical trials with cenicriviroc are still ongoing.

4.2 CCR5 and inflammation

CCR5 is largely expressed on resting T-lymphocytes with memory/effector phenotype, and on monocytes, macrophages and immature dendritic cells[80, 81], and is up-regulated by pro-inflammatory cytokines[82, 83].

CCR5 plays an important role in the fate of $CD8^+$ T cells. Indeed, upon entering the lymph nodes, naive $CD8^+$ T cells are activated and quickly express CCR5 on the cellular membrane. The subsequent contact between $CD8^+$ T cells and antigen-mediated dendritic cells promotes the development of $CD8^+$ T cells responses and the acquisition of immune memory. The effector T cells which express CCR5, subsequently exit from the lymph nodes and are recruited into sites of inflammation and/or infection by chemokines that are released by innate immune cells[84-87].

Moreover, CCR5 plays an important role in the fate of monocytes, macrophages and dendritic cells. Indeed, upon infection, tissutal macrophages produce a large amount of chemokines, among which some lead to CCR5 activation. This contributes to the survival of macrophages during inflammation and infection, and drives and retains the macrophages and dendritic cells in inflammed tissue[88].

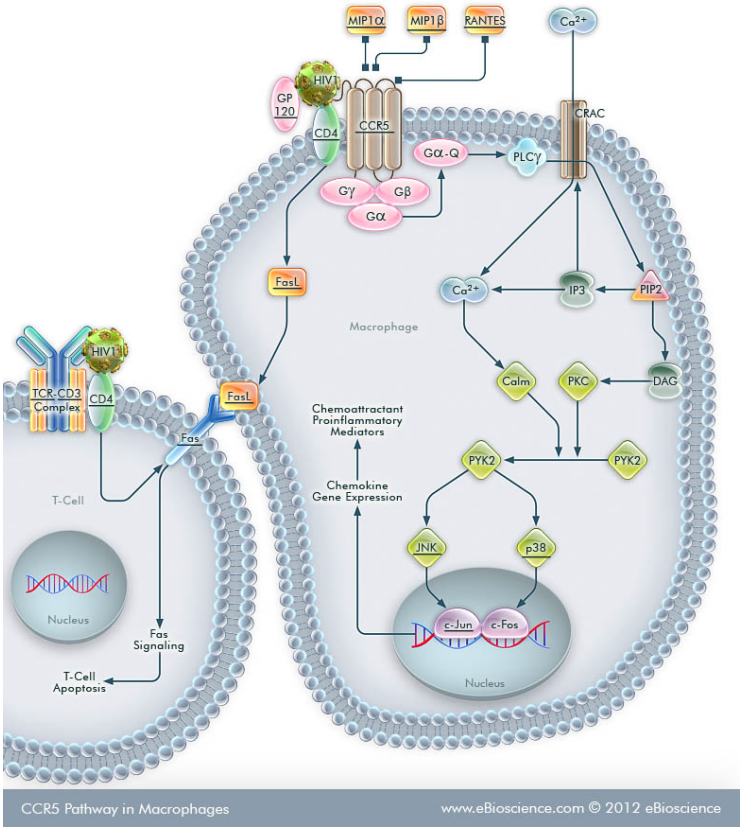


Figure 4. CCR5 effects on immune cells

Infections

The role of CCR5 in infection is well documented. First, $CCR5^{-/-}$ mice develop normally in a pathogen free-environment, but exhibit a partial deficiency in macrophage function resulting in: (i) a reduction of Listeria infection clearance; (ii) a delay in hypersensitivity reactions and (iii) an increase of humoral responses to T-cell- dependent antigenic challenge[89, 90].

In particular, CCR5 plays an important role in human immunodeficiency virus (HIV) infection. In detail, CCR5 is the co-receptor used by M-tropic HIV, while CXCR4 is the co-receptor used by T-tropic HIV[91, 92](Figure 5). In the first stage of HIV infection, the gp120 glycoprotein of HIV interacts with CD4 and, after a conformal change, it binds to CCR5.

In the second stage, the interaction between gp120, CD4 and CCR5 (or CXCR4) leads to exposure of the gp41 subunit on the virus envelop which anchors the membrane of the host and mediates the HIV fusion process crucial for the infection[93-96].

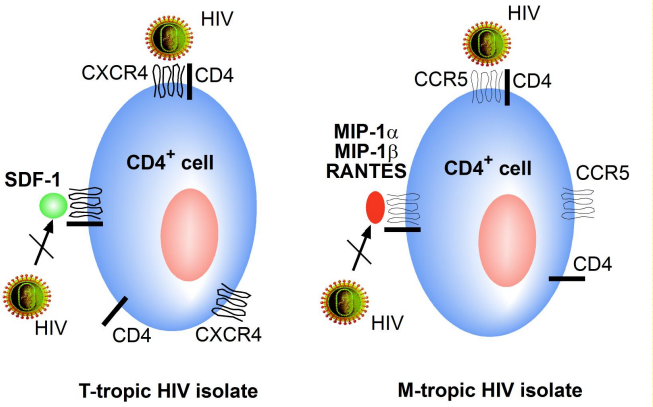


Figure 5. Scheme of HIV infection *(hivbook.com)*

Furthermore, CCR5 delta 32 (CCR5Δ32) is a mutant allele of CCR5 frequent in populations of European origin, and encodes a non-functional truncated protein that is not transported to the cell surface. Homozygotes for the Δ32 allele exhibit a strong resistance to HIV R5 strain infection, whereas heterozygotes display delayed progression to acquired immunodeficiency syndrome (AIDS). Many other alleles affect the primary structure of CCR5 or its promoter, some of which lead to a non-functional receptor or otherwise influence AIDS progression[97-101].

Autoimmune diseases

To evaluate the CCR5 mediated effects, most of the studies have taken advantage from the CCR5 KO mouse model and from pharmacogenetic analysis of patients (*i.e.* CCR5 Δ32 mutants).

For example, the mutant Δ32 allele is associated: (i) to a milder phonotype in rheumatoid arthritis[102] (ii) to a decrease risk of rejections in renal

transplantation[103], (iii) to a decrease risk of atherosclerotic plaque formation and a protection against an early episode of myocardial infraction[104, 105].

Neuroinflammation

Epidemiological studies reveal that CCR5Δ32 allele is associated with less severe forms of multiple sclerosis, but do not affect the risk of patients to develop the disease, suggesting that antagonism of CCR5 might reduce multiple sclerosis progression[106] but also that CCR5 is not involved in its development.

4.3 CCR5 and cancer

Large amount of evidence describes CCR5 as crucial in cancer pathology, although most of these refer to pre-clinical studies conducted on cancer cells and tumour-bearing mouse models.

CCR5/RANTES axis activation may have a double role in cancer: on one hand, it induces an immune response against cancer cells[107], on the other hands, it appears to promote cancer progression and metastasis formation[108]. These contradictory effects may be explained, at least in part, with the ability of RANTES to also bind receptors (*e.g.* CCR1 and CCR3) other than CCR5.

The activation of CCR5 leads to cancer cell proliferation, angiogenesis, modulation of extracellular matrix, and immune evasion mechanisms. Indeed, exogenous RANTES stimulates cell migration and invasion through the activation of PI3K/AKT and αvβ3integrins[109], and by releasing MMP-2 and 9 through NF-κB, ERK and Rac signalling pathways[110-113].

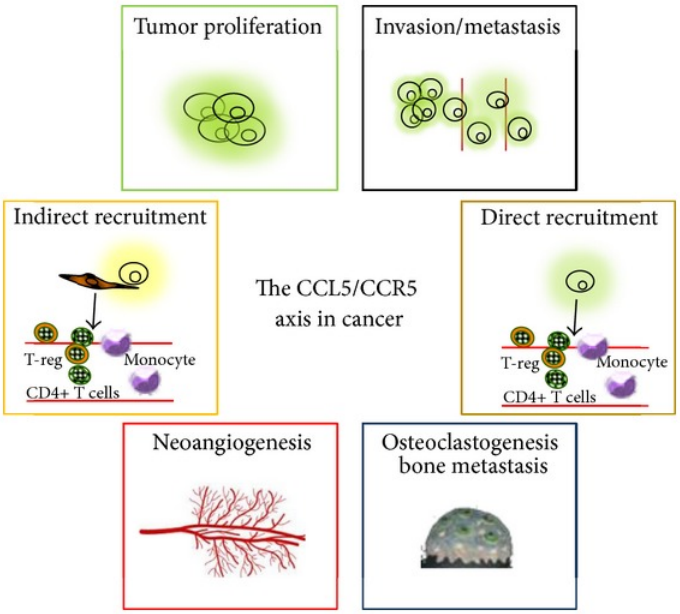


Figure 6. CCR5 in cancer[114]

Increase in glucose uptake and ATP production and enhanced glycolysis are consequences of CCR5 stimulation in cancer cells, favouring their proliferation[115, 116].

Moreover, different types of cancer, such as breast, melanoma, colorectal, ovarian and prostate cancer, over-express both CCR5 and RANTES[117-123], a selection advantage that contributes to increase ability of cell migration and invasiveness. Specifically, in primary melanoma and some cutaneous metastasis RANTES and CCR5 have been found over-expressed compared to normal melanocytes and their expression correlates with malignancy state[118, 119]. To understand the role of RANTES/CCR5 axis in melanoma, Song *et al.* generated a melanoma model in CCR5 knockout ($CCR5^{-/-}$) and wild type ($CCR5^{+/+}$) mice and observed that melanoma growth was delayed in CCR5-deficient mice due to an increased cell death and activation of apoptotic signalling[124].

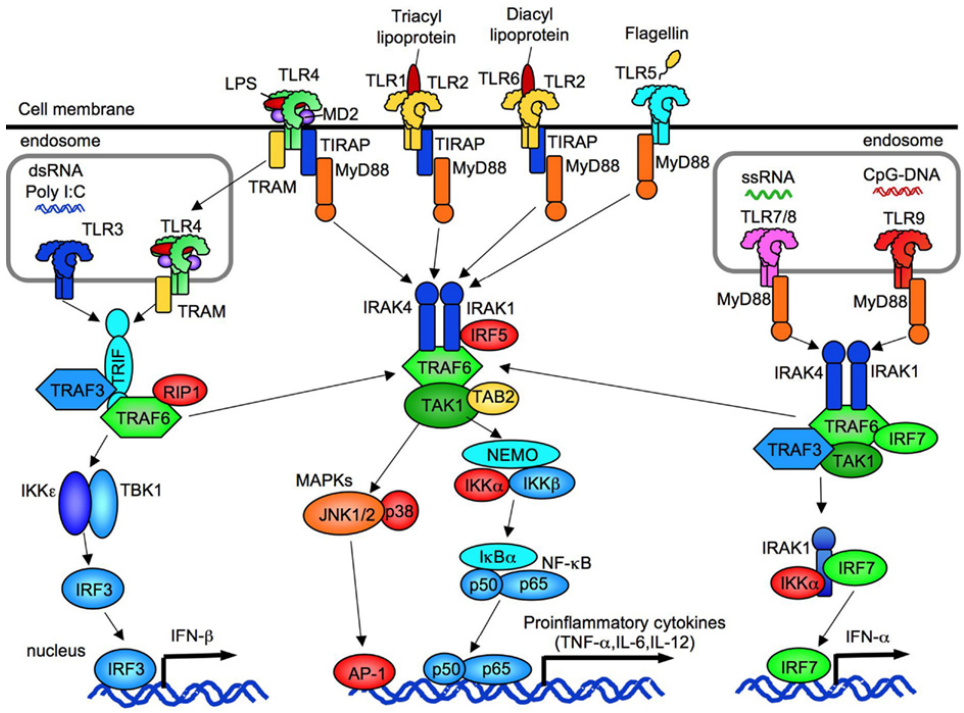
RANTES/CCR5 may also perturb the equilibrium of tumour infiltrating cells in the tumour microenvironment. In fact, RANTES promotes the recruitment

of tumour-promoting cells such as tumour associated macrophages (TAMs) and myeloid derived suppressor cells (MDSCs), that leads to immuno-suppressing effects and reduce the antitumor T-cell activities[117, 125, 126].

Last, few information are available regarding cancer patients and CCR5 signalling. Yet, in breast cancer, Niwa *et al.* and Bieche *et al*. found a correlation between RANTES and cancer progression, relapse and metastasis formation compared to patients in remission[127, 128]. Moreover, Luboshits *et al.* showed that RANTES release was mostly due to cancer cells, but also infiltrating leucocytes and mesenchymal stem cells (MSCs), present in the tumour microenvironment, contribute to increase RANTES levels in patients[129].

5 TLR4: toll-like receptor 4

Toll-like receptors (TLRs) are type I transmembrane proteins and belong to pattern recognition receptor (PRR) families. Their extracellular domain contains leucine-rich repeats (LRRs) and binds to a specific ligand unique to bacteria, fungi and viruses: lipopeptides are recognised by TLR2, lipopolysaccharide by TLR4 and flagellin by TLR5, while virus compounds such as RNA single- and double-strand are recognised by TLR8[130-133]. The activation of TLR receptors induces an inflammatory response through the cytosolic domain, which is conserved to all TLR receptors. The intracellular regions are similar to the cytosolic domain of IL-1 receptor and it is called Toll/IL-1 receptor (TIR) domain. Moreover, TLRs are able to heterodimerise with other types of TLRs, TLR2 dimerises with TLR6 or with TLR1 to respond to diacylated or triacylated lipoproteins[134].



Scheme 1. TLRs signalling[135]

5.1 Signalling of TLR4

Endogenous agonists:

TLR4 is activated by a variety of pathogen-associated molecular patterns (PAMPs), among which the most characterized is lipopolysaccharide (LPS). LPS is not the only ligand of TLR4, but there are also glucoronoxylomannan, mouse mammary tumour virus envelope protein, fibrinogen, heat shock protein (HSP) 60 and 70, high-mobility group box 1 protein (HMGB1) and others. In addition to PAMPs, TLR4 can be also activated by damage-associated molecular patterns (DAMPs) derived from damaged tissues, such as oligosaccharides of hyaluronic acid, fibronectins, and fatty acids in response to cellular damage[136-139].

Yet, LPS remains the main agonist of TLR4 and it is composed from 3 different regions: the endotoxin (or lipid A), the core composed by oligosaccharides and

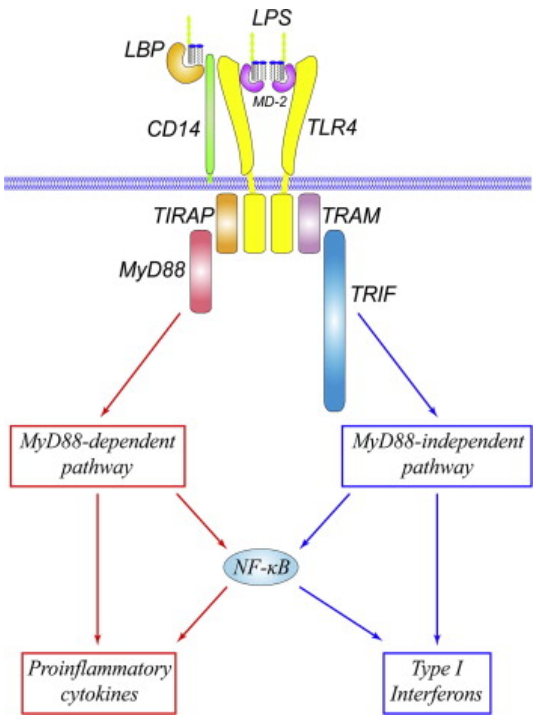
the O-antigen region[140]. The endotoxin is the only part of LPS recognised by TLR4, and the binding of endotoxin with TLR4 requires some different adaptor or accessory molecules, such as lipopolysaccharide-binding protein (LBP), CD14 and MD-2[141-143].

LPB: it is produced in the liver and in the lung and present into the blood. LPB does not participate in the TLR4 binding, but enhances the sensibility to LPS[141]. Indeed, LPB is a lipotransferase that facilitates the transfer of LPS onto CD14. Indeed, mice lacking LPB ($LPB^{-/-}$) showed a reduction of LPS responses[144, 145].

CD14: it exists in 2 forms: a GPI-linked and a soluble form. CD14 lacks in a transmembrane and in a cytoplasmic region and it is not able to produce an intracellular signalling. Otherwise, soluble CD14 plays an important role in increasing the sensitivity to LPS in cells[143].

MD-2: it is essential to LPS responses. Indeed, the lack of MD-2 in cells leads to a lack of LPS response. In this context, different research groups have achieved conflicting results when they transfect TLR4 in HEK293T cells, indeed, some report showed a response of LPS in these cells[146], while some others did not observed any responses. These conflicting results were due to a lack of MD-2 in HEK293T, while subsequent studies demonstrated that MD-2 is also a soluble TLR4 co-receptor and its presence in serum might be sufficient to make cells responsive to LPS[146-149].

The activation of TLR4 induced by MD-2/LPS leads to a recruitment of four different TIR-domain-containing adapter molecules, which lead to activation of two different pathways: (i) the MyD88-dependet pathway and (ii) the MyD88-independet pathway.



Scheme 2. Mechanism of LPS activation of TLR4[150]

(i) The MyD88-dependent pathway requires the recruitment of myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter protein (TIRAP), which are involved in the early activation of NF-κB and the consequent production of different cytokines, such as IL-12 and IL-6[151-154]. MyD88 recruits IL-1R-associated kinase-1 (IRAK1) and the adaptor molecule TNF Receptor-Associated Factor 6 (TRAF6)[155]. The fundamental role of MyD88 was demonstrated by Shizuno *et al.* that showed that macrophages obtained from MyD88-deficient mice were insensitive to LPS-induced death and were not able to release some cytokines such as IL-6 and TNFα[156]. In brief, the activation of MyD88 triggers the activation of TRAF6 which in turn induces transforming growth factor-β-Activated Kinase 1 (TAK1). All these events culminate with the activation of MAPK and IκB kinase (IKK) that promotes NF-κB translocation into the nucleus.

(ii) The MyD88-indipendent pathway requires the recruitment of TIR-domain-containing adaptor inducing interferon-β (TRIF) and TRIF-related Adaptor Molecule (TRAM). The activation of TRIF results in a strong activation of interferon (IFN)-regulator factor 3 (IRF3), involved in the *late* NF-κB activation, which culminates with the production of IFN-β and the expression of IFN-inducible genes[134, 153, 157, 158].

Antagonists:

The antagonists of TLR4 interfere both with receptor dimerization or with the adapter recruitment, leading to a reduction in NF-κB and MAPK activation and to a decreased production of pro-inflammatory cytokines[159, 160].

To date, no endogenous antagonists of TLR4 are known, however recently several TLR4 inhibitors obtained from plant extracts have been identified. Particularly, many herbs used in Traditional Chinese medicine (TCM) and Ayurvedic medicine resulted rich in molecules acting as antagonists of TLR4[161]. These include green tea, *Glycyrrhiza uralensis* (licorice), Magnolia officinalis, ginger, *Salvia miltiorrhiza*, curcumin and *Ganoderma lucidum*[159, 160, 162-164]. In detail, the main natural compounds that act as an antagonist of TLR4 are paclitaxel, morphine, opioids, sulforaphane, caffeic acid phenethyl ester and glycyrrhizin[163, 165-168].

Some synthetic compounds, such as naloxone, naltrexone, amitriptyline and imipramine, have been described as TLR4 antagonists[169, 170].

As alternative strategy, analogues of lipid A which act as antagonists of TLR4 have been synthesized, such as LPS-RS and eritoran, which is currently undergoing clinical trials for use in treating Gram-negative endotoxemia and sepsis[171, 172].

5.2 TLR4 and diseases

Since the major ligand of TLR4 is bacterial LPS, it is clear that TLR4 covers an important role in inflammatory diseases, in particular in infections. However, TLR4 is related also with non-infection diseases such as cancer, cardiac disease, obesity and diabetes.

Metabolic diseases

TLR4 is expressed in many cell components of insulin target tissues, including liver, adipose tissue, skeletal muscle, vasculature, pancreatic β cells, and brain[173, 174].

Saturated Fatty Acids (SFAs) activation of TLR4 is an attractive link between obesity, insulin resistance and inflammation. SFAs are acyl components of LPS, which bind directly to TLR4/MD2/LPS and activate the receptor *in vitro*[175, 176]. However, recent studies document endotoxin contamination of experimental reagents, which generate false positive[177-179].

Extensive literature suggests that high fat diet-augmented postprandial endotoxemia is a possible mode by which dietary SFAs induce inflammation through TLR4 in diet-induced obesity models[180]. Moreover, insulin resistant diet-induced obesity and genetically obese mice exhibited elevated plasma LPS levels and endotoxemia, and it is correlated with insulin resistance. In addition, genetically obese mice treated with a LPS inhibitor showed a reduction in inflammation and metabolic abnormalities compared to untreated mice, suggesting a correlation of obesity with the TLR4 signalling[181, 182].

All these evidences suggest a correlation between obesity, inflammation and insulin resistance with the activation of TLR4. In this sense, different studies show that the alteration of TLR4 gene in mice confers protection from obesity-induced inflammation and insulin resistance. In particular, using TLR4 deficient mice (with loss-of function or deletion/mutations in the TLR4 gene),

different studies demonstrated that TLR4 signalling in macrophages and adipose tissue regulates whole body glucose homeostasis thought the effects on adipose, muscle, and liver tissues. Moreover, TLR4 deletion also improved insulin sensitivity, with higher rates of glucose disposal into skeletal muscle and adipose tissue[183-187].

Cardiac diseases

An ischemia/reperfusion injury (I/R injury) is a hypoxic condition in which there is an interruption of the blood supply and a subsequent restoration. In addition to the damage caused by ischemia, the reperfusion of blood supply may cause an acute inflammatory response that leads to an extensive tissue injury named "reperfusion injury"[188]. Moreover, hypoxia induces the release of different damage-associated molecular patterns (DAMPs) from cells in response to a cell damage or tissue injury. DAMPs induce the activation of macrophages, which release pro-inflammatory cytokines, and the recruitment of neutrophils, monocytes, and lymphocytes in ischemic area[189]. Different studies, some of them conducted also in mice, demonstrated that DAMPs bind directly to TLR4 and activate its downstream pathways. Chong AJ *et al*. generated a TLR4 mutant mouse model, and observed a reduction of infarct size in I/R injury compered to wild type groups. Moreover, they reported a reduction in IL- 6 release and NF-κB and AP-1 activation[190]. Likewise, Shimamoto *et al.* demonstrated that the inhibition of TLR4 in a murine model of myocardial I/R injury results in a reduction of tissue damage[172].

Inflammation diseases

The polymorphism studies conducted on the human TLR4 gene revealed diversifications among individuals, which determine different infection disease outcome.

Two different TLR4 polymorphisms, the Asp299Gly and Thr399Ile variant alleles, were found to cause a reduction in LPS response[191]. Indeed, individuals

carrying these mutations showed a decreasing responsiveness to inhaled Escherichia coli LPS. Interestingly, Lorenz *et al.* observed in a comparative study between patients with septic shock, that patients with Asp299Gly and/or Thr399Ile polymorphisms were more susceptible compare to wild type. Moreover, these patients showed an increased sensitivity to gram-negative bacterial infections[192]. Unfortunately, other studies failed to confirm these data and the results were conflicting. Indeed, different research groups failed to observe any difference between patients with wild-type TLR4 and mutated TLR4. Ferwerda *et al.* reported that the primary cells obtained from Asp299Gly/Thr399Ile haplotypes patients did not show any different in LPS responsiveness compered to wild-type patient's primary cells[193, 194].

Ferwerda *et al.*, in a review, supposed that an erroneous study methodology can influence the results[194]. Indeed, they observed that four haplotypes exist for TLR4 genes: wild type/wild type, Asp299Gly /wild type, Thr399Ile /wild type, and Asp299Gly / Thr399Ile[194]. Moreover, recent data, obtained from the same research group, showed that only the Asp299Gly polymorphism differs from wild-type, indeed the blood samples obtain from Asp299Gly patients showed an increased TNF-α response after LPS-stimulation[193].

In addition, another single nucleotide polymorphism rs11536889 in 3′-untranslated region of TLR4 was identified, and different studies reported a correlation between this polymorphism and periodontitis or organ failure in sepsis[195]. Subsequent studies showed that this polymorphism causes a reduction in cell surface expression of TLR4 and IL-8 production in response to LPS treatment[196].

Cancer

A possible implication of TLR4 in tumour proliferation, metastasis formation and apoptosis inhibition has been supposed.

First, an overexpression of TLR4 was found in many different types of cancer cells, such as lung cancer A549 and H460 cell lines[197], cervical squamous

epithelial cells[198], colon cancer cells and MDA-MB-231 human breast cancer cell line[199].

Moreover, a role of TLR4 in chemotherapy resistance and in pro-proliferative and pro-invasive stimulation of the tumour has been demonstrated. Indeed, Wang *et al.* reported in ovarian cancer a possible role of TLR4 in paclitaxel chemotherapy resistance[200], while Hua *et al.* demonstrated that the silencing of TLR4 reduced cell invasion, survival, and tumorigenicity of prostate cancer[201]. Additionally, different studies observed that the TLR4 and MyD88 expression correlates with survival, metastasis formation and the expression of different chemokine such as CCL2 and CCL5[202]. Moreover, in H7402 (human liver cancer) and HepG2 (hepatoblastoma), LPS stimulation increased cell proliferation, chemotherapy resistance and NF-κB activation, which in turn leads to an increase of TNF, IL-6 and IL-8 release[203-205].

While on the one hand the expression of TRL4 in tumour cells is linked to a poor prognosis, on the other hand the expression of TLR4 on immune and inflammatory cells in tumour microenvironment leads to a production of pro-inflammatory and immunosuppressive cytokine and angiogenetic mediators. This results in a polarization of tumour-associated macrophages (TAM), tumour-promoting cancer-associated fibroblasts (CAF), and the activation and accumulation of myeloid-derived suppressor cells (MDSCs)[206-208].

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Chapter 2

During my Ph.D., I worked in two separate fields of research: (i) the investigation of novel chemicals as putative tubulin inhibitors; and (ii) the generation of data towards the identification of the eNAMPT receptor. Given that the articles describing novel tubulin agents have now been published[1], my thesis work will concentrate on the more challenging, and so far unpublished, data attempting to unravel the eNAMPT receptor.

In this latter aspect, I focused my attention on:

(i) evaluating whether CCR5 could mediate the effects of eNAMPT, starting from the report that an interaction between eNAMPT and CCR5 determined by Surface Plasmon Resonance occurred[2].

(ii) Confirming the hypothesis emerged by Camp et al. that TLR4 may mediate part of the effects elicited by eNAMPT[3].

(iii) setting up different methodologies to discover new eNAMPT interactors, via fluorescent probes and cross-linking techniques.

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Chapter 3

To be submitted:

Extracellular nicotinamide phopshoribosyltransferase (eNAMPT) modulates RANTES-mediated calcium signalling in cancer cells

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KEYWORDS

NAMPT, CCR5, calcium signalling, migration, cancer

ABSTRACT

Extracellular nicotinamide phosphoribosyltransferase (eNAMPT, PBEF, Visfatin) is a metabokine released by various cell types with pro-tumoural and pro-inflammatory properties. In cancer, eNAMPT regulates tumor growth and metastasis formation through the activation of intracellular pathways, suggesting that eNAMPT acts through a putative receptor, although the nature of this receptor is still elusive. Recently, using Surface Plasma Resonance (SPR) it has been demonstrated that eNAMPT binds to the C-C chemokine receptor type 5 (CCR5), although the physiological meaning of this finding is still unknown.

The aim of the present work was therefore to evaluate whether eNAMPT could act through the CCR5 receptor to explicate its effects in cancer cells. To study this phenomenon, B16 melanoma cells and a stable cell line that over-expressed murine CCR5 (HeLa-CCR5) were used. eNAMPT, unlike RANTES, did not induce MAPK activation and calcium responses, two typical CCR5 signalling pathways, suggesting that eNAMPT does not act as an agonist of the receptor. Surprisingly, pre-treatment with eNAMPT resulted in the blockage of RANTES-dependent calcium signalling. In addition, eNAMPT did not modify other calcium signalling pathways triggered by ATP and carbachol, suggesting that the effect of eNAMPT may be specific for CCR5. This modulation in calcium signalling culminates with a delay in migration of melanoma cells in wound healing assays *in vitro*. Yet, eNAMPT does not act as an allosteric modulator of CCR5 since it does not modify CCR5 internalization.

Our work does not support the hypothesis that CCR5 is the elusive receptor for eNAMPT, but shows that exists a functional link between eNAMPT and CCR5 signalling.

INTRODUCTION

Extracellular nicotinamide phosphoribosyltransferase (eNAMPT), also known as PBEF or visfatin, is the secreted form of NAMPT, a key enzyme involved in maintaining the balance of NAD and ATP levels in cells[1]. This enzyme is now considered a metabokine secreted by different cells with pro-inflammatory and pro-tumoural activity [2-4]. Importantly, eNAMPT is over-expressed in cancer [5, 6], where eNAMPT controls angiogenesis, tumour growth and metastasis formation [7-9]. The stimulation of cancer cells with exogenous eNAMPT is sufficient to activate specific intracellular signalling pathways (*e.g.* STAT3, NF-κB, Akt, p38[10-13]) within minutes, which indicates that eNAMPT binds to and activates a cell surface receptor. Although the identity of this receptor remains unknown. The first receptor proposed was the insulin receptor, based on the insulin mimetic properties of eNAMPT[14], however this article has been retracted[15]. Nonetheless, at least two others putative eNAMPT receptors have been proposed. Camp et al., demonstrated that eNAMPT induces lung inflammatory via direct ligation of Toll-like receptor 4 (TLR4)[16]. Moreover, computational analysis demonstrated that eNAMPT and MD-2, a TLR4-binding protein, share ~30% sequence identity[16]. In 2012, was reported that eNAMPT selectively inhibits infection of macrophages by human immunodeficiency virus (HIV), this activity was linked, using SPR, to a direct interaction with the C-C chemokine receptor type 5 (CCR5; CD195)[17].

CCR5 is a seven transmembrane, G-protein coupled receptor (GPCR), belongs to the β-chemokine receptor, expressed by macrophages, T-cells, microglia, dendritic cells and cancer cells[18]. CCR5 is involved in inflammatory response, thought the regulation of trafficking and effector functions of immune cells and it also serves as a co-preceptor for the entry of HIV R5 strains[19, 20]. Moreover, CCR5 is over-expressed in several cancers (*e.g.* breast cancer, melanoma) and it was suggested that the activation of CCR5 controls tumour development by acting as growth factors, stimulating angiogenesis, inducing the

recruitment of additional stromal and inflammatory cells, and taking part in immune evasion mechanisms[21, 22]. For example, in melanoma CCR5 expression on stromal cells is necessary for the spread of B16 cells to the lungs[23] and in CCR5-deficient mice the B16 growth is delayed [24]. The activation of CCR5 receptor induces G-protein alpha-i family that results in a blockage of adenylate cyclase type I and then in a reduction of intracellular cAMP, PLC-γ activation and a calcium influx[21, 25]. To date, the CCR5 ligands include the agonists RANTES (CCL5), MIP-1α and 1-β and CCL2-8-11-14, and the natural antagonist MCP-3[26].

Starting from this proof of principle, the aim of our study was to shed light on whether eNAMPT may be a novel ligand for CCR5 in cancer cells.

MATERIALS AND METHOD

Cell culture

HeLa (human cervix carcinoma) and B16 (murine melanoma) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS), 2 mg/ml glutamine, 10 U/ml penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO, USA). Cells were maintained in a humidified incubator supplied with 5% CO_2/95% air at 37°C.

Recombinant eNAMPT preparation

Wild-type murine full-length and H247E NAMPT (ORF GenBank BC018358) was cloned in pET28a (NdeI/EcoRI). Recombinant eNAMPTs were expressed in E. Coli (ClearColi, BL21(DE3)), inducted with IPTG 0.5 mM for 3 h at 20°C, and purified by His-tag affinity chromatography with NiNTA Superflow resin (Qiagen).

Generation of stable HeLa-CCR5 cell line

Murine CCR5 was cloned in the pLV lentiviral vector. Correct insertion and sequence were confirmed by DNA sequencing. The lentiviral particles were produced as described elsewhere[7] in HEK293T cells transfected with pMDLg/pRRE, pMD2.VSVG, pRSV-Rev and pLV-CCR5/pLV-empty plasmids. Briefly, after 48 h, cell medium was collected, filtrated and centrifuged for 1 h 30 min at 100 000 g. The viral particles, corresponding to the pellet fraction, were resuspended and used to infect HeLa cells, after virus titration. Stable scramble (HeLa-SCR) and HeLa-CCR5 were generated and CCR5 expression was monitored with Real Time PCR and FACS analysis.

CCR5 internalization assay

5×10^5 cells were plated in 96-well plates and treated with vehicle, RANTES (Peprotech) and/or eNAMPT for 15, 30 or 60 minutes at 37°C or 4°C. Cells

were washed twice in PBS and resuspended in 100 μL of Phosphate Buffer saline (PBS) and stained with anti-CCR5 PE for 20 min at 4°C. Cells were then washed twice in PBS and resuspended in PBS. Cell surface expression of CCR5 analysis was determined with a BD Accuri FACS.

Immunofluorescence

Cells were fixed in 4% formaldehyde. Subsequently, primary and secondary antibodies were applied in 0.2% gelatin. Fluorescence images were acquired using a Leica Confocal microscopy.

Fura-2 imaging

Cells were loaded with 2 μM Fura-2-AM in KRB solution (Krebs-Ringer modified buffer: 125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4) supplemented with 2 mM $CaCl_2$, 0.01% pluronic acid and 5μM sulfinpyrazone. After washing and de-esterification (30 minutes), the coverslip was mounted in a chamber and placed on the stage of a Leica epifluorescent microscope equipped with a S Fluor 40×/1.3 objective. Cells were stimulated with the indicated treatments and excited at 340/380 nm by the monochromator Polichrome V (Till Photonics, Munich, Germany) and the fluorescent signal was collected by a CCD camera (Hamamatsu, Japan) through bandpass 510 nm filter; the experiments were controlled and images analysed with MetaFluor (Molecular Devices, Sunnyvale, CA, USA) software. To quantify the difference in the amplitude of Ca^{2+} transients, the ratio values were normalized according to the formula $(\Delta F)/F_0$ (referred to as normalized (norm.) ratio). The cells with norm. ratio above 0.2 were considered as responders and used for further analysis.

Western blot analysis

5×10^5 cells were treated at the indicated time-points and the cells were lysed in 80 µL in lysis buffer composed of 20 mM HEPES, 100 mM NaCl, 5 mM EDTA, 1% Nonidet P-40+ Protease & Phosphatase Inhibitor Cocktail (Sigma). Proteins quantification was performed with BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA), and proteins were resolved on SDS–PAGE. Densitometry analysis was performed with Quantity One program (Bio-Rad, Hemel Hempstead, UK).

Wound-healing assay

In vitro wound assays were performed using IBIDI Culture-Inserts according to Shih *et al.*[27]. Briefly, when confluent monolayers of B16 cells were established on IBIDI dishes (35 mm with high culture-insert coating), cells were washed twice with PBS to remove residual cell debris. Cells were then incubated with RANTES, eNAMPT and maraviroc or the combination of three for 24 h and pictures of a defined wound spot were made at different time points. The area of the wound in the microscopic pictures was measured using Image J software (National Institutes of Health, MD) at different time points. The percentage wound healing after 4, 6, 8 and 10 hours was calculated in relative to the total wound area at t=0 h of the same wound spot.

Binding experiment

1×10^5 cells suspension were incubated with 2U/1×10^5 cells of Heparinise I and III for 1 hour at 4°C. Then, cells were washed and incubated with eNAMPT (27 µg) or maraviroc (10 µM) for 20 minutes at 4°C in complete medium. After, biotin-RANTES (25ng) conjugated with PE were added to cells suspension for 2 hours at 4°C. Then, cells were washed 3 times in ice cold PBS, resuspended in FACS Buffer (Hanks' Balanced Salt solution HBSS + 0.5% BSA) and samples were analysed using a FACS (BD accuri).

Reagents

Rabbit polyclonal antibody anti-p42/44 MAPK was from Cell Signalling Technology,; mouse anti-tubulin was from Sigma; peroxidase-conjugated secondary antibodies were from Biorad; maraviroc, ATP and carbachol were from Sigma, PE-anti mouse CCR5 from Biolegend. Recombinant RANTES/CCL5 was from Peprotech; biotinylated-reconbinant-RANTES was from ChemoTactis. DRAQ5 was from Thermo Fischer Scientific

RESULTS

It has been demonstrated that eNAMPT binds to CCR5 through Surface Plasmon Resonance technique and that it is capable of inhibiting infections by R5 HIV in monocytes, although the relevance of this interaction in cancer has not been evalueted[17]. 4r

To understand whether CCR5 could be the receptor for eNAMPT, at first, we decided to employ HeLa cells with stable over-expressing of CCR5 (HeLa-CCR5) comparing them with scrambled HeLa (HeLa-SCR). We first evaluated whether this cytokine parallels the effects of RANTES, a known potent agonist, in CCR5-overexpressing HeLa cells. As it can be seen in Figure 1A-B, RANTES 25ng/ml was able to elicit a marked induction of p42/p44 ERK phosphorylation in a time-dependent manner. Yet, we were unable to see an effect with eNAMPT, in a range of concentrations between 250-1000 ng/ml, in the activation of this pathway (Figure 1A-B; concentrations of 250 ng/ml and 1000 ng/ml not shown). It is therefore likely that the receptor usually associated with eNAMPT is not CCR5.

Calcium signalling has also been associated with CCR5 and we also evaluated this pathway. As it can be seen in Figure 1C-D, RANTES was able to induce calcium increases while eNAMPT was unable to do it. In support of this data, we investigated the ability of eNAMPT to induce CCR5 internalization, a common feature of CCR5 agonists. As expected RANTES induced a time and concentration dependent internalization of the receptor, however eNAMPT alone failed to modify the pattern of CCR5 (Figure 2A, B).

In light of these results, it would not appear that CCR5 is the receptor responsible for the effects commonly associated to eNAMPT and that eNAMPT is not an agonist of CCR5. On the contrary, the pre-treatment of eNAMPT partially obstructed the RANTES-mediated CCR5 internalization, suggesting a possible antagonistic role.

We next tested whether eNAMPT was able to modulate the responses to RANTES. Co-incubation or pre-treatment of cells with eNAMPT did not modify the phosphorylation pattern of p42/p44 ERK. The effect of RANTES, as expected, was antagonized, by maraviroc, a known antagonist of CCR5 (Figure 3A-B). Surprisingly, pre-incubation, but not co-incubation, with eNAMPT at both 250 and 500 ng/ml reduced significantly the calcium mobilization induced by RANTES. The effect of eNAMPT at the higher dose was quantitatively similar to maraviroc (Figure 4A-B). To evaluate whether the effect of eNAMPT on calcium signalling was specific, we evaluated the effect of the same concentration of eNAMPT on Ca^{2+}-signalling induced by ATP or carbachol, whose receptors are present both in wild-type and HeLa-CCR5 cells. As shown in Figure 4C-F, the antagonistic effect of eNAMPT was specific for CCR5 signalling, as the responses to ATP or CCh were unaffected. All these data suggest that eNAMPT blocks selectively the RANTES-dependent calcium signalling. To test if this inhibition is mediated by the enzymatic activity or via binding to the receptor, we investigated the effect of a mutated and inactive form of eNAMPT called H247E eNAMPT. The pre-incubation with H247E eNAMPT (500 mg/ml) reduced the calcium mobilization induced by RANTES (91.5 ± 3.28 % of responsive cells) in the same manner of the WT form (47.16 ± 11.47 % of responsive cells for H247E vs 45.58 ± 8.47 % of responsive cells for WT) (Figure 5 A-B). This data suggests that the reduction in calcium signalling by eNAMPT is mediated by a putative interaction with the receptor and not *via* its enzymatic activity. Moreover, the effect of eNAMPT could not be mimicked by the buffer in which the protein was dissolved, or by proteins isolated in the same manner such as nicotinamide mononucleotide adenylyltransferase (NMNAT) at identical concentrations (Figure 5).

The effect observed was obtained in an artificious system, and we therefore decided to investigate the interaction between CCR5 signalling and eNAMPT in a system that has been reported to respond to both. In this respect, we chose

a melanoma cell line, B16, in which we have previously shown an effect of eNAMPT[7]. As expected, in this system, RANTES was able to induce a Ca^{2+}-response, which was blunted by the eNAMPT pre-incubation (Figure 6A).

RANTES has been shown to promote migration in these cells, possibly via the calcium signalling pathway, and we confirmed this via the wound-healing assay. Briefly, RANTES, at a concentration of 200 ng/ml promoted wound closure compared to control in a time dependent manner (Figure 6B-C). This effect, as expected was antagonized by maraviroc and by eNAMPT, that *per se* had no effect on migration of these cells.

Considering these results, it would appear that eNAMPT acts as a natural antagonist of CCR5. However, from our results we were not yet able to determinate whether eNAMPT binds directly to CCR5 or interferes with RANTES-mediated calcium signalling through another receptor.

Therefore, we tested whether eNAMPT could directly bind to CCR5, taken advantage from a competitive binding assay. As it shown in Figure 7, pre-incubation of eNAMPT reduced cell surface binding of biotinylated-RANTES conjugated with avidin PE in HeLa-CCR5 cells. The effect of eNAMPT was similar to maraviroc. Specifically, eNAMPT reduces the percentage of RANTES-PE positive cells of 24%, while maraviroc reduction was of 29% (Figure 7A). The analysis of the mean florescence intensity (MFI) confirmed these results (Figure 7B).

A binding assay performed with a labelled iodine CCL3, preferred to radioactive CCL5 because of nonspecific aggregation, in collaboration with professor Massimo Locati (Humanitas research centre) is still on going to confirm the data described above obtained using RANTES-PE.

Since the crystal structure of RANTES in complex with CCR5 has been resolved, we performed an *in silico* analysis. Uniport alignment revealed similar amino acid sequence between eNAMPT and RANTES considering the chemical and physical properties of amino acids (Figure 8A). Surprisingly, these data were partially confirmed analysing the structure of eNAMPT and

RANTES using PyMol software. We identified a N-terminal alpha-helix of RANTES (black, Figure 8B), crucial for the binding with CCR5 (green, Figure 8B), which was aligned with a loop of eNAMPT (orange, Figure 8B). Moreover, RANTES has three β-strands and one α-helix that co-participate to the binding with CCR5. These motifs are also present in eNAMPT (yellow, Figure 8B). Despite of eNAMPT is a 55 kDa protein with a greater steric hindrance compared to RANTES (9 kDa), the alignment with PyMol demonstrated that eNAMPT could interact with CCR5. Mutations in these emerged regions might be useful to evaluate the structure–function relationship between eNAMPT and CCR5.

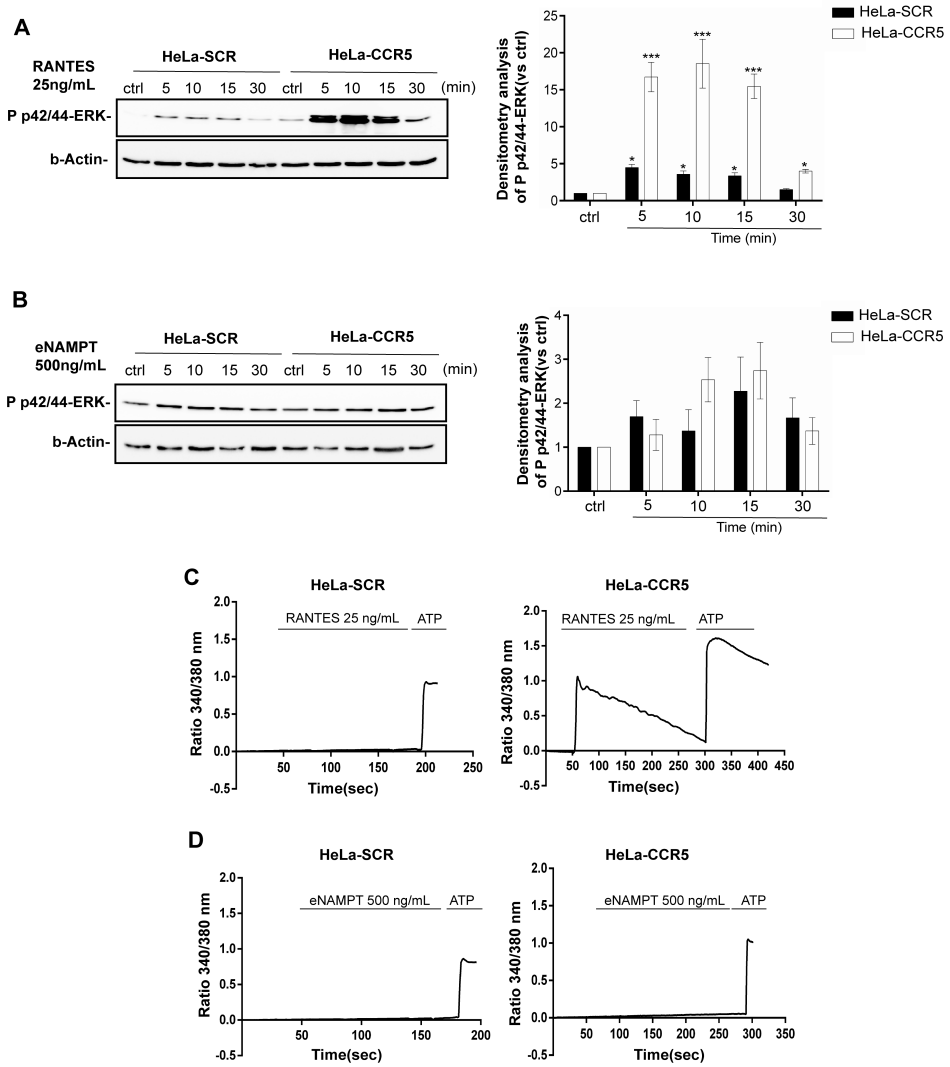

Figure 1. (**A**) Western blot and densitometry analysis of phosphorylated p42/44 ERK after 2 hours of starvation followed by treatment for 5 minutes with recombinant RANTES (25ng/mL) in serum-free condition in HeLa-SCR and HeLa-CCR5 cell lines. Representative data of 4 separate experiments. (**B**) Western blot and densitometry analysis of phosphorylated p42/44 ERK after 2 hours of starvation followed by treatment for 5 minutes with recombinant eNAMPT (500ng/mL) in serum-free condition in HeLa-SCR and HeLa-CCR5. Representative data of 3 separate experiments. (**C**) Calcium traces in HeLa-SCR and HeLa-CCR5 loaded with FURA-2AM and stimulated with RANTES (25ng/mL). Representative traces of peak calcium concentrations reached are shown for 98–110 cells from 5 independent experiments. (**D**) Calcium traces of HeLa-SCR and HeLa-CCR5 loaded with FURA-2AM and stimulated with eNAMPT (500ng/mL). Representative traces of peak calcium reached (90–102 cells from 7 independent experiments).

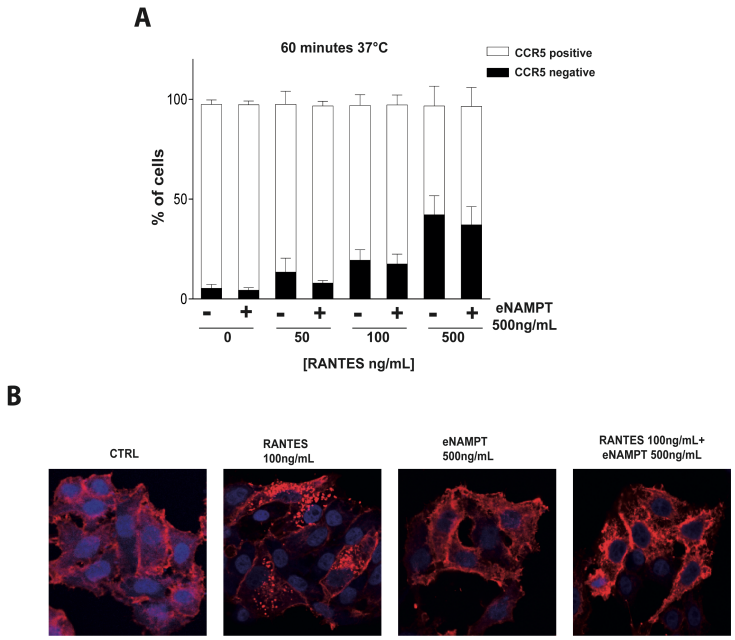

Figure 2. (**A**) Flow cytometry analysis of HeLa-CCR5 cells treated with RANTES (50, 100, 500 ng/mL) for 60 minutes in presence or absence of eNAMPT at 37°C, stained with anti-mouse CCR5-PE and analysed using FACS BD accuri. The data are summarized in histograms and expressed as mean ± S.E.M. of 12 determinations from 4 separate experiments. (**B**) Confocal images of HeLa-CCR5 cells treated with RANTES (100 ng/ml) for 60 minutes in presence or absence of eNAMPT (500ng/ml) at 37°C, stained with anti-mouse CCR5-PE (Red) and DRAQ5 (nuclear marker, blue). Representative images of 6 determinations of 3 separate experiments.

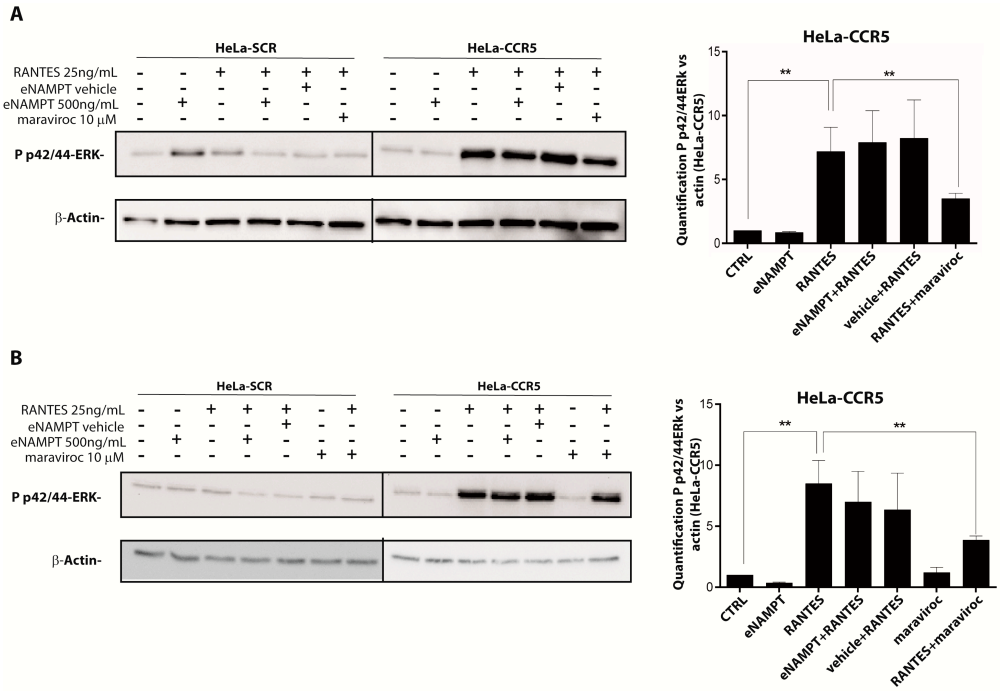

Figure 3. (**A**) Western blot and densitometry analysis of phosphorylated p42/44 ERK after 2 hours of starvation followed by co-treatment for 5 minutes with recombinant RANTES (25 ng/mL) in presence of eNAMPT (500ng/mL) or maraviroc (10 μM) or eNAMPT vehicle in serum-free condition in HeLa-SCR and HeLa-CCR5 cell lines. Representative data of 4 separate experiments. (**B**) Western blot and densitometry analysis of phosphorylated p42/44 ERK after 45 minutes of eNAMPT (500ng/mL) or maraviroc (10 μM) or eNAMPT vehicle pre-incubation followed by treatment for 5 minutes with recombinant RANTES (25 ng/mL). Representative data of 4 separate experiments.

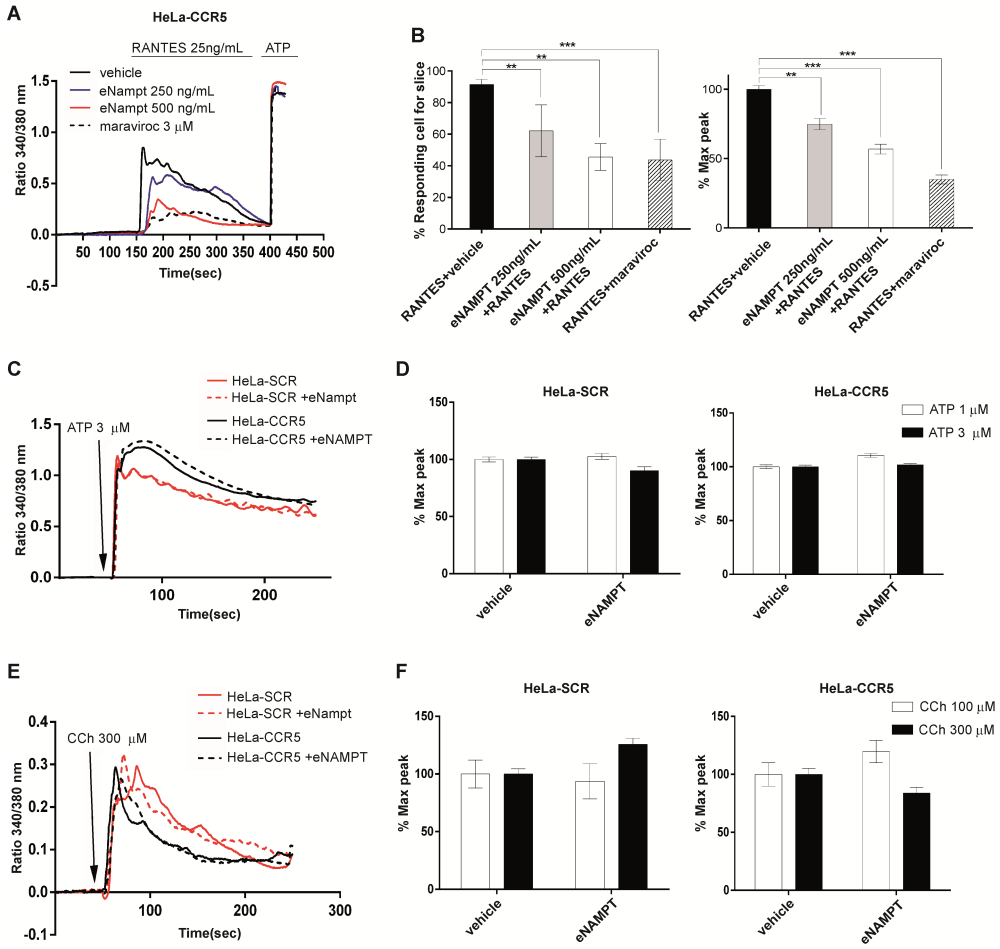

Figure 4. (**A**) Representative calcium traces of HeLa-CCR5 loaded with FURA-2AM and treated with RANTES (25 ng/ml) alone or pre-treated with eNAMPT (250ng/mL or 500 ng/ml) or maraviroc (3 μM) for 100 sec. High dose of ATP (333 μM) was used to verify the ability of the cells to evoke normal calcium traces. (**B**) Histograms of responding cells (left panel) and % of max peak (right panel) as mean ± S.E.M. of % responding cell for slice. (248–410 cells from 6 to 11 independent experiments) **P < 0.01, ***P<0.001 versus ctrl. (**C**) Representative calcium traces of HeLa-SCR and HeLa-CCR5 loaded with FURA-2AM and treated with ATP (3 μM) alone or pre-treated with eNAMPT (500 ng/ml) for 5 minutes. (**D**) The data are summarized in histograms and expressed as mean ± S.E.M. of % max peak calcium response (120–190 cells from 5 to 9 independent experiments). (**E**) Representative calcium traces of HeLa-SCR and HeLa-CCR5 loaded with FURA-2AM and treated with CCh (300 μM) alone or pre-treated with eNAMPT (500 ng/ml) for 5 minutes. (**F**) The data are summarized in histograms and expressed as mean ± S.E.M of % max peak calcium response (105–185 cells from 5 to 9 independent experiments).

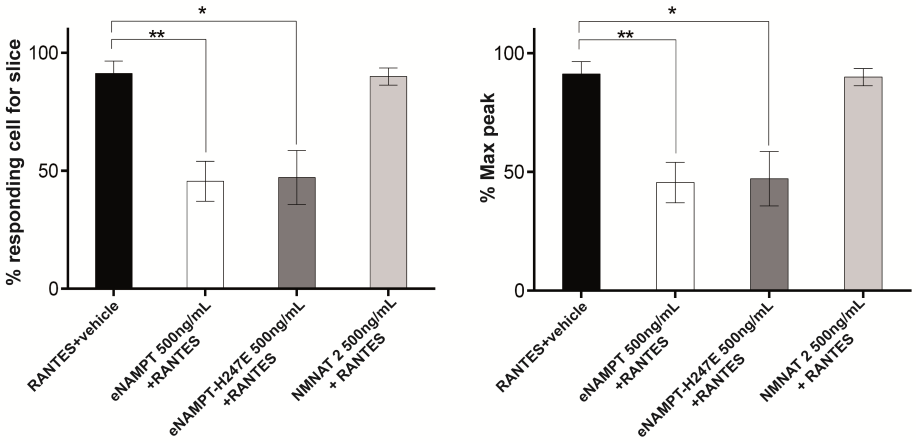

Figure 5. HeLa-CCR5 cells were loaded with FURA-2AM and treated with RANTES (25 ng/ml) alone or pre-treated with eNAMPT (500 ng/ml) or eNAMPT-H247E (500 ng/ml) or NMNAT 2 (500 ng/ml) for 100 sec. Histograms of responding cells (left panel) and % of max peak (right panel) as mean ± S.E.M. of % responding cell for slice. (180–210 cells from 3 independent experiments) *P<0.05, **P < 0.01, versus ctrl.

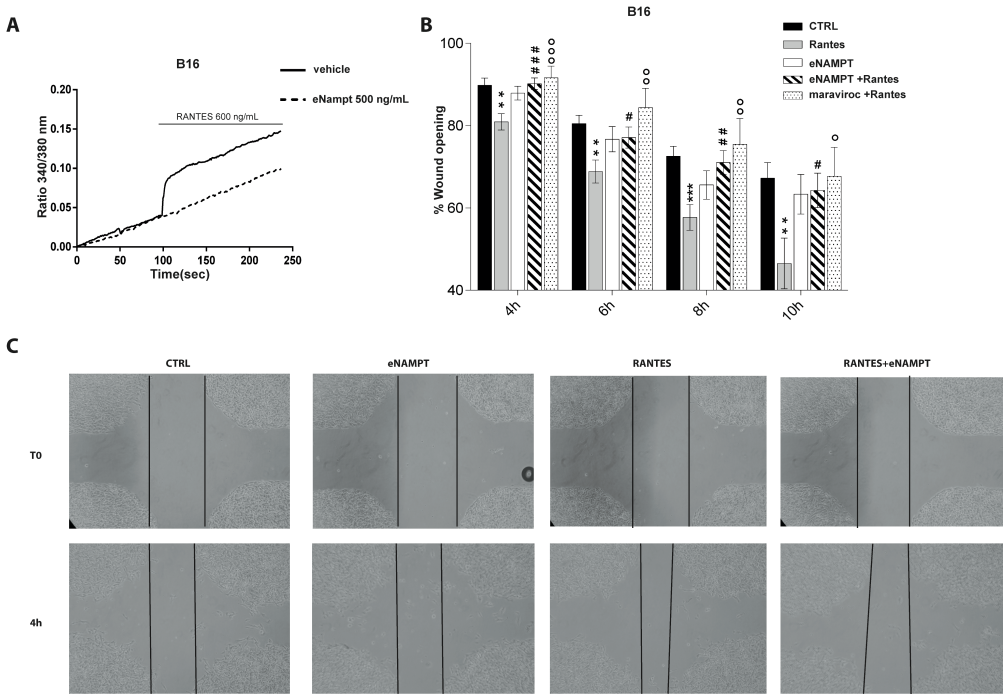

Figure 6. (**A**) Representative calcium traces of B16 cells loaded with FURA-2AM and treated with RANTES (600 ng/ml) alone or pre-treated with eNAMPT (500 ng/ml) for 100 sec. (**B**) Time course of wound opening in B16 cells treated or not with RANTES (200 ng/ml), eNAMPT (500 ng/ml) or maraviroc (10 μM). Mean ± S.E.M. of 12 determinations from 4 separate experiments. (**C**) Representative images of wound healing assay at 4 hours of treatment with RANTES (600 ng/ml) and/or eNAMPT (500 ng/ml) *P<0.05, ** P<0.01 ***P<0.001: * RANTES vs CTRL; + RANTES vs eNAMPT + RANTES.

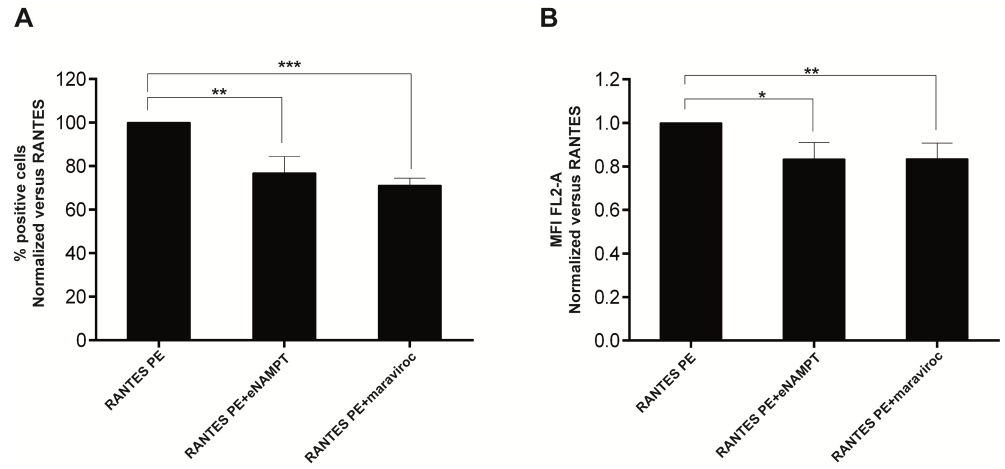

Figure 7. Percentage of positive cell (**A**) and the mean florescence intensity (**B**) of RANTES-PE binding on HeLa-CCR5 cells incubated or not with eNAMPT (27 µg, 100-fold increase compared to RANTES molar concentration) or maraviroc (10 µM). Mean ± SEM of 5 separate experiments.

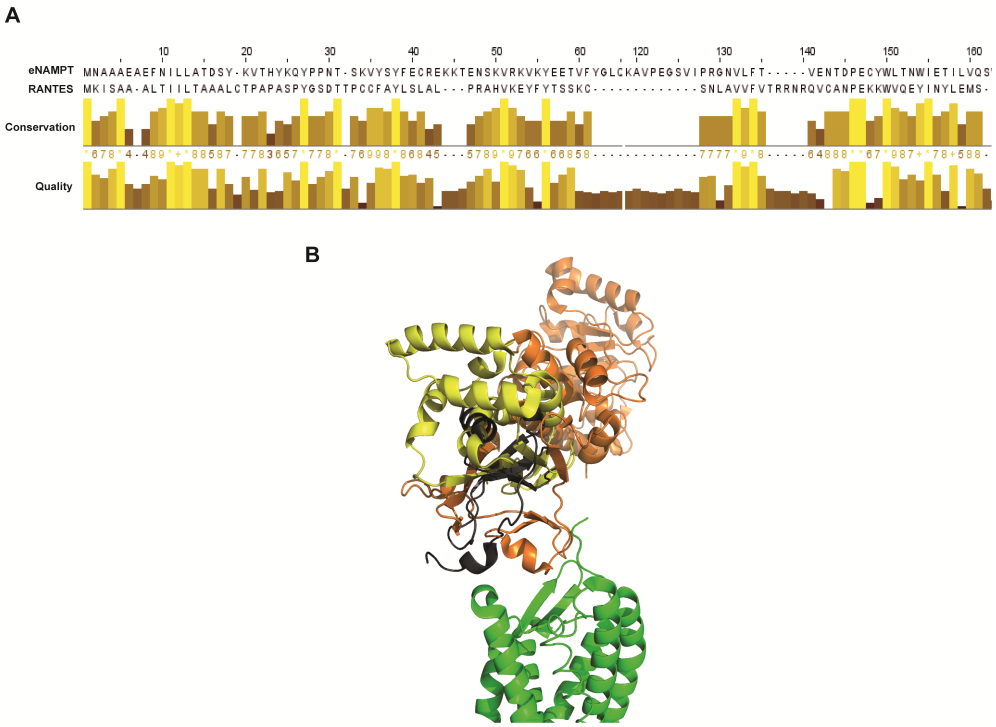

Figure 8. (A) Uniprot alignment of amino acid sequence between murine NAMPT and RANTES. (**B**) Analysis of NAMPT and RANTES using PyMol software. Full-length RANTES in black; CCR5 in green; NAMPT residues from 1 to 60 in yellow; NAMPT residues from 60-491 in orange.

Key finding:

- eNAMPT is not an agonist of CCR5, since it is unable to induce ERK phosphorylation, calcium signalling and internalization of CCR5.
- eNAMPT is an antagonist of CCR5, as it reduces RANTES-mediated calcium signalling and migration.
- eNAMPT does not affect the internalization rate of CCR5 induced by RANTES.
- eNAMPT binds to CCR5 and competes with RANTES.
- Bioinformatics suggests structural similarities between RANTES and eNAMPT.

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Chapter 4

UNPUBLISHED RESULTS

Is the Toll-Like Receptor 4 (TLR4) the eNAMPT receptor?

Keywords: eNAMPT, membrane receptor, TLR4, NF-κB pathway

ASTRACT

Camp *et al*. sustains that eNAMPT induces the activation of NF-κB through the direct binding to the Toll-like Receptor 4 (TLR4), demonstrated via the Surface Plasma Resonance (SPR) technique. In addition, using *in silico* model, they suggest that eNAMPT does not require MD-2 binding complex to activate TLR4. In this paragraph we tried to recapitulate Camp's observations, even more experiments are necessary to draw a conclusion.

To study the ability of eNAMPT to bind to and activate TLR4 in presence or absence of MD2, Jurkat $CD4^+$ T cells and a stable cell line that over-expressed human TLR4 (HeLa-TLR4) were used. eNAMPT and LPS did not induce NF-κB activation in MD2 lacking cells (HeLa-TLR4). Surprisingly, eNAMPT was able to induce NF-κB activation in $CD4^+$ T cell line Jurkat, (which expressed MD-2) after 1 hour of treatment. These data suggest that eNAMPT probably required MD2 to explicate its effects on TLR4. Moreover, gene expression analysis reveals similarities in genes up-regulation evoked by eNAMPT and LPS, but results are not superimposable.

Our work does not support the hypothesis that TLR4 is the elusive receptor for eNAMPT, but shows that exists a functional link between eNAMPT and TLR4 signalling.

INTRODUCTION

One mechanism by which the immune system senses pathogens is through the Toll-like receptors (TLRs), which recognize microbial components[1-4]. TLRs are type I transmembrane proteins expressed on the membranes of immune cells, including macrophages, B and T cells and dendritic cells, but also fibroblast and endothelial cells. In humans, to date 11 functional TLRs have been identified, and their stimulation induces different patterns of gene expression which lead first to the activation of the innate immune system and second to the development of antigen-specific acquired immunity[5].

TLR4 plays an important role in controlling innate immune responses after infection, but has been found involved also in other disorders including cancer, cardiac disease, obesity and diabetes.

TLR4 is activated by a variety of pathogen-associated molecular patterns (PAMPs), among which the most characterized is lipopolysaccharide (LPS). In addition to PAMPs, TLR4 can be also activated by damage-associated molecular patterns (DAMPs) derived from damaged tissues, such as oligosaccharides of hyaluronic acid, fibronectins, and fatty acids in response to cellular damage[7-10].

Recently, Camp *et al.*, reported through the Surface Plasmon Resonance (SPR) technique that the extracellular nicotinamide phosphoribosyltransferase (eNAMPT) binds to TLR4 and it is able to activate NF-κB downstream[32]. They demonstrated that eNAMPT induced the activation of NF-κB and the expression of NF-κB signalling genes in pulmonary endothelial cells and that this effect was reverted by the pre-treatment with TLR4 inhibitors (*e.g.* RS-LPS, CLI-095 and OxPAPC). Moreover, through the use of monoclonal antibodies against eNAMPT and TLR4, they have demonstrated the dependence of TLR4 in eNAMPT-mediated NF-κB activation. In support of this, eNAMPT-induced lung inflammation was reduced in $TLR4^{-/-}$ mice compared to wild type mice[32].

eNAMPT is a cytokine released by various cell types with autocrine and paracrine effects[27, 28]. The first evidence of a relevant role of eNAMPT in immunity was the identification of its capacity to synergize with IL-7 and SCF to stimulate B-cell differentiation[29]. From this first observation, several studies have been conducted and now we know that several inflammatory stimuli induce the secretion of eNAMPT, especially from innate immune cells, such as monocytes, neutrophils, and macrophages. Furthermore, eNAMPT *per se* has pro-inflammatory properties. For example, eNAMPT controls the differentiation of resting monocytes, proliferation and polarization of macrophages[27]. In these cells, the treatment with recombinant eNAMPT induces the activation of different pathways such as STAT3, NF-κB, Akt and ERK1/2, suggesting the presence of a receptor that mediates these effects[30, 31]. Starting from these evidences, we decided to further investigate the possibility that TLR4 was the eNAMPT receptor.

MATERIALS AND METHODS

Cell culture

HeLa (human cervix carcinoma) and Jurkat ($CD4^+$ T cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mg/ml glutamine, 10 U/ml penicillin and 100 µg/ml streptomycin (Sigma). Cells were maintained in a humidified incubator supplied with 5% CO_2/95% air at 37°C.

Recombinant eNAMPT preparation

Wild-type murine full-length NAMPT (ORF GenBank BC018358) was cloned in pET28a (NdeI/EcoRI), expressed in E. Coli (ClearColi, BL21-DE3), induced with IPTG 0.5 mM for 3 h at 20°C) and purified by His-tag affinity chromatography with NiNTA Superflow resin (Qiagen).

Limulus amebocyte lysate (LAL) test

Endotoxin levels were determined with the ToxinSensor Chromogenic LAL Endotoxin Assay kit according to manufacturer's instructions. Briefly, reconstitute LAL was added to eNAMPT samples and incubate 40 minutes at 37°C. Then, chromogenic substrate solution was added to each sample and incubate for 6 minutes at 37°C. Subsequent, the Colour-stabilizer was added to stop the reaction and absorbance of samples were acquired at 545 nm using the spectrophotometer Victor3 (Perkin Elmer).

Generation of stable HeLa-TLR4 cell line

Human TLR4-YFP plasmid was purchased from AddGene. HeLa cells were transfected using lipofectamine 2000 with Human TLR4-YFP or PCDNA-empty vector and after 48 h cells were maintained in medium supplemented with neomycin at 500 ng/mL for cells selection for the generation of stable HeLa-TLR4 and HeLa-SCR (scramble) cell lines. The relative expression of TLR4-YFP was determined by flow cytometry (BD accuri).

Western blot analysis

5×10^5 cells were treated at the indicated time-points with or without eNAMPT or LPS (581-009-L002 Alexis Biochemicals) and then lysed in 80 μL in lysis buffer (20 mM HEPES, 100 mM NaCl, 5 mM EDTA, 1% Nonidet P-40$^+$ Protease & Phosphatase Inhibitor Cocktail (Sigma)). Proteins quantification was performed with BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA), and proteins were resolved on SDS–PAGE. Densitometry analysis was performed with Quantity One program (Bio-Rad, Hemel Hempstead, UK). Mouse anti-tubulin was from Sigma; peroxidase-conjugated secondary antibodies were from Biorad. P-NF-κB (p65) at Ser^{536} was from Cell Signalling Technology.

Peritoneal exudate cells (PECs) isolation

Peritoneal exudate cells (PECs) were elicited by injecting i.p. 1 ml of thioglycolate (3% in H_2O) into 8-weeks old C57BL/6 mice. After 4 days, cells were recovered from the peritoneal cavity by lavage using 5 ml of PBS. Cells were plated in 6-well plates for 1 h at 37°C. Non adherent cells were removed by washing the wells three times with ice-cold PBS. Macrophages were treated with eNAMPT or LPS for indicated time points.

RT-PCR

Total mRNA was extracted using QIAzol Lysis Reagent (Quiagen, Milan, Italy) and retro-transcribed to cDNA using ImProm-II RT system (Promega, Milan, Italy). Real-time PCR was performed using SYBR Green according to manufacturer's instructions (Biorad) on a SFX96 Real-time system (Biorad, Segrate, Italy). For normalization of the raw qPCR data, S18 ribosomal subunit housekeeping gene was used.

Results

Camp *et al*., demonstrated that eNAMPT induces the activation of NF-κB through the binding to TLR4. In addition, they suggested that eNAMPT, conversely to LPS, does not require MD-2 binding complex to activate TLR4. Indeed, using an *in silico* model, they demonstrated that a similarity exists between the loop region of eNAMPT and MD-2 involved in TLR4 binding[32].

First of all, we decided to produce a recombinant eNAMPT protein in endotoxin-free bacteria to avoid unspecific activation of TLR4 due to a probable LPS contamination. After the purification of eNAMPT, the endotoxin content was determined by LAL test and as expected the level of endotoxins was <0.1 EU.

We generated a stable HeLa cell line overexpressing TLR4 (HeLa-TLR4) and scramble HeLa (HeLa-SCR) cell line as control. It is known that HeLa cells lack of MD-2 expression and we decided to use this model to investigate the ability of eNAMPT to induce NF-κB activation, monitored through the p65 phosphorylation[33, 34].

As expected, in MD-2-deficient cells (HeLa-SCR; HeLa-TLR4) the treatment with LPS (100 ng/ml) was not able to induce p65 phosphorylation (Figure 1A-B). Unfortunately, we failed to observe a significant phosphorylation of p65 upon eNAMPT-treatment at different time points (Figure 1A-B).

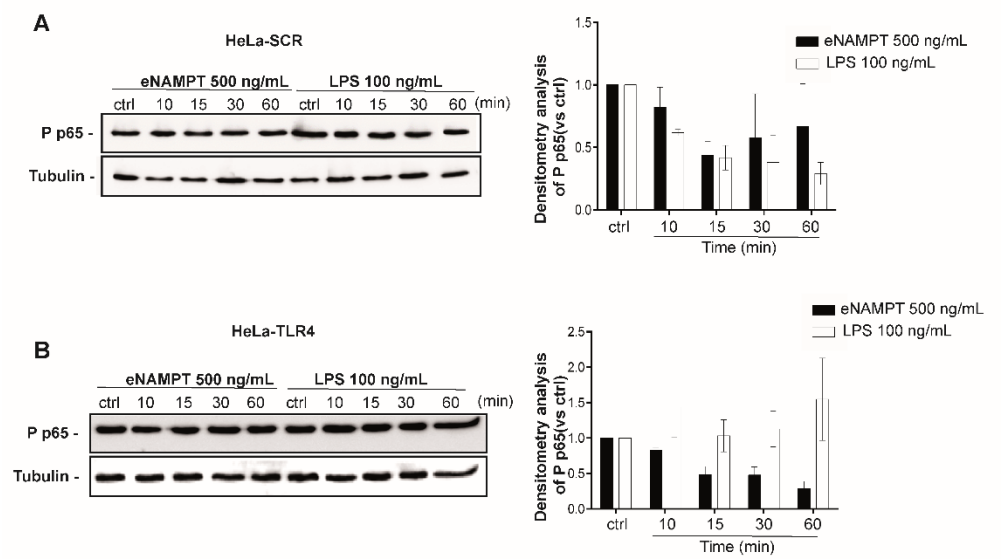

Figure 1. Western blot (left) and densitometry (right) analysis of phosphorylated p65 after 2 hours of starvation followed by treatment for indicated time points with recombinant eNAMPT or LPS in serum-free condition in HeLa-SCR (**A**) and HeLa-TLR4 (**B**) cells. Representative data of 4 separate experiments. (t test * P<0.05; **P<0.01; ***P<0,001)

We next evaluated whether the inability of eNAMPT to induce NF-κB in TLR4-overexpressing cells was due to the absence of MD-2. To do this, we next tested the ability of eNAMPT to induce NF-κB in $CD4^+$ T cell line Jurkat, which expressed MD-2.

The stimulation of LPS (100ng/mL) of Jurkat cells was able to elicit a marked increase of p65 phosphorylation in a time-dependent manner (Figure 2A). On the other hand, as shown in Figure 2B, eNAMPT treatment induced the p65 phosphorylation at 15, 45 and 60 minutes, however the p65 phosphorylation was statistically significant only after 60 minutes of stimulation (Figure 2C).

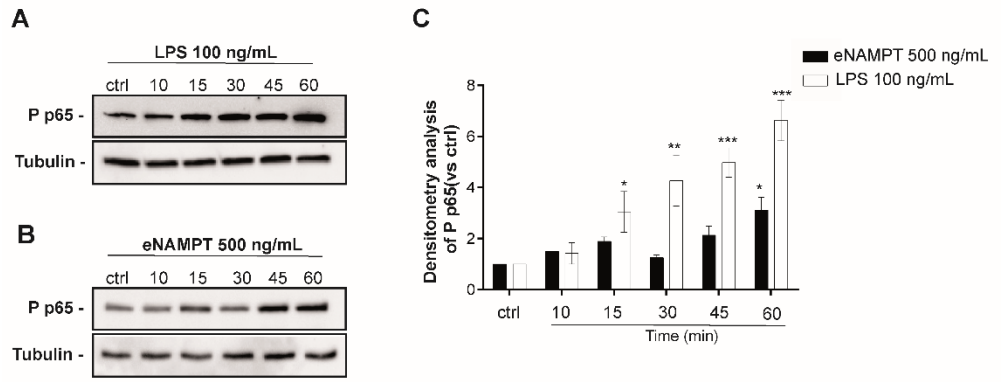

Figure 2. Representative Western blot of phosphorylated p65 after 2 hours of starvation followed by treatment for indicated time points with recombinant LPS (**A**) or with recombinant eNAMPT (**B**) in serum-free condition in Jurkat cell line. (**C**) Densitometry analysis of phosphorylated p65 after LPS or eNAMPT treatment. Representative data of 4 separate experiments. (*P<0.05, **P < 0.01, ***P<0.001 versus ctrl).

These data, although not fully convincing, are unable to support TLR4 as the receptor for eNAMPT. A number of explanations may be provided for these negative findings, including the possibility that eNAMPT binds to TLR4 with lower affinity compared to LPS or that it is responsible for the late activation of NF-κB. However, our results are largely inconclusive.

Interestingly, genome-wide gene expression analysis reveals similarities in genes up-regulation evoked by eNAMPT and LPS[32]. Therefore, to shed light on this aspect, we moved to *ex vivo* experiments using mouse peritoneal macrophages (PEC), which expressed both TLR4 and MD-2. As expected, the treatment of macrophages with LPS (100 ng/ml for 4 hours, Figure 3A) was able to increase several pro-inflammatory cytokines (*i.e.* iNOS, COX-2, IL12p40, CXCL9, CXCL10, IFNβ, IL-6, IL-1β, TNF, IL23p19), however eNAMPT induced the up-regulation only of CXCL9, CXCL10, IL-6, and IL-1β (Figure 3B).

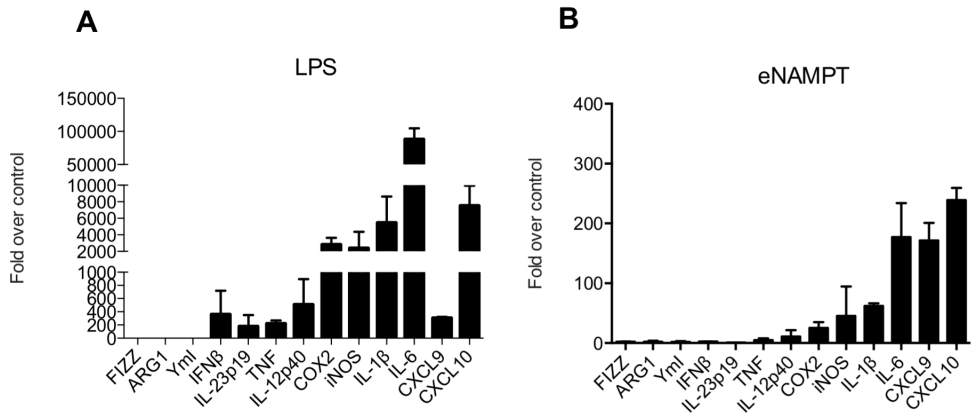

Figure 3. RT-PCR analysis of M1-polaritazion-associated genes (iNOS, COX-2, IL12p40, CXCL9, CXCL10, IFNβ, IL-6, IL-1β, TNF, IL23p19) and M2-polarization-associated genes (FIZZ, ArgI, YmI) in peritoneal exudate cells (PECs) isolated from C57BL6 mice and stimulated for 4h with LPS (100ng/ml) (**A**) and eNAMPT (500ng/ml) (**B**). Representative data of 3 separate experiments.

In conclusion, the experiments conducted in this section are very preliminary and inconclusive. They represent a small part of my PhD work, but I believe that investigating this aspect based on Garcia's finding was relevant and essential for the objective of my thesis. Unfortunately, the time spent for them was insufficient to obtain strong results, but this is still an open project in the laboratory of Armando Genazzani.

Key findings:

- Data on TLR4 and eNAMPT from my work are largely inconclusive;
- We failed to see an activation by eNAMPT of TLR4 in MD-2 deficient cells or a strong activation in Jurkat cells, which express MD-2;
- We observed a moderate and late increase in p65-phosphorylation in MD-2 expressing cells;
- Similarities exist between LPS and eNAMPT in the pattern of up-regulation of genes in peritoneal macrophages, but results are not superimposable.

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Chapter 5

Investigation of a putative receptor for extracellular Nicotinamide Phosphoribosyltransferase (eNAMPT) using fluorescent probes

INTRODUCTION

The identity of the eNAMPT receptor is still unknown although there is sufficient evidence that suggests that a plasma membrane receptor exists and mediates at least part of the effects of eNAMPT. We have demonstrated that eNAMPT binds to CCR5 acting as natural antagonist, but this interaction is unlikely to explain many of the effects observed with eNAMPT. To identify other putative eNAMPT receptors, we therefore decided to use a different experimental approach.

In 1970 Lefkowitz RJ *et al.* first described an innovative assay to study and characterize receptor-ligand interactions using radiolabelled proteins[1]. In subsequent years, radioligand binding assays were widely used to discover new receptors and/or investigate new ligands. Given that this technique is perceived as costly, risky and dangerous to the users, has an environmental impact and requires a custom infrastructure, researchers have been encouraged to develop ligand-binding assays not based on radioactivity, but rather based on fluorescent-labelled ligands. One of the first fluorescent-ligand binding assays was developed in 1988 by Yamasaki K *et al.* They have taken advantage from fluorescence probe techniques to discover the IL-6 receptor. In their strategy, they coupled FITC to the IL-6 protein and evaluated binding by using FACS analysis on COS7 cells transfected with different cDNAs. They observed that FITC-IL6 was able to bind on the cell surface of COS7 transfected with pBSF2R.236 cDNA plasmid, identifying the coding sequence of the IL-6

receptor. In addition, they demonstrated that cells that lacked mRNA for the sequence they identified (*e.g.* Jurkat cells) were unable to bind FITC-IL6[2].
In the last years, different types of fluorophores have been developed with different spectra of excitation and emission to improve the emission intensity and to reduce the quenching phenomena and the effect of temperature, pH and ionic strength on fluorescent emission. For example, the Alexa Fluor® 488 dye, which has the same excitation and emission spectra of fluorescein, is brighter and more photostable than fluorescein. In addition, the intensity fluorescence of the Alexa Fluor® 488 dye is insensitive to pH between 4 and 10 as opposed to fluorescein. We decided to develop a fluorescent eNAMPT labelled with the Alexa Fluor® 488 dye to investigate new interactors.

MATERIALS AND METHODS

Cell culture

B16 (murine melanoma) and 4t1 (murine mammary carcinoma) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS), 2 mg/ml glutamine, 10 U/ml penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO, USA). Cells were maintained in a humidified incubator supplied with 5% CO_2/95% air at 37°C.

Recombinant eNAMPT preparation

Wild-type murine full-length NAMPT (ORF GenBank BC018358) was cloned in pET28a (NdeI/EcoRI). Recombinant eNAMPT was expressed in E. Coli (ClearColi, BL21 DE3) (inducted with IPTG 0.5 mM for 3 h at 20°C) and purified by His-tag affinity chromatography with NiNTA Superlow resin (Qiagen).

eNAMPT 488 preparation

eNAMPT was labelled with Alexa Fluor®488 Protein Labelling Kit (Molecular Probes) according to manufacture manual. Briefly, 1 mg of eNAMPT was incubated with Alexa Fluor®488 for 1 hour at room temperature and free dye was removed by column resin. The labelled eNAMPT-488 was collected and the concentration and the degree of labelling were controlled using a spectrophotometer.

Purification of membrane fraction from tissues of mice

Heart, lung, brain, kidney, liver, adipose tissue, spleen and bone marrow obtained from C57BL/6 mice were weighed and cut with scalpel. Five volumes of homogenizer buffer (20 mM Hepes pH 7.2, 0,5 mM EGTA, 0.32 M sucrose, 1 mM β-mercaptoethanol) were added and tissues were lysate in a potter with 30 strokes and centrifuged at 600g 10 min at 4°C to remove debris and intact cells. The supernatants were collected and centrifuged at 10000g for 10 min at 4°C, to remove the nuclei. The supernatants were collected and subsequently centrifuged at 40000g for 45min at 4°C to obtained membrane fractions. Membranes were washed twice and resuspended in homogenizer buffer.

Competitive binding assay

Spectrophotometer analysis: labelled eNAMPT-488 (5 µg) with or without unlabelled eNAMPT (500 µg) was added to 50 or 100 µg of membrane extracts in binding buffer (137 mM NaCl, 2.7 mM KCL, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.2) and the mixture was incubated for 1h at 4°C on a wheel. Then, membranes were washed 3 times with binding buffer and centrifuged at 20000g 15min 4°C. The pellet was then solubilized in binding buffer and transferred in a black plate. Fluorescence was acquired using a Viktor3 plate-reader.

SDS-page analysis: labelled eNAMPT-488 (5 µg) with or without unlabelled eNAMPT (500 µg) was added to tissue membranes in binding buffer and the

mixture was incubated for 1h at 4°C on a wheel. Then, the membranes were washed 3 times and centrifuged at 20000g for 15min at 4°C in binding buffer. Pellets were then resuspended in 50 µL of binding buffer and loaded on SDS-page gels. Images were acquired using Chemidoc Biorad with the Alexa Fluor 488 program.

Pull down with NI-NTA beads and cross-linking

Recombinant NAMPT (50 µg) was incubated with 100 µL of NI-NTA beads for 15 minutes at 4°C. Then, beads were washed 3 times in wash buffer (50 mM NaH_2PO_4 pH 7.5, 0.5 M NaCl, 40 mM imidazole) and added to kidney or liver membranes (700 µg) for 1h at 4°C. Subsequently, beads were washed 3 times in wash buffer, and 0.01% of glutaraldehyde was added for 45 min at 4°C. The reaction was quenched with Tris-HCl (1M, pH 8) and the samples were loaded on SDS-page. Proteins were analysed via western blot and/or silver stain. The gel bands were analysed by Liquid chromatography–mass spectrometry (LC-MS) in collaboration with the Laboratory of Prof. Emilio Marengo, University of Piemonte Orientale.

Western blot analysis

Membranes were lysed in 80 µL in lysis buffer composed of 20 mM HEPES, 100 mM NaCl, 5 mM EDTA, 1% SDS + Protease & Phosphatase Inhibitor Cocktail (Sigma). Protein quantification was performed with BCA Protein Assay (Thermo Fisher Scientific, Waltham, MA, USA), and proteins were resolved on SDS–PAGE. Densitometry analysis was performed with Quantity One program (Bio-Rad, Hemel Hempstead, UK).

FACS analysis

3×10^5 cells were trypsinised, wash 3 times in cold PBS, and suspended in PBS. eNAMPT-488, at different concentrations, was added to cells and incubated at 4°C for the indicated time points. Cells were washed 3 times in

ice cold PBS and resuspended in FACS Buffer (Hanks' Balanced Salt solution HBSS + 0.5% BSA). Samples were analysed using a FACS (BD accuri).

Immunofluorescence

2×10^4 B16 cells were seeded on a 13mm coverslip. Cells were washed in PBS and incubated with 10 µg of eNAMPT-488 in Locke's solution (134 mM NaCl, 5mM KCl, 4 mM $NaHCO_3$, 10 mM HEPES, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Dextrose, pH 7.4) for 1 hour at 4°C. Then, cells were cross-linked with 2mM of BS^3 (Bissulfosuccinimidyl suberate) for 2h at 4°C, and after quenching with Tris-HCl (1M, pH 7.4), cells were washed with PBS. Subsequently cells were fixed with PFA 4% for 15 minutes at 4°C and stained with DRAQ5 (2.5 µM) for 20 minutes. Then, cells were washed in PBS and mounted onto cover slips to be visualized with confocal microscopy (Leica).

Reagents

Mouse monoclonal antibody anti-NAMPT was purchased from AdipoGen. Mouse monoclonal antibody anti-HSP90 and Mouse monoclonal antibody anti-CoxIV were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-H2A was purchased from Abcam. Rabbit polyclonal anti-E-cadherin and was purchased from cell signalling. Peroxidase-conjugated secondary antibodies were from Biorad. Glutaraldehyde from Sigma, NI-NTA beads were purchased from Quiagen. Bissulfosuccinimidyl suberate (BS^3) and Pierce™ Silver Stain for Mass Spectrometry were purchased from Thermo Fisher Scientific. Glutaraldehyde was purchased from sigma. DRAQ5 was purchased from Invitrogen™ Life technology.

Results

Starting from the idea of Yamasaki K et al., we decided to generate a fluorescent eNAMPT with Alexa Fluor 488 (eNAMPT-488) to evaluate the ability of eNAMPT-488 to bind on cell surface. We started from the murine melanoma B16 and mammary carcinoma 4T1 cell lines, in which we previously demonstrated that the administration of recombinant eNAMPT induced an activation of phospho- ERK1/2[5] and phospho-STAT-3 (not shown). As shown in Figure 1A-B, FACS analysis reveals that the incubation of eNAMPT-488 (30ng, 500ng and 1 μg) on B16 cells was able to induce a shift in positive FL-1 area (indicative of 488 intensity) in a dose-dependent manner. The percentage of B16-positive cells was in a range between 5-20%, while in 4T1 cells the percentage was between 2-5%. Moreover, in 4T1 cells the binding of eNAMPT occurs only with very high amounts of eNAMPT-488 (1μg), suggesting that the expression of the putative receptor in these cells is lower than in B16 cells. Anyway, the strong binding of eNAMPT-488 observed on B16 cell surface convinced us to investigate more deeply these findings. Therefore, we evaluated the localization and the binding intensity of eNAMPT-488 via confocal microscopy.

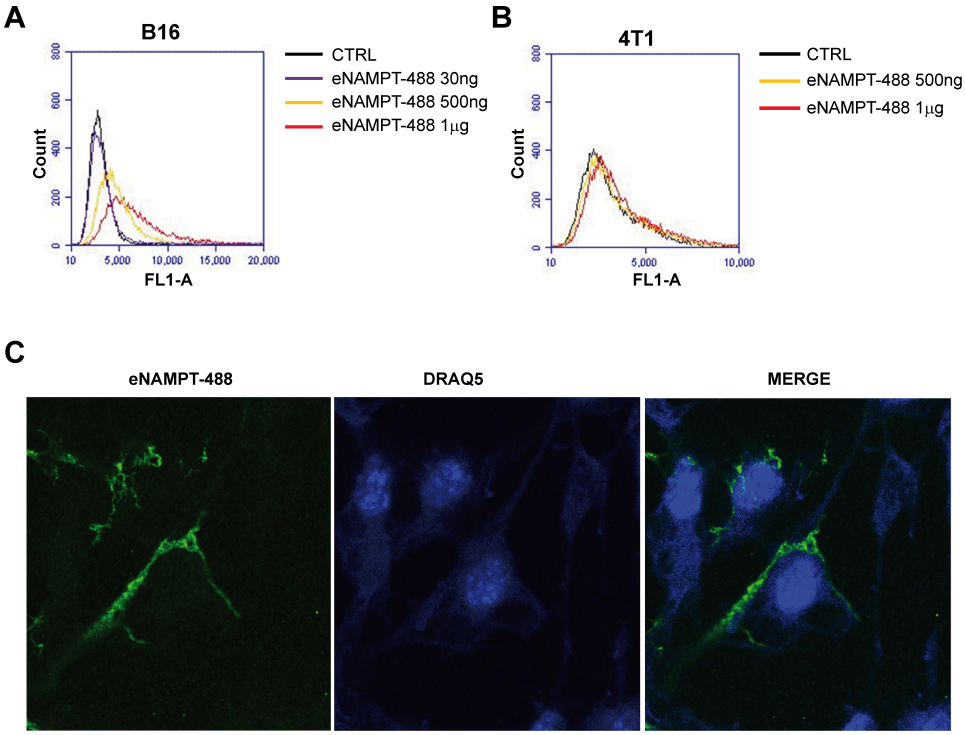

Figure 1. eNAMPT-488 bind to the cell surface of B16 and 4T1 cells. Representative flow cytometry analysis of B16 cells (**A**) and 4T1 cells (**B**) incubated with eNAMPT-488 (30ng, 500ng, 1 μg) for 60 minutes and analysed using FACS BD accuri. (**C**) Confocal images of B16 cells treated with eNAMPT-488 (10 μg, green) for 60 minutes, stained with DRAQ5 (nuclear marker, blue). Representative images of 3 determinations of 3 separate experimental days.

B16 cells were treated with 10 μg of eNAMPT-488 and crossed-linked with BS^3, in order to avoid a possible dissociation of the protein-receptor binding during the different steps of the protocol. Specifically, the BS^3 cross-linker is membrane-impermeable, helping the study of cell surface interactions, and has a space arm of 11.4 angstrom, that reduces the unspecific cross-linking. The cells were then fixed and analysed by confocal microscopy. As shown in Figure 1C, eNAMPT-488 was linked to the cell surface membrane of B16 cells, confirming the data obtained by flow cytometry.

These data confirm our hypothesis that eNAMPT is able to bind a putative receptor and that the receptor is present in melanoma cells.

To better characterize these evidences and to improve the probabilities to obtain a functional competitive binding assay by increasing the percentage of

positive cells linked to eNAMPT-488, we moved to membranes obtained from whole tissues.

We generated membrane fractions from heart, lung, brain, kidney, liver, adipose tissue, spleen and bone marrow of C57BL/6 mice. To confirm the purity of the membranes in the isolation process, we performed a western blot analysis of membrane fractions for E-cadherin and Cox-IV (membrane markers), for HSP90 (cytosolic marker) and H2A (nuclear marker) (Figure 2).

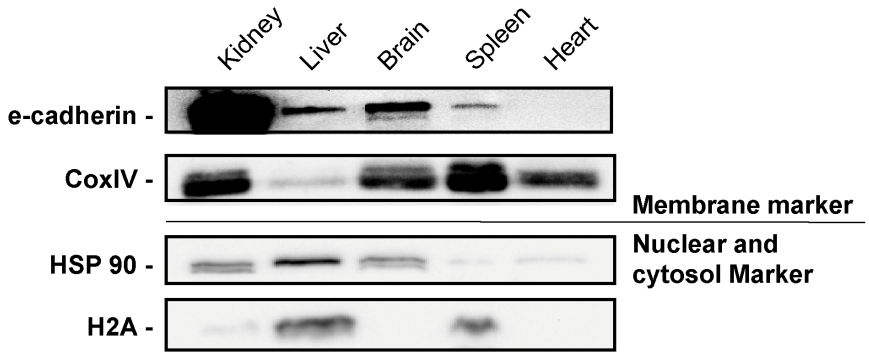

Figure 2. Isolation of tissue membranes. Western blot analysis of HSP90, H2A, E-cadherin and CoxIV levels in different tissues. Representative data of tissue membrane preparation.

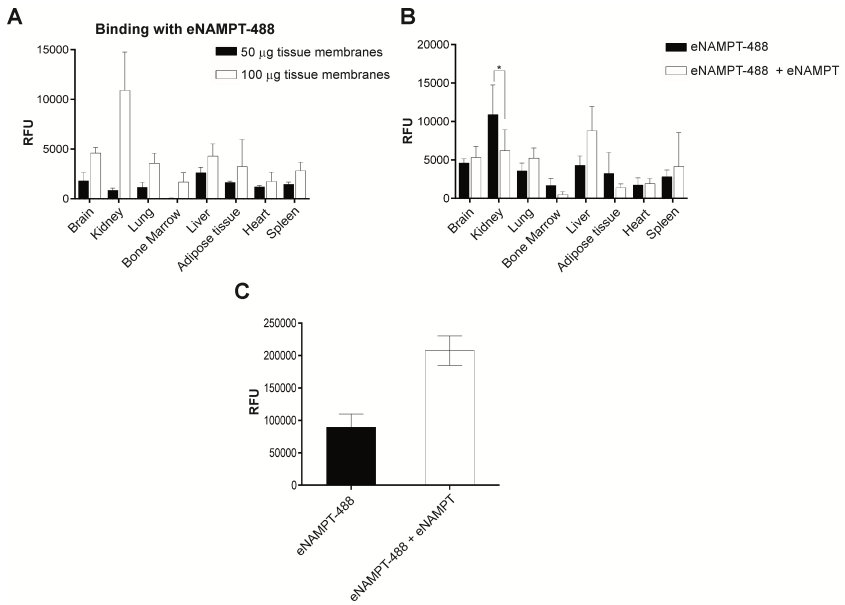

Figure 3. eNAMPT-488 binds to tissue membranes. (A) Tissue membranes (50, 100 µg) were incubated with eNAMPT-488 (5 µg) in binding buffer for 1 hour at 4°C. Then, the fluorescence was acquired using Victor3. Histograms of relative fluorescent intensity as mean ± S.E.M. of 5-6 independent experiments. (**B**) Tissue membranes (100 µg) were incubated with eNAMPT-488 (5 µg) in presence or absence of unlabelled eNAMPT (500 µg) in binding buffer for 1 hour at 4°C. Histograms of relative fluorescent intensity as mean ± S.E.M. of 5-7 independent experiments. (**C**) Histograms represent the fluorescence intensity of eNAMPT-488 (5 µg) with or without eNAMPT (500 µg) after 1 hour at 4°C of incubation in absence of tissue membranes. Histograms of relative fluorescent intensity as mean ± S.E.M. of 5 independent experiments.

To evaluate whether labelled eNAMPT-488 was able to bind to tissue membranes, we performed a fluorescent binding assay in the presence or absence of an excess amount of unlabelled eNAMPT, and the fluorescent intensity was acquired using Victor3 spectrophotometer.

As shown in Figure 3A, eNAMPT-488 (5 µg) was able to bind to all tissues tested. In the competitive binding assay, in which unlabelled eNAMPT (500 µg) was added together with labelled eNAMPT (5 µg), the binding of eNAMPT-488 was inhibited significantly only in kidney (Figure 3B). Unfortunately, in some tissues we observed an increase of fluorescent intensity when labelled and unlabelled eNAMPT were incubated together (*e.g.* liver). Overall, while most of the data suggested that the binding of eNAMPT on

membranes occurred, the inability of unlabelled eNAMPT to displace the fluorescence raised doubts on the phenomenon.

Thus, we proceeded in verifying whether an interaction occurred between labelled and unlabelled eNAMPT in the absence of membranes. To our surprise, we observed that the fluorescence intensity was significantly higher in the presence of unlabelled eNAMPT (Figure 3C). Moreover, we performed a repeated acquisition of fluorescence intensity at different time points and the fluorescence intensity changed over time, resulting in a high variability (not shown). The above data were drawback, as they suggest that standard displacement protocols, usually employed in binding assay, could not be performed. The reason is unclear and might be attributed to a change in protein folding as well as to the formation of labelled-unlabelled dimers. In either case, fluorescence cannot be reputed linear with membrane binding, a pre-requisite for quantitative approaches.

We therefore decided to change the conditions of the binding experiments, moving to a static system, *i.e.* SDS-Page gels, in order to avoid possible dynamic conformational changes or protein-protein interactions that might compromise fluorescence linearity. We decided to start this novel approach analysing the binding in two organs that, in the preliminary experiments yielded the highest signal, kidney and liver. Briefly, eNAMPT-488 with or without the unlabelled eNAMPT was incubated with membrane fractions and samples were then loaded into a SDS-page gel to resolve the proteins. The fluorescence of the bands on the gel was then analysed via the Chemidoc equipment.

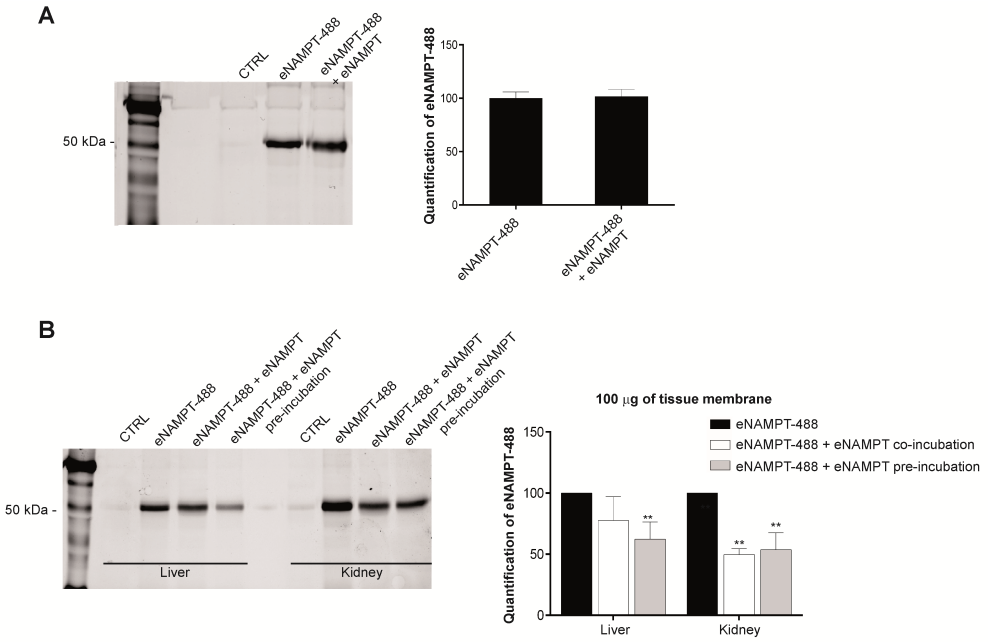

Figure 4. Competitive binding assay. (A) eNAMPT-488 (500 ng) was incubated in presence or absence of unlabelled eNAMPT (50 µg) for 1 hour at 4°C. Then samples were resolved in SDS-page and fluorescence intensity was acquired using Chemidoc (Bio-Rad) with Alexa fluor 488 program. Representative SDS-page gel (left panel) and histogram (right panel) of eNAMPT-488 fluorescence intensity. (**B**) Liver and kidney tissue membranes (100 µg) were pre-incubated or co-incubated with unlabelled eNAMPT (500 µg) in presence or absence of eNAMPT-488 (5 µg) in binding buffer for 1 hour at 4°C. Representative SDS-page gel (left panel) and histogram (right panel) of eNAMPT-488 fluorescence intensity. (5 independent experiments) **P < 0.01, ***P<0.001 versus control.

As shown in Figure 4A, a fluorescent band corresponding to the correct molecular weight of eNAMPT was evident in this procedure. Pre-incubation with an excess of unlabelled eNAMPT resulted in both tissues in a reduction of the fluorescence intensity, which was significant in both cases as determined by densitometry. Both co- and pre- incubation resulted in a reduction of the displacement, as would be expected (Figure 4B).

These new evidences convinced us of the existence of a competitive binding for eNAMPT in kidney and liver membranes, which could be translated in a concrete possibility of the presence of an eNAMPT receptor in these preparations. We next decided to identify its nature through the pull-down of the complex and subsequent analysis of LC-MS.

Towards this aim, we took advantage of the histidine tag of recombinant eNAMPT, which is specifically recognized by NI-NTA beads. Briefly, eNAMPT was incubated with NI-NTA beads, added to tissue membranes and cross-linked with glutaraldehyde. Samples were then loaded in SDS-page gel and developed with antibody against NAMPT. Subsequently, the bands of interest were analysed by LC-MS.

As shown in Figure 5A, we were able to recognise the monomeric and dimeric forms of cross-linked eNAMPT alone at expected molecular weights (50 kDa for the monomer and 110 kDa for the dimer). When eNAMPT was cross-linked with membranes, we observed the appearance of bands with molecular weight higher than 110 kDa (Figure 5A, band B). In contrast to the above bands, we are unable to determine whether these bands contain the receptor or whether sufficient amounts are present to be detectable by LC-MS.

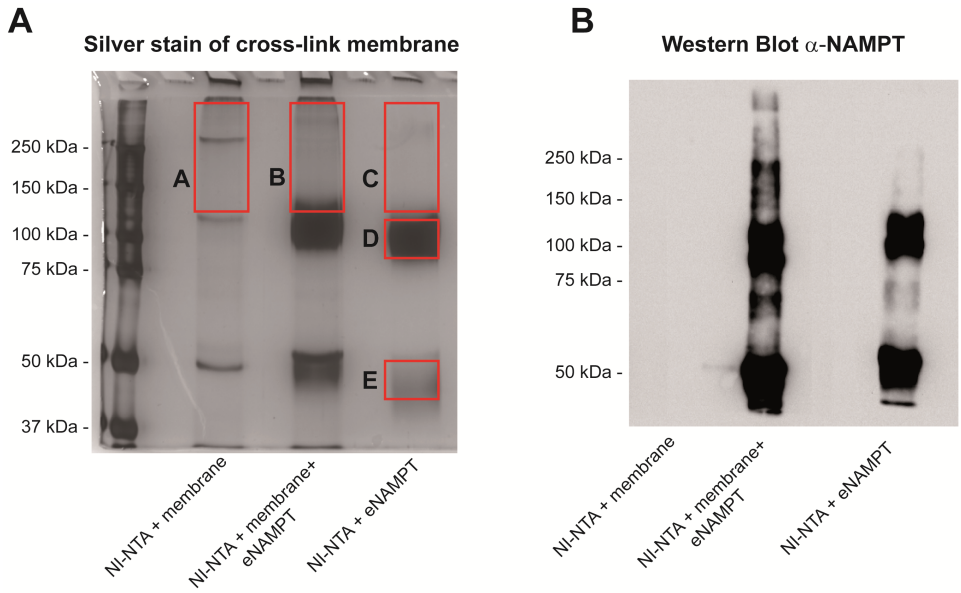

Figure 5. Pull down assay of eNAMPT interactors from membranes. eNAMPT (50 µg) was incubated with NI-NTA beads in presence or absence of liver and kidney tissue membranes (700 µg) in binding buffer for 1 hour at 4°C. Then samples were cross-link and the proteins were resolved by SDS-Page. Representative silver stain (**A**) and western blot (**B**) of eNAMPT alone and in presence or absence of membranes.

We performed LC-MS analysis in collaboration with Dr. Marcello Manfredi (University of Piemonte Orientale, Alessandria) of the bands named A, B, C, D and E in Figure 5. D and E bands were analysed as controls to verify the presence of eNAMPT in the samples. In band B, LC-MS identified two interested proteins such as glyceraldehyde-3-phosphate dehydrogenase, sodium/potassium-transporting ATPase subunit alpha 1 and 3 and obviously eNAMPT (Table 1). Table 1 shows the proteins identified by this technique. It must be noted that LC-MS also revealed in band B cytosolic and nuclear proteins, such histones, nuclear receptor coactivator 2, calmodulin and actin, suggesting that the membrane preparations were contaminated with cytosolic and nuclear proteins.

Band B			
protein description	**prot_score**	**prot_mass**	**N° peptide**
10853|H2B1F_MOUSE Histone H2B	411	13928	8
Q6GSS7|H2A2A_MOUSE Histone H2A	168	14087	2
Q99KQ4|NAMPT MOUSE	121	55698	3
P16858|Glyceraldehyde-3-phosphate dehydrogenase	55	36072	3
Q6PIC6|Na+/k+ -transporting ATPase subunit alpha-3	41	113045	2
Q8VDN2|Na+/k+ -transporting ATPase subunit alpha-1	24	114221	2
Q61026|Nuclear receptor coactivator 2	22	159221	1
Q99P72-1|Isoform 3 of Reticulon-4	21	22452	1
Band C			
protein description	**prot_score**	**prot_mass**	**N° peptide**
P36369|K1B26_MOUSE Kallikrein 1-related peptidase	142	224807	3
Q3TD16|K226L_MOUSE Uncharacterized protein	68	1246366	6
Q3UH68-2|LIMC1_MOUSE Isoform 2 of LIM	14	101706	3
Band D			
protein description	**prot_score**	**prot_mass**	**N° peptide**
sp|Q99KQ4|NAMPT_MOUSE	229	55698	2
Q61846|MELK_MOUSE Maternal embryonic	29	73709	1
Q3UV17|K22O_MOUSE Keratin, type II cytoskeletal	27	63319	1
Band E			
protein description	**prot_score**	**prot_mass**	**N° peptide**
sp|Q99KQ4|NAMPT_MOUSE	76	55698	2
sp|Q8BJH1-2|ZC21A_MOUSE	40	31891	1
sp|Q61846|MELK_MOUSE	27	73709	1

Table 1. eNAMPT interactors identified through LC-MS analysis

We also performed a further analysis with StavroX program, which is specifically programmed for Cross Linking-Mass Spectrometry Analysis. Indeed, cross-linking creates an analysis bias by which cross-linked proteins would not be recognized by expected molecular weights. The analysis with StavroX of band B, revealed that eNAMPT was in complex with glyceraldehyde-3-phosphate dehydrogenase, sodium/potassium-transporting ATPase subunit alpha 1 and 3 and with the same eNAMPT with a significance <0.05 compared to the internal control of decoy analysis. The program also suggested a putative sequence of interaction of eNAMPT with glyceraldehyde-3-phosphate dehydrogenase and sodium/potassium-transporting ATPase (Table 2).

Analisys with StavroX				
Score	Peptide 1	Protein 1	Peptide2	Protein 2
105	[LNKYLK]	Q99KQ4|NAMPT_MOUSE	[TGVFTTMEKAGAHLK]	P16858|G3P_MOUSE
105	[RKNBLVK]	ATPase subunit alpha-1 and alpha-3	[NKILIFGL]	ATPase subunit alpha-1 and alpha-3
102	[SSKIM]	ATPase subunit alpha-1 andalpha-3	[VLDILGKKF]	Q99KQ4|NAMPT_MOUSE
102	[ILLQGK]	ATPase subunit alpha-3	[VLDILGK]	Q99KQ4|NAMPT_MOUSE
102	[GKVVT]	Q99KQ4|NAMPT_MOUSE	[TVLKVLDILGK]	Q99KQ4|NAMPT_MOUSE
102	[NBSFK]	Q99KQ4|NAMPT_MOUSE	[GIALIKK]	Q99KQ4|NAMPT_MOUSE
101	[VLDILGK]	Q99KQ4|NAMPT_MOUSE	[QPRNPKT]	ATPase subunit alpha-1
100	[YLKGKV]	Q99KQ4|NAMPT_MOUSE	[LRMYPLK]	ATPase subunit alpha-1 and alpha-3
100	[SILLHGK]	ATPase subunit alpha-1	[AKRMARK]	ATPase subunit alpha-1 and alpha-3
99	[KNGKVTK]	Q99KQ4|NAMPT_MOUSE	[TVKAENGKLVINGK]	P16858|G3P_MOUSE
99	[KEVAM]	ATPase subunit alpha-3	[FNSTNK]	ATPase subunit alpha-1 and alpha-3
97	[SGTSFDK]	ATPase subunit alpha-3	[SEHGDKKGKK]	ATPase subunit alpha-1
97	[FEBREK]	Q99KQ4|NAMPT_MOUSE	[ASAHLVNFK]	Q99KQ4|NAMPT_MOUSE
97	[NGEKmSIN]	ATPase subunit alpha-1	[LMRERNK]	ATPase subunit alpha-3
96	[KTENS]	Q99KQ4|NAMPT_MOUSE	[VKFBR]	ATPase subunit alpha-1 and alpha-3
96	[ADPNK]	Q99KQ4|NAMPT_MOUSE	[YDNSLKIVSNAS]	P16858|G3P_MOUSE
96	[YYGTK]	Q99KQ4|NAMPT_MOUSE	[YDNSLKIVSNA]	P16858|G3P_MOUSE
96	[KYYGT]	Q99KQ4|NAMPT_MOUSE	[YDNSLKIVSNA]	P16858|G3P_MOUSE

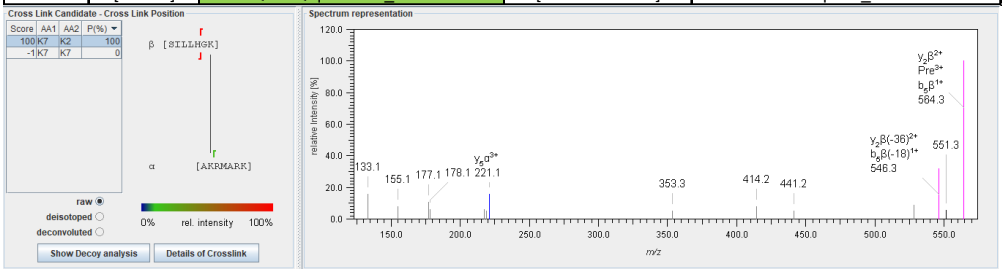

Table 2. Peptides corresponding to sequence of interactions identified using StavroX tool.

Key finding:

- The fluorescence binding assays is an efficient technique to track membrane proteins that bind to eNAMPT;
- While artefacts may occur when determining binding in cells via spectrophotometer, SDS-page appears to be effective. This technique must therefore be useful if a protein purification strategy is sought to identify the eNAMPT receptor or to confirm the identity of the receptor;
- Cross-linked fluorescent eNAMPT coupled with LC-MS allows for the identification of a number of eNAMPT binding proteins, some of which have been validated via other means (see discussion).

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Chapter 6

Discussion

In 1966, Dietrich *et al.* reported for the first time the enzymatic activity of nicotinamide phosphoribosyltransferase (NAMPT), which catalyses the formation of nicotinamide mononucleotide (NMN) starting from nicotinamide (NAM), 5-phosphoribosylpyrophosphate (PRPP) and adenosine triphosphate (ATP)[1]. Nowadays, NAMPT is considered an important regulatory enzyme since it is the rate-limiting enzyme in the nicotinamide adenine dinucleotide (NAD) salvage-pathway in mammals[2].

In 1994, Samal *et al*. identified NAMPT as a secreted protein that enhanced the proliferation and differentiation of lymphocytes B[3]. From that time on, the extracellular form of NAMPT (eNAMPT) was classified as a *cytokine*, a factor released from cells with paracrine and autocrine effects. Large amount of data have demonstrated that eNAMPT has the ability to activate intracellular pathways such as NF-κB, Akt, p38 and MAPK, and leads to the control of angiogenesis, cellular proliferation, cell differentiation and migration[4-6].

To date, the information available in the literature reveal that the extracellular form of NAMPT has the same sequence and structure of the intracellular form, maintaining its ability to dimerize[7]. This insinuates the doubt if its function in the extracellular space is due to its ability to form NMN or to the binding to a plasma membrane receptor. Some evidences, even if not fully convincing, on the identity of eNAMPT receptor are present in literature. After the retraction by Fukuhara of the identification of the insulin receptor as a candidate[8, 9], other groups started to tackle the issue. In 2012, Van den Bergh *et al.* demonstrated that eNAMPT binds to CCR5 through the technique of Surface Plasmon Resonance[10], however they did not characterize the biological role of this interaction.

I decided to start from this evidence to develop my PhD project: discover of the receptor of eNAMPT. I started from the validation of Van den Bergh's evidence at first by verifying the interaction between eNAMPT and CCR5. The

choice to concentrate on CCR5 was mainly due to the fact that the report on TLR4 had not been released at the time.

Initially, I generated an experimental model by over-expressing murine-CCR5 in HeLa cells in which perform most of the experiments. Through a competitive binding assay in which the pre-treatment of the cells with recombinant eNAMPT led to a reduction of the binding of the natural ligand RANTES to CCR5 receptors, I confirmed effectively an interaction between NAMPT and CCR5. Then, I moved to characterize the biological role of this interaction. It is known that CCR5 triggers the activation and consequent phosphorylation of ERK and the efflux of calcium from endoplasmic reticulum. However, in our hands, eNAMPT, tested at different concentrations, was not able to induce neither ERK activation nor calcium efflux in HeLa CCR5 cells, suggesting that eNAMPT was not behaving as an agonist on this receptor. Surprisingly, the pre-incubation with high doses of recombinant eNAMPT was able to inhibit the RANTES-mediated calcium transients in HeLa-CCR5 cells.

Coming back on Van den Bergh's report, they showed that NAMPT was able, similar to the CCR5 antagonist maraviroc, to reduce HIV R5 strand (which use CCR5 as a co-receptor) infection in macrophages, on the contrary it was not able to reduce HIV X4 strand infection (which use CXCR4 as a co-receptor)[10]. Indeed, I started to think that eNAMPT might be considered an antagonist of this receptor, binding to it to interfere with the RANTES stimulus.

Maraviroc is a well-known competitive antagonist of CCR5. This molecule has antiretroviral properties and it is also classified as an entry inhibitor and used in the treatment of HIV positive patients[11-14].

I then decided to compare the effects of maraviroc to eNAMPT on CCR5-mediated phenomena. Effectively, the pre-treatment of eNAMPT was able to reduce RANTES-mediated calcium signalling in the same manner of maraviroc. In support of this, eNAMPT was not able to internalize the receptor, phenomenon that occurs only for CCR5 agonists[12].

This antagonistic activity seemed to be specific for RANTES-mediated CCR5 activation. In fact, ATP-induced calcium transients and carbachol-induced calcium transients were not affected by eNAMPT treatment, demonstrating that it was not a general action on calcium stores, but a specific activity on CCR5.

It is well known that RANTES treatment induces migration and chemotaxis in a calcium-dependent manner. Cell migration requires local calcium pulses to activate myosin and to modulate focal adhesions. In this context, our data demonstrated, once again, that eNAMPT was interfering with CCR5 by reducing RANTES-mediated migration in the same manner of maraviroc. We performed these experiments on B16 melanoma cells as HeLa cells have a reduced tendency to migrate. To corroborate these data, we confirmed the antagonistic effects of eNAMPT on RANTES-mediated calcium signalling even in B16 cells.

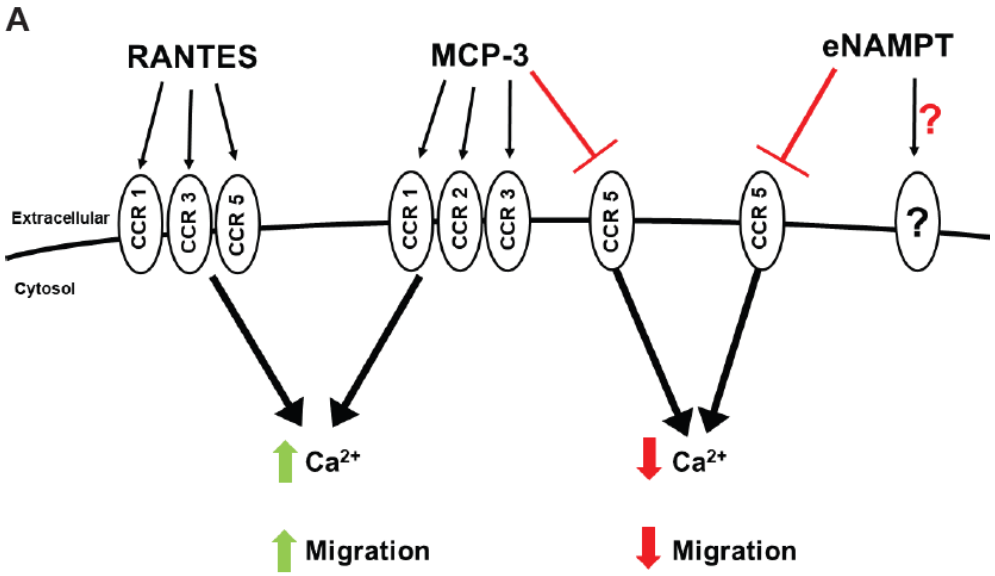
All together these evidences demonstrate that eNAMPT can act as an antagonist against CCR5.

A concrete example of this antagonistic effect on CCR5 signalling is the chemokine monocyte chemotactic protein-3 (MCP-3), also named CCL7. MCP-3 is the unique endogenous antagonist proposed for CCR5[15]. It abolishes CCR5-dependent calcium signalling and cell migration, while at lower doses it is an agonist of CCR1, CCR2 and CCR3[16]. Indeed, it has been reported that the chemokines MCP-1, MCP-2, MCP-3 and RANTES induced a migration response with bimodal concentration dependence, with different efficacy and curve trend[17].

Therefore, my hypothesis is that eNAMPT might have the same behaviour of MCP-3 protein: it may be an antagonist of CCR5 receptor at a certain dose and in a specific type of cell, while it may activate other pathways at similar or different concentrations or in a different context (Figure 1A).

In support of my hypothesis, I performed a preliminary *in silico* analysis using PyMol and Uniprot software, which revealed a common structure conformation and conserved sequencing between eNAMPT and MCP-3.

As shown in Figure 1B, the Pymol analysis of the NAMPT (cyano) and MCP-3 (orange) structures showed a strong homology. Specifically, MCP-3 random coil (highlighted by the red square), which is used by the protein to bind to CCR5, is superimposable with a portion of the NAMPT structure (amino acids 420-430). Moreover, the chemical-physical features of the amino acids of this portion are comparable to the steric hindrance of MCP-3 in the same region.



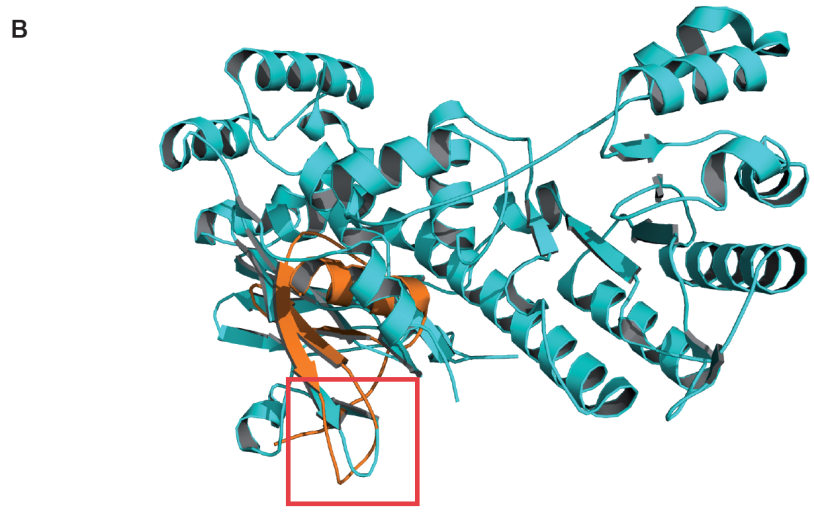


Figure 1. A) Scheme of RANTES, MCP-3 and eNAMPT signalling; **B**) PyMol analysis of alignment of eNAMPT (ciano) and MCP-3 (orange) sequences. Red square indicates the random coil in common between NAMPT and MCP-3, which leads to bind to CCR5.

Thus, our experimental data and our assumption derived from the *in silico* model can support the role eNAMPT as antagonist of CCR5 and its similarity with the natural antagonist MCP-3, both in the structure and in the functions.

During the second year of PhD, an innovative paper was published by Camp *et al*. They reported that eNAMPT binds to and activates Toll-like receptor 4 (TLR4), inducing NF-κB activation in human lung endothelium. Through the antibodies against eNAMPT and TLR4, and the use of TLR4 KO mice, they validated this hypothesis[18]. Of course, I decided to devote part of my experiments to validate their hypothesis also in my models.

Taken together, my results only partially recapitulate the evidence of Camp *et al.* In my hands, we failed to observe an activation of TLR4 in MD-2 deficient cells, maybe suggesting that eNAMPT requires MD-2 for TLR4 activation, contrarily to what affirmed. Yet, eNAMPT seems to bind to TLR4 because it induces a mild and late increase in p65-phosphorylation in MD-2 expressing cells (Jurkat). Moreover, eNAMPT up-regulated four genes (CXCL9,

CXCL10, IL-6, and IL-1β) involved in TLR4 signalling in *ex vivo* peritoneal macrophages.

Therefore, this evidence on TLR4 brings further support to the idea that more than one receptor may be recognized by eNAMPT, yielding therefore a complex pattern. This is further supported by the notion that NF-κB is not the only intracellular pathway activated by eNAMPT from literature[4-6, 19]. Moreover, our results were not able to convince us that eNAMPT actions were fully comparable to those of LPS, even if we recognize that many other experiments should be carried to fully elucidate this issue.

A crucial element regarding the experiments with TLR4 is the absolute necessity to avoid any endotoxin contamination in eNAMPT preparations. We have paid incredible attention to ensure that recombinant proteins (in our case eNAMPT) used for cell treatment and all the reagents and tools used in the experiments were completely endotoxin-free. For example, our eNAMPT were prepared from ClearColi BL21 (DE3) kit, in which the bacterial strain has been modified to express an LPS modified form not recognised by the receptor and the preparations were subjected to the LAL test. Our eNAMPT was comparable to most commercial available recombinant proteins, which puts us in a situation at par with others, but does not give a 100% guarantee of totally LPS-free preparations. Only the use of samples from the TLR4 KO mouse model could inform us without further doubt that the gene expression changes peritoneal exudates can be attributed to the activation of TLR4.

Taken together, the evidences that I obtained regarding CCR5 and TLR4 as eNAMPT receptor candidates, are not fully convincing and conclusive. At present, the working hypothesis is that eNAMPT antagonizes CCR5, mildly activates the TLR4 and that further receptors may be involved in explaining the full spectrum of effects. In this setting, there is room for actions attributable also to its enzymatic activity and to NMN.

In this scenario, I decided to pursue a fishing strategy to identify further receptors. As other researchers have attempted to identify the eNAMPT receptor by using the yeast two-hybrid technique and by screening cDNA libraries without being able to achieve appreciable results, it persuaded us to move in a different direction[4].

I set up and validated a method to identify new membrane interactors of eNAMPT by using fluorescent probes. Our idea arises from the evidences of Yamasaki K *et al.*, which discovered the IL-6 receptor using a fluorescence IL-6 on COS7 transfect cells[20]. We paralleled their experiments by generating a labelled eNAMPT-488 to analyse its binding to the membranes.

Despite initial problems with artefacts in our model, we demonstrated an effective binding of eNAMPT to the membranes of both B16 melanoma and 4T1 mammary carcinoma cells, both with FACS analysis and confocal imaging.

To improve the quality of our results and allow this technique to support future identifications, we required an increased amount of protein coupled with homogeneity of our preparations, and for this we moved to mouse tissues. A competitive binding assay, in which unlabelled NAMPT was used to compete with the binding of eNAMPT-488, revealed that in kidney and liver membranes a partial displacement occurred, indicating an effective binding to a competitive site.

The following step was to pull-down the interactors present in kidney and liver preparations and to identify them through LC-MS analysis. For this, we took advantage from the cross-linking technique, via a BS^3 linker, which allows to fix and stabilize the interaction between proteins and to improve the quality and accuracy of the results.

Our very preliminary data indicate two possible interesting proteins: GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) and Na^+/K^+ ATPase subunit 1 and 3 (Sodium/potassium-transporting ATPase).

While the presence of GAPDH might at first appear to be an artefact, a member of my lab (Ambra Grolla; personal communication) has strong evidence that this interaction occurs and may be relevant both in a physiological and pathological perspective. This provides therefore reassurance on the validation of the method.

On the other hand, despite the fact that GAPDH has been demonstrated to be also at plasma membrane[21], its presence in our preparations may be translated in a partial contamination with the cytosolic fraction and this may mean that further characterizations of the membrane preparations may be required in the future.

Since 1994, when eNAMPT was described for the first time as a cytokine, no one has reported convincing evidence on the identity of its receptor(s)[3]. A possibility to explain the difficulty that has challenged researchers and that has probably been over-looked deals with the large variability of eNAMPT preparations that have been used. Indeed, most results in the literature have been recombinant NAMPT produced in bacteria, which might not be superimposable, for post-translational modifications, to mammalian eNAMPT. Members of my lab are at present tackling this issue (Cristina Travelli, Michele Bianchi, personal communication) and are finding striking differences between eukaryotic and prokaryotic recombinant eNAMPT preparations. Future studies should possibly use recombinant protein obtained from HEK293T or CHO cells, which may be more reliable and relevant. Such a drawback, which somehow may explain part of my negative results, is shared by the entire literature: even Camp *et al*., in the material and methods section of the manuscript reporting the TLR4 interaction, reported "Commercially available recombinant NAMPT/PBEF exhibits batch/lot variability on NF-κB signalling, possibly via post-translational modifications or loss of bioactivity"[18].

In conclusion, the scheme of Figure 2 summarizes all the evidences that I obtained during my PhD on eNAMPT, that has allowed me to define eNAMPT as an antagonist of CCR5 and to hypothesize that this protein may be a promiscuous ligand for multiple receptors.

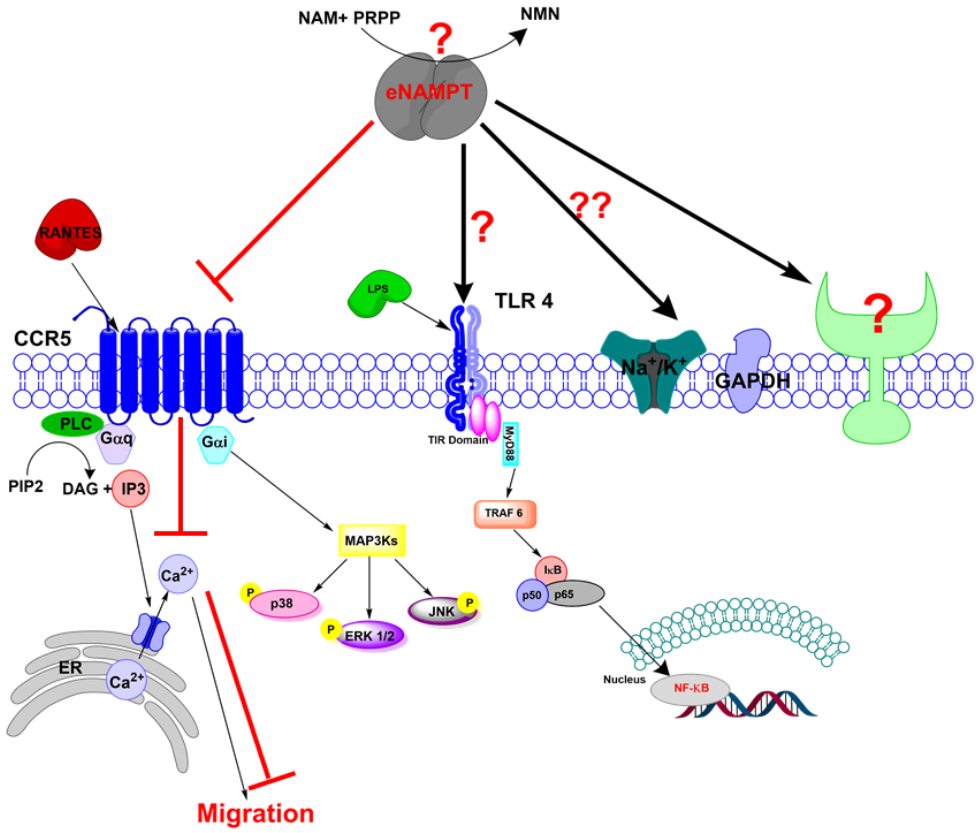


Figure 2. Summarizing scheme of eNAMPT findings

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Chapter 7

List of publications

1. Marset A.; Caprioglio,D.; Torretta S; Appendino GB; Minassi A. Synthesis of colchifulvin, a colchicine-griseofulvin hybrid. TETRAHEDRON LETTERS. Doi: 10.1016/j.tetlet.2016.02.086.

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