Atherosclerosis 243 (2015) 204–210

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Increased expression of NAMPT in PBMC from patients with acute coronary syndrome and in inflammatory M1 macrophages

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ARTICLE INFO

Article history:
Received 26 March 2015
Received in revised form
2 September 2015
Accepted 2 September 2015
Available online 8 September 2015

Keywords:
Macrophage polarization
Atherosclerosis
NAMPT
Acute coronary syndrome
Inflammation

ABSTRACT

Aim: The aim of the present study were to elucidate the role of NAMPT in atherosclerosis, by examine NAMPT expression in peripheral blood mononuclear cells (PBMC) in patients with coronary artery disease (CAD) and healthy controls and by examining the regulation and effect of NAMPT on macrophage polarization, hypothesizing that it could influence the polarization to inflammatory and resolving macrophages.

Method and Results: We analyzed RNA levels of NAMPT in PBMC from CAD and healthy controls and found NAMPT to be increased in PBMC from patients with acute coronary syndrome (n = 39) compared to healthy controls (n = 20) and patients with stable CAD (n = 22). Within the PBMC NAMPT was correlated to several inflammatory cytokines and the antioxidant enzyme superoxide dismutase 2. *In vitro* cell experiments revealed that NAMPT is increased both intracellular and extracellular in inflammatory M1 macrophages compared to in anti-inflammatory M2 macrophages. In addition, inhibiting NAMPT enzymatic activity inhibited M1 polarization in macrophages.

Conclusion: Based on our *in vivo* and *in vitro* findings we suggest that NAMPT could contribute to systemic and plaque inflammation in atherosclerotic disorders at least partly through effect on macrophages.

1. Introduction

Inflammation is an essential response that enables survival during infection or injury, and maintains necessary homeostasis during harmful conditions [1]. However, if this response is not properly regulated, it can result in persistent inflammation and tissue damage such as seen in various autoimmune and autoinflammatory disorders [2]. Studies suggest that macrophages play a key role in controlling and terminating such a state of non-resolving inflammation [3].

Atherosclerosis is characterized by accumulation of lipids and immune cells within the vascular wall, creating an atherosclerotic

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http://dx.doi.org/10.1016/j.atherosclerosis.2015.09.010

lesion that may eventually become prone to plaque rupture [4]. As in other inflammatory conditions, macrophages are of major importance in regulating atherosclerotic-related inflammation, and various sub-types of macrophages have different phenotypes with different effects on initiation, progression and maintenance or resolution of inflammatory status [5]. Both inflammatory and anti-inflammatory or resolving macrophages are present in the atherosclerotic lesion, and the balance between these cellular sub-types is of major importance for the fate of the atherosclerotic plaque [6–9]. Within the lesion, several factors may modulate the functional polarization of macrophages, and the picture is further complicated by the fact that polarized macrophages of the atherosclerotic plaque retain their plasticity and are capable of changing phenotypes upon changes in the microenvironment [10–12]. Thus, while several studies support the importance of macrophage polarization in atherogenesis, the regulation of these processes is not fully understood.

Nicotinamide phosphoribosyl transferase (NAMPT) has been detected in atherosclerotic plaques [13], primarily located to lipid-loaded macrophages. NAMPT has also been found to modulate lipid accumulation and the inflammatory status in these cells [14], but its effect on macrophage function is far from clear. Moreover, while enhanced NAMPT expression has been reported in carotid atherosclerosis [13], whether NAMPT is also regulated within circulating leukocytes, before entering the lesion, is largely unknown.

To further elucidate the role of NAMPT in atherosclerosis we in the present study examined NAMPT expression in peripheral blood mononuclear cells (PBMC) from patients with coronary artery disease (CAD) and healthy controls and its relation to expression of inflammatory markers within the same cell samples. We also examined the regulation and effect of NAMPT on macrophage polarization, hypothesizing that it could influence the polarization to inflammatory (i.e., M1) and resolving (i.e., M2) macrophages.

2. Methods

2.1. Study population and PBMC isolation

Patients with CAD verified by coronary angiography (>50% stenosis) were included in the study. Patients was classified into either stable angina pectoris (SAP) referred to elective coronary angiography (n = 22), unstable angina pectoris (UAP) or non-ST elevation myocardial infarction (MI) (NSTEMI) referred to urgent coronary angiography (n = 19), or patients with ST-elevation MI (STEMI) (n = 20). SAP was defined as episodes of reversible ischemic chest pain with significant CAD. UAP was defined as angina at rest or crescendo angina with a clinical indication of urgent (within 48 h) coronary angiography. NSTEMI was defined according to the universal definition of MI as typical rise and fall of the cardiac specific biomarker Troponin T (TnT) with at least one value above the 99th percentile of the upper reference limit in patients presenting with symptoms of ischemia without any ST elevation of EKG [15]. STEMI was defined by the same rise in TnT and ischemic symptoms together with new ST elevation at the J point in two contiguous leads with the cut-points: ≥0.1 mV in all leads other than leads V2–V3 where the following cut points apply: ≥0.2 mV in men ≥40 years; ≥0.25 mV in men <40 years, or ≥0.15 mV in women. *or* new left bundle-branch block [16]. Clinically unstable patients with cardiac arrest, cardiogenic shock, hypotension or pulmonary congestion were not eligible for inclusion. Patients with active malignant disease, chronic inflammatory disease (eg. inflammatory bowel disease, rheumatic arthritis, systemic lupus erythematosus), intercurrent infection or use of glucocorticosteroids, were also excluded. Age matched controls were recruited from a healthy population based on medical history and clinical evaluation.

The study protocols were approved by the Regional Health Authorities of South-Eastern Norway. The study confirms with the principles outlined in the Declaration of Helsinki for use of human tissue or subjects. Signed informed consent was obtained from all participants.

2.2. Blood sampling and isolation of peripheral blood mononuclear cells (PBMC)

Blood was sampled for isolation of PBMC and for analysis of biomarkers including TnT. Venous blood was drawn immediately before coronary angiography in patients with SAP or UAP/NSTEMI and arterial blood was drawn in STEMI patients from radial or femoral artery cannulation before coronary angiography and percutaneous coronary intervention (PCI) was performed. PBMCs were isolated from heparinized blood from healthy controls and CAD patients by Isopaque-Ficoll (Lymphoprep; Fresenius Kabi Norge AS, Oslo, Norway) gradient centrifugation within 1 h after blood collection and stored in −80 °C as cell pellets before RNA isolation.

2.3. Primary human macrophages

Primary monocytes were obtained from buffy coats (The Blood Bank, Oslo University Hospital, Oslo, Norway). PBMC, isolated as described above, were seeded in cell plates (10×10^6/ml; Sigma–Aldrich) for 2 h before the media was removed and cells were washed. The adherent monocytes were then added RPMI 1640 (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal bovine serum (Gibco), 1% Penicillin-Streptomycin (Sigma–Aldrich) and Macrophage-colony stimulating factor (M-CSF; 50 ng/ml; R&D Systems, Minneapolis, MN) for 7 days. Medium was changed every 3 day. The last 18 h lipopolysaccharide (LPS) from *E. coli* 026:B6 (100 ng/ml, Sigma–Aldrich) and interferon (IFN)γ (20 ng/ml, R&D Systems) for M1 differentiation and interleukin (IL)-4 (20 ng/ml, R&D Systems) for M2 differentiation was added before harvesting.

2.4. Murine bone marrow-derived macrophages

Mice were euthanized by cervical dislocation and bone marrow cells were harvested from mice femur and tibia from C57BL6N mice as described [17,18]. The cells were seeded at 3×10^6/ml in culture medium (1640 RPMI [PAA Laboratories], 1% penicillin, 10% fetal calf-serum [FCS], 0.1% gentamycin [Sigma–Aldrich]) and incubated for one week with M-CSF (10 ng/ml; Pepro Tech, Rocky Hill, NY) for macrophage differentiation. Cells were seeded in 12-well plates (10^6/ml), and stimulated with either IFNγ (4 ng/ml, Pepro Tech) or IL-4 (20 ng/ml Sigma–Aldrich) for polarization to M1 and M2 macrophages, respectively.

All use of animals were approved and registered by the Norwegian Animal Research authority and conform with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

2.5. THP-1 cells

The human monocytic cell line THP-1 (American Type Culture Collection, Rockville, MD) were grown in cell medium (1640 RPMI [PAA Laboratories], 1% penicillin, 10% FCS, 0.1% gentamycin [Sigma–Aldrich]) at 37 °C with 5% CO_2. Before experimental start, the cells were differentiated into macrophages by incubation with phorbol myristate acetate (PMA, 100 nM, Sigma Aldrich) for 6 h. Thereafter, the cells were stimulated with either IFNγ (5 ng/ml, R&D System) and LPS (10 ng/ml, Sigma–Aldrich) or IL-4 (25 ng/ml, R&D System) and IL-13 (25 ng/ml, R&D System) for M1 or M2

polarization, respectively. In a separate set of experiments, the cells were in addition co-stimulated with the NAMPT enzyme inhibitor FK866 (100 nM; Axon MedChem) or recombinant NAMPT (200 ng/ml, Adipogene, San Diego, CA). At different time points, cell-free supernatants and cell pellets were harvested and stored at −80 °C. In all experiments, the vehicle of the stimulus was added as control.

The selection and concentrations of the different stimuli that were used for M1 and M2 polarization were based on optimizing experiments in primary human macrophages, THP-1 macrophages and murine bone marrow-derived macrophages (BMDM). Verification of macrophage polarization was done with gene expression of appropriate marker [19–23], i.e., human M1 macrophages: tumor necrosis factor (TNF) and IL-6, murine M1 macrophages: TNF and inducible nitric oxide synthase (iNOS), human M2 macrophages: CD163 and peroxome proliferator-activated receptor (PPAR) γ and murine M2 macrophages: arginase 1 (Online Supplemental Data Table 1).

2.6. RNA analysis

Isolation of RNA was performed using the RNeasy Mini Kit from Qiagen (Hilden, Germany). RNA was treated with DNase (Ambion, Austin, TX) to make sure no DNA was left to contaminate, and RNA was eluted in 30 μl RNase free H_2O. Isolated RNA was stored at −80 °C until further analysis. cDNA synthesis was carried out using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantification of mRNA was performed using qPCR Master Mix for SYBR Green I (Applied Power Master Mix) and the ABI 9700 (Applied Biosystem) with the accompanying software SDS 2.3. Primers were designed with the use of primer express software version 1 (Applied Biosystems), and primer sequences can be given upon request. Gene expression of the housekeeping gene GADPH was used for normalization.

2.7. Measurements of NAMPT and inflammatory and anti-inflammatory mediators

Supernatants were analyzed using multiplexing bead technology. Detection of IL-1 receptor antagonist (IL-1ra), IL-4, IL-6, IL-10, IL-13, TNF (Bio-Plex pro human cytokine assay, Bio-Rad Laboratories, Hercules, CA) and NAMPT (Visfatin kit from the Human Diabetes panel, Bio-Rad) were analyzed on a Bio-plex suspension array system using Bio-Plex Manager 6.0 (Bio-Rad Laboratories) according to the manufacturer's instructions and according to their respective instruction manuals. Plasma levels of myeloperoxidase (MPO) were measured by enzyme immunoassay obtained from Hycult biotech (Uden, The Netherlands).

2.8. Statistics

When comparing more than two groups of individuals, the Kruskal–Wallis test was used *a priori*. If significant, Man–Whitney *U* test was used to calculate the difference between each pair of groups. In the *in vitro* experiments, t-test and Man-Whitney *U* test were used for univariate comparison as appropriate. Correlations were calculated by the Spearman rank test. P values < 0.05 were considered statistical significant.

3. Results

3.1. NAMPT expression is increased in PBMC from patients with CAD

We have previously found increased NAMPT expression in unstable rupture-prone compared to stable atherosclerotic carotid plaques. Staining of these lesions showed that NAMPT was located to macrophages [13]. To investigate if NAMPT is increased also in circulating hematopoietic cells in atherosclerotic disorders, we measured NAMPT mRNA levels in PBMC isolated from patients with stable angina pectoris (SAP, n = 20), patients with unstable angina pectoris (UAP)/NSTEMI (n = 19) and patients with STEMI (n = 20) as well as healthy controls (n = 20) (Table 1). As shown in Fig. 1, patients with UAP/NSTEMI and STEMI, but not those with SAP, had increased NAMPT mRNA levels in PBMC as compared with healthy controls. For STEMI patients, mRNA levels were also elevated as compared to SAP, but with no difference between unstable angina/NSTEMI and STEMI patients (Fig. 1).

Table 1
Clinical and biochemical characteristics of patients with stable coronary artery disease (CAD), unstable angina pectoris/Non-ST-elevation myocardial infarction (NSTEMI/UAP) or ST-elevation myocardial infarction (STEMI) referred to coronary angiography.

	SAP(N = 22)	NSTEMI/UAP(N = 19)	STEMI(N = 20)
Age (years)	62 (51, 68)	63 (54, 71)	60 (52, 64)
Gender (male)	19 (86%)	14 (74%)	16 (80%)
Hypertension (treated)	14 (78%)	11 (58%)	8 (40%)
Diabetes (treated)	9 (41%)	8 (42%)	3 (15%)
Prior PCI[a]	13 (72%)	6 (32%)	3 (15%)
Prior CABG[b]	5 (28%)	3 (16%)	1 (5%)
Smoking (current)	4 (22%)	4 (22%)	8 (40%)
Aspirin	16 (89%)	19 (100%)	2 (35%)
Clopidogrel	2 (11%)	17 (90%)	2 (10%)
Statin	17 (94%)	15 (79%)	6 (30%)
β-blocker	12 (67%)	15 (79%)	6 (30%)
ACE/ARB[c]	11 (61%)	6 (32%)	5 (25%)
Warfarin	2 (11%)	0	0
Heparin before blood sampling	2 (11%)	15 (79%)	13 (65%)
Height (cm)/weight (kg)	176/84	172/80	178/80
sBP (mmHg)	159 (132, 172)	145 (126, 158)	140 (120, 150)
dBP (mmHg)	90 (77, 97)	81 (70, 90)	80 (80, 92)
BMI (kg/m^2)	27	27	25
Peak Troponin T (ng/L)	10 (10, 12)	80 (46, 631)	3394 (1032, 6804)
S-Creatinine (μmol/L)	75 (64, 84)	70 (61, 99)	70 (63, 92)

Categorical data presented as number of total, n (%). Continuous data are presented as median (interquartile range) unless otherwise noted.
[a] PCI, percutaneous coronary intervention.
[b] CABG, coronary artery bypass grafting.
[c] ACE: angiotensin converting enzyme, ARB: Angiotensin receptor blocker.

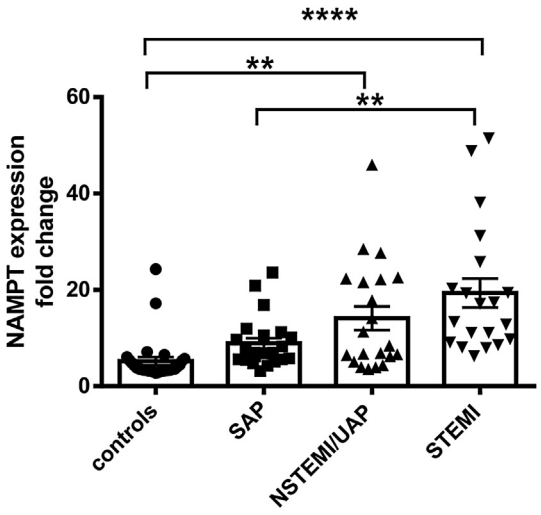

Fig. 1. NAMPT is increased in PBMC from patients with acute coronary syndrome. The Figure shows NAMPT mRNA levels in PBMC from healthy controls (n = 20), patients with stable angina pectoris (SAP, n = 20), patients with unstable angina pectoris (UAP)/NSTEMI (n = 19) and STEMI patients (n = 20). mRNA levels were quantified by qPCR and data are given in relation to the control gene GAPDH. Data are presented as mean and SEM and single values from each individual. **p < 0.005, ****p < 0.0001.

3.2. NAMPT expression correlates with inflammatory markers and redox regulation in PBMC from CAD patients

NAMPT is found to be regulated by inflammatory cytokines [13,14]. Within the CAD group as a whole, NAMPT mRNA levels were correlated with mRNA levels of the inflammatory cytokines IL-6 and TNF as well as the monocyte markers CD14 and CD163 within the same PBMC, although the correlation with TNF did not reach statistical significance (Fig. 2). MPO is an important mediator in atherosclerosis and particularly in plaque destabilization. However, we found no significant correlation between plasma levels of MPO and NAMPT expression within PBMC in the CAD group ($r = 0.19$, $p = 0.12$). Neutrophils are known as important cellular sources of MPO, potentially being the main contributor to plasma levels of MPO. However, both human and murine monocytes express relatively large amounts of MPO [24,25]. We therefore also analyzed correlation between NAMPT and MPO expression in PBMC from the present CAD population However, similar to plasma levels, we found no correlation between mRNA levels of MPO and NAMPT within PBMC from the CAD group ($r = 0.02$, $p = 0.85$). NAMPT could be involved in the regulation of cellular redox status. We therefore finally analyzed the expression of central enzymes involved in redox regulation in PBMC from patients with CAD and examined their relation to NAMPT expression in the same cells. While there was no correlation between NAMPT *and* CuZn superoxide dismutase 1 (SOD1) ($r = -0.18$, $p = 0.15$), mainly found in the cytosol, or catalase ($r = 0.08$, $p = 0.5$), NAMPT expression was strongly correlated with Mn SOD (SOD2) ($r = 0.75$, $p < 0.001$), mainly located within the mitochondria.

3.3. NAMPT expression is increased in inflammatory (M1) macrophages

Macrophages in the atherosclerotic plaque exert great plasticity. The best characterized macrophages in the human atherosclerotic plaque are the inflammatory M1 macrophages and the anti-inflammatory M2 macrophages [26]. To investigate if NAMPT

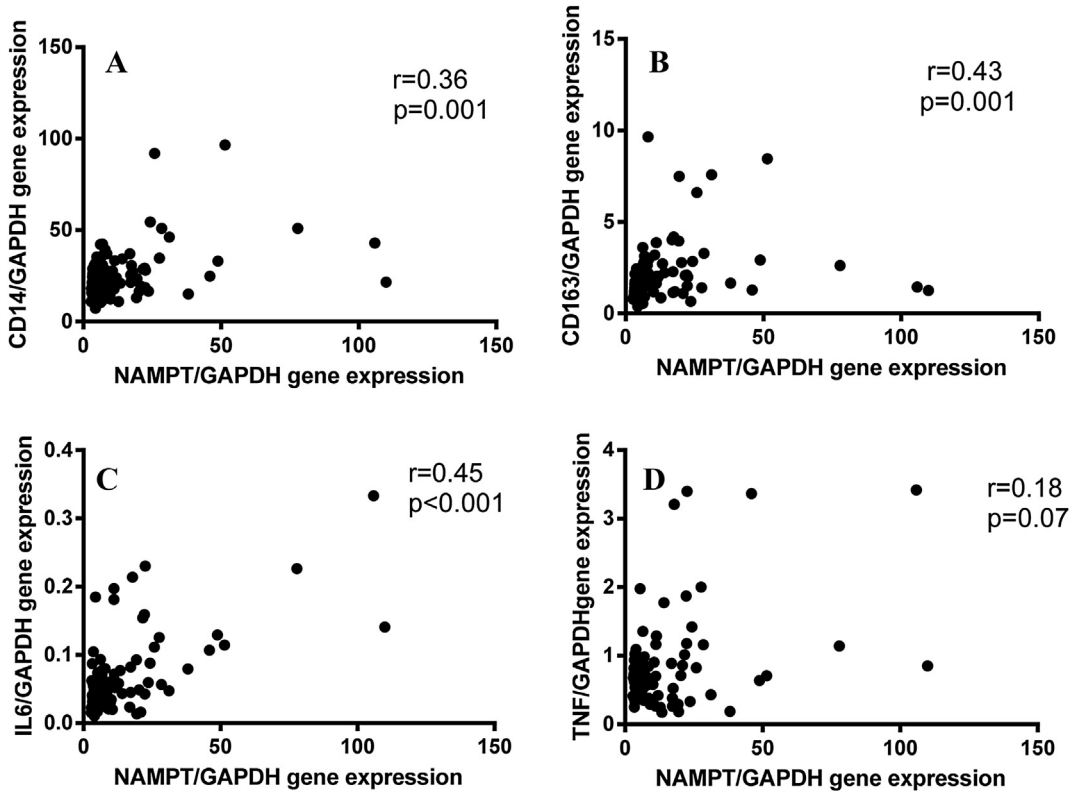

Fig. 2. NAMPT expression correlates with expression of inflammatory markers in PBMC from CAD patients. The panels show correlation between mRNA levels of NAMPT and CD14 (**A**), CD163 (**B**), IL-6 (**C**) and TNF (**D**) within PBMC from same individuals. mRNA levels were quantified by qPCR in PBMC from the whole CAD population (n = 59) and data are given in relation to the referece gene GADPH.

expression is dependent on macrophage polarization we generated M1 (LPS/IFNγ polarized), and M2 (IL-4 polarized) macrophages from primary human monocytes and from BMDM from C57BL/6N mice. NAMPT was highly up-regulated in the inflammatory M1 macrophages compared to non-polarized control cells and M2 macrophages, while NAMPT expression in M2 macrophages was not different from control cells (Fig. 3). The same pattern was seen in both human monocyte-derived macrophages and in BMDM in mice (Fig. 3A–B).

3.4. NAMPT expression increases during macrophage polarization

To study the regulation of NAMPT during macrophage polarization in more detail, we examined the time course of NAMPT expression during THP-1 macrophage polarization. As previously shown, NAMPT expression increases during PMA differentiation from monocytes to macrophages [14], and interestingly, NAMPT expression increased even further during the first 3 h of M1 polarization, and stayed at that level during 24 h polarization (Fig. 4). Concordant with the findings in Fig. 3 NAMPT levels did not markedly change during M2 polarization, with only modest or no increase after PMA macrophage differentiation (Fig. 4). For both M1 and M2 differentiation, the same pattern was seen at the mRNA (Fig. 4A) and the protein level (measurements in cell supernatants, Fig. 4B), demonstrating regulation of both intracellular and extracellular NAMPT during M1 polarization.

3.5. NAMPT inhibition during polarization pushes the macrophage towards a less inflammatory phenotype

Our data show that NAMPT is up-regulated during M1 polarization. To investigate if this is only a secondary phenomenon (i.e., reflect M1 polarization) or if NAMPT itself could modulate macrophage polarization, we polarized THP-1 macrophages towards the inflammatory M1 (LPS/IFNγ) phenotype with and without FK866, a non-competitive inhibitor of intracellular NAMPT enzyme activity. As seen in Fig. 5A, NAMPT inhibition down-regulated both TNF and IL-6 mRNA levels and secretion, with no effects on IL-4, IL-10, IL-1 receptor antagonist (IL-1Ra) and IL-13, representing typical M1 and M2 related cytokines, respectively. In contrast, PPARγ and CD163, representing additional markers of M2 macrophage polarization, was up-regulated during FK866 exposure, although the increase in CD163 did not reach statistical significance (Fig. 5B). In contrast to the effect of enzyme inhibition (i.e., FK866), recombinant extracellular NAMPT and the NAMPT substrate vitamin B3, had no effect on polarization markers (data not shown).

4. Discussion

NAMPT is elevated in symptomatic atherosclerotic carotid plaque [13], and increased serum levels of NAMPT have been associated with recent ischemic cerebral events in patients with carotid atherosclerosis [27]. Our findings in the present study suggest that increased NAMPT expression in atherosclerotic disorders is not restricted to immune cells within the atherosclerotic lesion, but is also found in circulating mononuclear cells. Thus, we found that patients with acute coronary syndrome (ACS) have increased NAMPT expression in PBMC as compared with those with stable CAD and healthy controls, with particularly high levels in STEMI patients, correlated with inflammatory markers and SOD2 expression in these cells. Together with our *in vitro* findings suggesting a link between NAMPT and inflammatory M1 macrophages, these findings suggest that NAMPT could promote an inflammatory phenotype in monocytes/macrophages both systemically and within the lesion, potentially contributing to plaque progression and destabilization. A recent study by Nencioni et al. showing that the NAMPT inhibitor FK866 attenuate plaque inflammation in atherosclerotic mice ($ApoE^{-/-}$) on western diet further support such a notion [28].

Macrophages show great plasticity and the macrophage phenotype ranges from inflammatory to anti-inflammatory, usually referred to as M1 and M2 macrophages, respectively. We have previously found that NAMPT is localized to macrophages in carotid plaques [13], and we and others have shown that NAMPT could promote inflammatory responses in this cell population [13,29]. In the atherosclerotic lesion NAMPT seems to be located in the rupture-prone, macrophage rich areas [13]. These areas have been suggested to be dominated by pro-inflammatory M1 macrophages [22]. In this study we show that NAMPT is markedly up-regulated during M1 polarization as opposed to no or only modest changes during M2 polarization. This was shown in three different model systems, i.e., THP-1 macrophages, primary human monocyte-derived macrophages and murine BMDM. These findings further link NAMPT to an inflammatory macrophage phenotype, potentially being one of several characteristics of these macrophages.

Macrophage polarization is a fairly new field of research. The alternative activation of murine macrophages was first proposed by

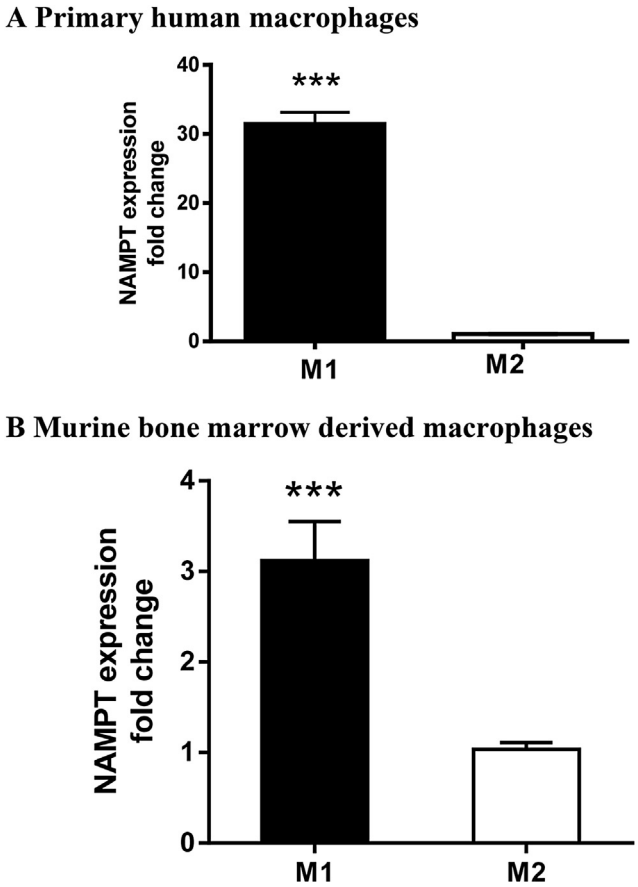

Fig. 3. NAMPT gene expression is increased in inflammatory M1 macrophages. The panels show NAMPT mRNA levels in macrophages derived from human monocytes (buffy coates) (**A**) and in bone-marrow derived macrophages from C57BL/6N mice (**B**). Macrophages were polarized to M1 macrophages by LPS (100 ng/ml) and IFNγ (20 ng/ml), in murine macrophages only IFNγ (4 ng/ml) was used, and to M2 macrophages by IL-4 (20 ng/ml) after culturing for 18 h mRNA levels of NAMPT were analyzed by qPCR in relation to expression of the reference gene GAPDH. The concentrations of the different stimuli were based on preliminary dose–response experiments. Data are given as mean and SEM (n = 3) in relation to NAMPT expression in control cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus M2 macrophages and controls.

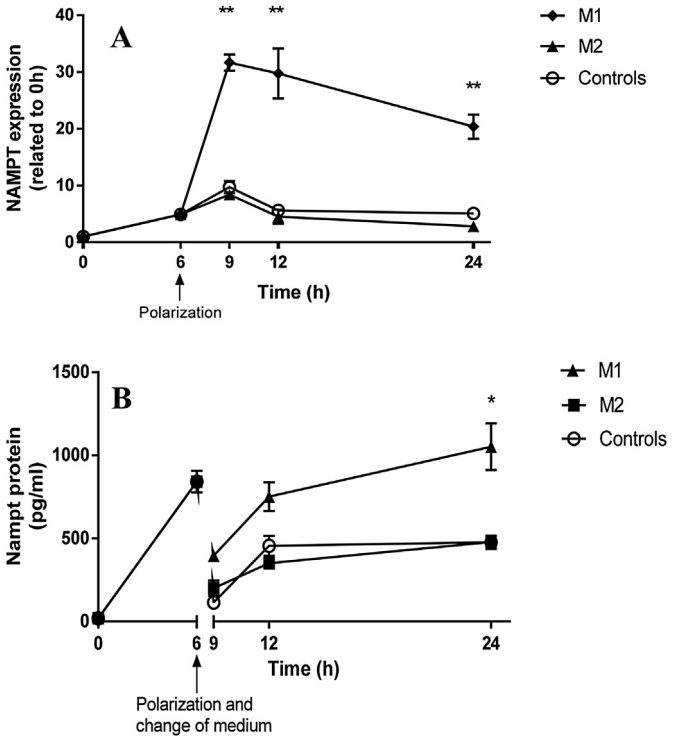

Fig. 4. NAMPT expression and secretion increases during M1 polarization. NAMPT expression was analyzed in PMA differentiated THP-1 macrophages before and during polarization to M1 macrophages by LPS (10 ng/ml) and IFNγ (5 ng/ml) and to M2 macrophages by IL-4 (20 ng/ml) and IL-13 (25 ng/ml). Control cells were not exposed to polarization stimuli. Panel **A** shows NAMPT mRNA levels as assessed by qPCR in relation to the reference gene GAPDH. Panel **B** shows protein levels of NAMPT in supernatants. Note that the cell culture media was exchanged before polarization. Data are given as mean and SEM (n = 3). h, hours. *p < 0.05 and **p < 0.01 versus M2 macrophages and control cells.

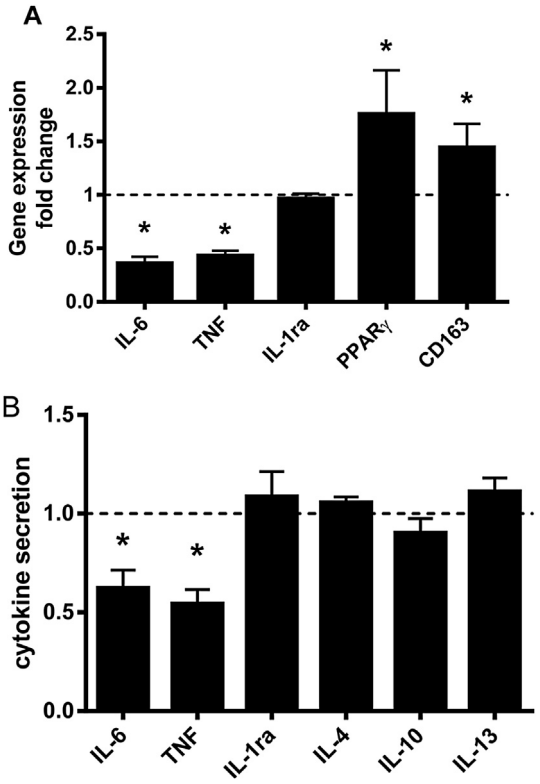

Fig. 5. Inhibition of NAMPT activity during M1 polarization pushes the macrophage towards a less inflammatory phenotype. We polarized THP-1 cells towards the inflammatory M1 phenotype by LPS (10 ng/ml) and IFNγ (5 ng/ml) with and without the NAMPT inhibitor FK866 (1000 nM) and cell pellet and cell supernatants were harvested after 24 h. Panel **A** shows mRNA levels in relation to the control gene GAPDH as assessed by qPCR and panel **B** shows protein levels in cell supernatants. Data are given in relation to levels without FK866 (mean and SEM, n = 3). P < 0.05 versus M1 polarized macrophages without FK866.

Munder et al. in 1998 [19], and the M2 macrophages were described in 2002 [30]. In the present study we show that NAMPT is not only up-regulated during M1 polarization, it seems to push the macropheages in a M1 direction. Thus, we found that inhibition of NAMPT enzymatic activity in macrophages by FK866 down-regulated the inflammatory cytokines IL-6 and TNF, with no effect on the anti-inflammatory mediators IL-1ra, IL-4, IL-10 and IL-13. Moreover, NAMPT inhibition also enhanced the expression of CD163 and PPARγ in these cells, thought to be markers of M2 macrophages. The regulation of macrophages polarization is not completely understood. Our findings suggest that NAMPT enzymatic activity could contribute to this process. Venter et al. have recently showed that FK866 promotes downregulation of metabolic activity in macrophages accompanied by impaired phagocytic and adhesion capacity in these cells [31]. Moreover, intracellular NAD levels have been shown to regulate TNF in a sirtuin-dependent manner in THP-1 macrophages [32]. These findings further link NAMPT enzymatic activity and macrophage-mediated inflammation.

NAMPT exists in both extracellular and intracellular forms, mediating cytokine-like and enzymatic activity, respectively. Intracellular NAMPT is the rate limiting enzyme in the salvage pathway of NAD generation and its mechanisms of action involve regulation of intracellular metabolic activity and interaction with various intracellular pathways such as sirtuins [33]. In contrast, the mechanism of action of extracellular NAMPT is poorly understood. In the present study we show that M1 polarization of macrophages increases both mRNA levels and the release of NAMPT protein into cell supernatants suggesting some common regulation. However, whereas inhibition of NAMPT enzymatic activity attenuated M1 polarization, no effect was seen of extracellular NAMPT. On the other hand, we and others have previously shown inflammatory effect of extracellular NAMPT in macrophages [13,29]. Moreover, Audrito et al. recently reported that extracellular NAMPT promotes M2 polarization in PBMC from patients with chronic lymphocytic leukemia (CLL) [34]. The reasons for these discrepancies are not clear but could involve differences in the NAMPT proteins that are used in these experiments (e.g., degree of glycosylation). Moreover, it is conceivable that cellular activation and metabolic status will affect the influence of NAMPT, and macrophages in patients with CLL will clearly differ from macrophages from healthy controls. Nonetheless, the mechanisms of action of extracellular NAMPT as well as its regulation need to be further clarified.

5. Conclusion

We have previously shown increased NAMPT expression in symptomatic carotid plaques, primarily located to macrophages [13]. Herein we show enhanced NAMPT expression in PBMC from patients with ACS (i.e., UAP/NSTEMI and STEMI) with particulary high levels in STEMI patients. Together with our *in vitro* (NAMPT promotes M1 polarization), these data may suggest that NAMPT could contribute to systemic and plaque inflammation in atherosclerotic disorders at least partly through its effect on macrophages. However, these results do not prove any causal relationship

between NAMPT and atherogenesis. Although anti-inflammatory effect was recently shown through NAMPT inhibition in ApoE$^{-/-}$ mice on western diet [28], further studies on the effect of NAMPT inhibition or overexpression in experimental models of atherosclerosis will be of major importance to prove any causal role of NAMPT in the development and progression of atherosclerotic disorders. Although we previously have found NAMPT to colocalized to lipid loaded macrophages in carotid plaque [13] and to modulate lipid loading [14]. The role of NAMPT and lipid loading are rater complex and this essential process in atherosclerosis has to be studied further.

Sources of funding

This work was supported by grants from the South-Eastern Norway Regional Authority.

Disclosures

None declared.

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2015.09.010.

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