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# Leukocyte overexpression of intracellular NAMPT attenuates atherosclerosis by regulating PPARγ–dependent monocyte differentiation and function

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**Running title**
Intracellular NAMPT in Atherosclerosis

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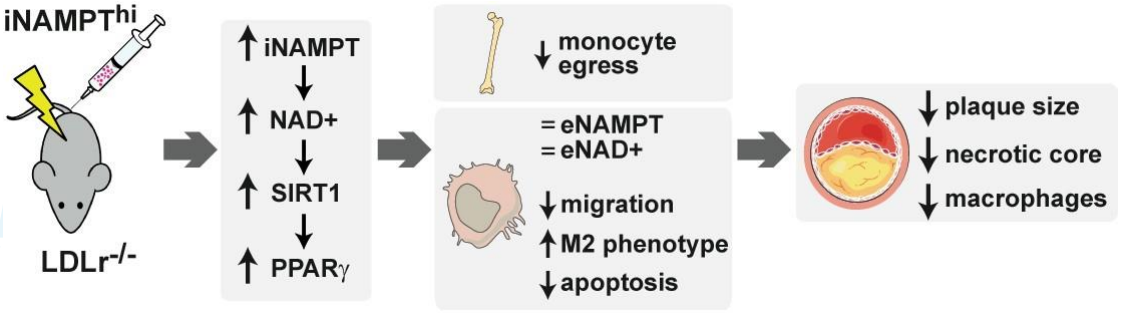
## Summary

**Objective:** Extracellular nicotinamide phosphoribosyltransferase (eNAMPT) mediates inflammatory and potentially pro-atherogenic effects, whereas the role of intracellular NAMPT (iNAMPT), the rate limiting enzyme in the salvage pathway of nicotinamide adenine dinucleotide $(NAD)^+$ generation, in atherogenesis is largely unknown. Here we investigated the effects of iNAMPT overexpression in leukocytes on inflammation and atherosclerosis.

**Approach and Results:** LDL receptor deficient mice with hematopoietic overexpression of human iNAMPT ($iNAMPT^{hi}$), on a western type diet, showed attenuated plaque burden with features of lesion stabilization. This anti-atherogenic effect was caused by improved resistance of macrophages to apoptosis, by attenuated CCR2-dependent monocyte chemotaxis, and by skewing macrophage polarization towards an anti-inflammatory M2 phenotype. The $iNAMPT^{hi}$ phenotype was almost fully reversed by treatment with the NAMPT inhibitor FK866, indicating that iNAMPT catalytic activity is instrumental in the atheroprotection. Importantly, iNAMPT overexpression did not induce any increase in eNAMPT, and eNAMPT had no effect on CCR2 expression and promoted an inflammatory M1 phenotype in macrophages. The iNAMPT-mediated effects at least partly involved sirtuin 1-dependent molecular crosstalk of NAMPT and peroxisome proliferator-activated receptor (PPAR)γ. Finally, iNAMPT and PPARγ showed a strong correlation in human atherosclerotic, but not healthy arteries, hinting to a relevance of iNAMPT/PPARγ pathway also in human carotid atherosclerosis.

**Conclusion:** This study highlights the functional dichotomy of intra- vs extracellular NAMPT, and unveils a critical role for the iNAMPT-PPARγ axis in atherosclerosis.

**Non-standard abbreviation and acronyms**

NAMPT: Nicotinamide phosphoribosyltransferase
$NAD^+$: Nicotinamide adenine dinucleotide
BMDM: Bone marrow derived macrophage
PPARγ: Peroxisome proliferator-activated receptor γ
CCR2: Chemokine (C-C motif) receptor 2
SIRT1: Sirtuin 1
TNF: Tumour necrosis factor
PBMC: Peripheral blood mononuclear cell

## Introduction

Nicotinamide phosphoribosyltransferase (NAMPT) was originally reported as a pre–B cell colony–enhancing factor, secreted by activated lymphocytes in bone marrow stroma[1]. Intracellular NAMPT (iNAMPT) is the rate–limiting enzyme in the salvage pathway of nicotinamide adenine dinucleotide ($NAD^+$) generation[2]. $NAD^+$ acts as coenzyme in oxidative phosphorylation and as critical signalling regulator[3]. Accumulating evidence has highlighted the importance of NAMPT-mediated $NAD^+$ recycling, in concert with $NAD^+$–dependent protein deacetylases (sirtuins [SIRT]), on the expression of peroxisome proliferator–activated receptors (PPARs), nuclear factor kappa B, and endothelial nitric oxide synthase[4, 5], and indirectly on cellular proliferation, differentiation, stress responses, aging, and metabolism[6].

NAMPT also is released by cells to give rise to an extracellular pool of NAMPT (eNAMPT), exerting cytokine-like activity[7]. We have previously shown increased expression of NAMPT within atherosclerotic carotid plaques [8], and we and others have reported that eNAMPT promotes inflammatory and matrix degrading responses[9]. Recently, hepatic NAMPT knockdown was seen to enhance atherosclerosis, at least partly mediated through eNAMPT [10], while concordant with this finding, global systemic inhibition of NAMPT with FK866 dampened plaque inflammation[11]. However, the effects of iNAMPT on atherogenesis are only poorly understood.

Here, we examined the effects of specific up-regulation of human iNAMPT in hematopoietic cells of LDL receptor deficient ($LDLr^{-/-}$) mice on plaque progression with special focus on myeloid cell function and phenotype.

## Materials and Methods

Materials and Methods, including Supplemental Figure I on the time line of the animal experiments, are available in the online-only Data Supplement.

## Results

### Hematopoietic human iNAMPT overexpression attenuates and stabilizes atherosclerotic lesions in $LDLr^{-/-}$ mice

In mice with hematopoietic lentiviral (LV)-iNAMPT overexpression ($iNAMPT^{hi}$ chimeras), neither body weight (Supplemental Figure IIA), total plasma triglyceride (Supplemental Figure IIB) nor cholesterol (Supplemental Figure IIC) levels were influenced during 12 weeks on a western type diet (WTD). At sacrifice, iNAMPT chimeras had similar plasma NAMPT levels as control mice (Figure 1A). However, spleen and bone marrow cells of the $iNAMPT^{hi}$ chimera displayed up-regulation of NAMPT mRNA and protein (Figures 1B-D and Supplemental Figure IIIA) an effect that was accompanied by a 2-fold increase in intracellular $NAD^+$ levels (Figure 1E). A similar increase in iNAMPT expression was also seen 24 hours after LV infection (Supplemental Figures IID-E). Apparently, LV gene transfer led to overexpression of iNAMPT, but not eNAMPT, providing a unique opportunity to specifically study iNAMPT phenotypic effects.

While early lesion development (6 weeks) in the aortic root tended to be reduced in $iNAMPT^{hi}$ and control chimeras (Figure 2A), this effect was more pronounced and reached statistical significance at 12 weeks on WTD (Figure 2B). At 12 weeks, necrotic core size and plaque macrophage content (Figure 2C) were more than 50% decreased in the $iNAMPT^{hi}$ chimera. The proportion of intimal apoptosis as assessed by activated caspase-3 staining was also reduced (Figure 2D). Plaques of $iNAMPT^{hi}$ and control chimeras showed equivalent neutrophil

(Supplemental Figure IVA), T-cell (Supplemental Figure IVB), vascular smooth muscle cell (Supplemental Figure IVC), as well as collagen (Supplemental Figure IVD) contents. To establish the involvement of NAMPT enzyme activity (and exclude off–target effects of the LV intervention) in the observed iNAMPT phenotype, we subjected a subgroup of iNAMPT$^{hi}$ chimeras to NAMPT inhibitor treatment (FK866, 10 mg/kg; twice/week) for 8 weeks. FK866 fully reversed the atheroprotective phenotype of iNAMPT mice, with plaque and necrotic core sizes not differing from those of LV-CTR (Supplemental Figure IVE). Likewise, FK866 treatment increased plaque macrophage (Supplemental Figure IVF) and caspase-3 positive cell (Supplemental Figure IVG) contents of iNAMPT$^{hi}$ mice that were similar to values in control mice. Unfortunately, however, we had no control with FK866 alone.

**Hematopoietic human iNAMPT overexpression alters myeloid stromal egress**

We did not detect any changes in the number of circulatory CD3$^{-}$B220$^{+}$, CD3$^{+}$CD4$^{+}$ or CD3$^{+}$CD8$^{+}$ lymphocytes; of splenic granulocytes or of monocytes in iNAMPT$^{hi}$ vs control chimeras (data not shown). However, bone marrow of iNAMPT$^{hi}$ chimeras were found enriched in CD11b$^{+}$Ly6G$^{+}$ granulocytes (Figure 3A) and CD11b$^{+}$Ly6G$^{-}$ monocytes (Figure 3B). While circulating granulocyte levels were not affected (Figure 3C), monocyte counts were significantly reduced in iNAMPT$^{hi}$ chimeras (Figure 3D). Analysis of bone marrow cells from iNAMPT$^{hi}$ mice demonstrated increased levels of total colony forming units (CFU) compared to cells from the controls (Supplemental Figure VA). This effect could be almost entirely ascribed to an increased frequency of CFU–GM, CFU–G, and CFU–M (Supplemental Figures VB-D), suggesting a myeloproliferative response to hematopoietic iNAMPT overexpression. However, circulating granulocytes were unchanged, whereas monocyte levels were reduced, suggestive of reduced stromal egress (monocytes) or increased stromal re-entry (granulocytes). Circulating (Figure 3E), but not bone marrow granulocytes (Figure 3F) were reduced in CD62L$^{lo}$CXCR4$^{hi}$ cells, representing activated, senescent granulocytes prone to home to bone marrow. This could reflect a diminished rate of neutrophil senescence and/or more effective stromal re-entry of senescent cells. Resident Ly6C$^{lo}$ monocytes appeared to be overrepresented in bone marrow, resulting in lower Ly6C$^{hi}$ and higher Ly6C$^{lo}$ monocyte content (Figure 3G). This effect was paralleled to the predominance of Ly6C$^{lo}$ monocytes in circulation (Figure 3H). Consistent with an inhibitory role of iNAMPT in bone marrow Ly6C$^{hi}$ monocyte mobilization, FK866 treated LDLr$^{-/-}$ mice exhibited reduced stromal bone marrow monocyte content (Supplemental Figure VE) and increased number of circulating monocytes (Supplemental Figure VF).

One of the key regulatory pathways in stromal monocyte egress is the CCR2–CCL2 axis[12]. Gene and protein analysis of CCR2 in bone marrow derived macrophages (BMDMs) from iNAMPT$^{hi}$ vs control chimera revealed a strong down-regulation (Figures 4A-B and Supplemental Figure IIIB). Moreover, while CCL2 mRNA expression remained unchanged (Figure 4C), CCL2 secretion was decreased in iNAMPT$^{hi}$ chimeras (Figure 4D). Remarkably, incubation of BMDMs from iNAMPT$^{hi}$ or control chimeras with eNAMPT did neither affect CCR2 and CCL2 mRNA levels nor CCL2 secretion (Figures 4A-D and Supplemental Figure IIIB). As baseline and fMLP elicited cell motility (Figure 4E) and directional migration (Figure 4F) were both sharply diminished in BMDMs of iNAMPT$^{hi}$ vs control chimeras, it is conceivable that iNAMPT overexpression compromises the general migratory capacity of monocytes. An inverse pattern was seen for BMDMs isolated from FK866 treated mice or treated with eNAMPT, which had augmented motility (Figure 4F). Importantly, overexpressed iNAMPT was not secreted (i.e., no increase in

eNAMPT; Figure 4G). Moreover iNAMPT$^{hi}$, but not eNAMPT, caused increases in intracellular, but not extracellular NAD$^+$ levels (Figure 4H). Our data again confirm that iNAMPT's activity in our model is strictly confined to the intracellular compartment.

**Hematopoietic human iNAMPT overexpression favours alternatively activated macrophage polarization**

In inflammation, Ly6C$^{hi}$ monocytes are recruited to the affected tissue via CCR2, amongst others, to give rise to classically activated M1 macrophages[13]. In iNAMPT$^{hi}$ chimeras, Ly6C$^{lo}$ monocytes were however found to be the predominant subset. We therefore hypothesized that iNAMPT overexpression is accompanied by skewing of macrophage differentiation towards alternatively activated M2 macrophages, known for their wound healing and resolution of inflammation activities[14]. In agreement with this hypothesis, iNAMPT$^{hi}$ BMDMs, polarized with IFNγ and LPS (M1) or IL-4 (M2), showed reduced or increased expression, respectively, of established M1 (tumour necrosis factor [TNF], IL-6, and inducible nitric oxide synthase [iNOS]) and M2 (IL-10, arginase [ARG]1, and to a lesser extent mannose receptor, C-type [MRC]1) markers relative to control BMDMs (Supplemental Figure VIA). In contrast, exposure of BMDMs from control mice to eNAMPT led to upregulated TNF and IL-6 expression at mRNA (Supplemental Figure VIB) and secretion (Supplemental Figure VIC) level, albeit these effects were less pronounced in iNAMPT$^{hi}$ BMDMs. It is worth noting that hematopoietic iNAMPT overexpression did not confer systemic anti-inflammatory effects, as judged by the unaltered plasma cytokine patterns observed in iNAMPT$^{hi}$ compared to control chimeras (Supplemental Figure VID).

**Hematopoietic human iNAMPT overexpression promotes PPARγ activation in a SIRT1 dependent manner**

PPARγ has been proposed as a key regulator of M2 polarization[15]. Interestingly, *in silico* pathway analysis (string-db.org) linked PPARγ to NAMPT (Supplemental Figure VIIA), pointing to a potential involvement of PPARγ in iNAMPT$^{hi}$ associated M2 polarization. In search of the molecular pathway of iNAMPT's anti-inflammatory activity, we assessed whether iNAMPT is able to interact directly with PPARγ or via its immediate effector enzyme, SIRT1, which is reported to regulate PPARγ activity[14]. BMDMs from iNAMPT$^{hi}$ chimeras featured up-regulated expression of PPARγ and its regulator SIRT1 as compared to control (Figures 5A-B and Supplemental Figure IIIC). However, conditional deletion of PPARγ (i.e., LysM–Cre/PPARγ$^{flox/flox}$), even if iNAMPT was overexpressed, or SIRT1 inhibition had no effects on iNAMPT expression or NAD$^+$ levels in BMDMs (data not shown). We further studied the effect of iNAMPT$^{hi}$ on polarization of WT vs PPARγ deficient macrophages. LysM–Cre/PPARγ$^{flox/flox}$ BMDMs infected with control and iNAMPT LV were used to determine whether the polarizing effects of iNAMPT overexpression were mediated via PPARγ. As expected, macrophage deficiency in PPARγ led to a profound increase in M1 marker expression (Figure 5C). Unlike in control BMDMs, however, iNAMPT$^{hi}$ overexpression was unable to alter M1 marker gene expression in LysM–Cre/PPARγ$^{flox/flox}$ BMDMs. Similar findings were obtained after inhibiting SIRT1 (Figure 5C). CCR2 and CCL2 were up-regulated in PPARγ deficient cells, indicating a regulatory link between PPARγ and the CCR2 axis (Figure 5D). Hematopoietic iNAMPT overexpression failed to suppress CCR2 expression in LysM–Cre/PPARγ$^{flox/flox}$ BMDMs, whereas it reverted CCL2 expression to levels observed in control and iNAMPT$^{hi}$ BMDMs from WT mice (Figure 5D). In keeping with the lack of effect on CCR2, iNAMPT$^{hi}$ did not have any effects on LysM–

Cre/PPARγ$^{flox/flox}$ BMDMs migratory capacity (Figure 5E). Unlike iNAMPT, eNAMPT did not impact on PPARγ or SIRT1 expression (data not shown), once again confirming the divergent activity profile of iNAMPT and eNAMPT. Collectively, these data reveal the SIRT1-PPARγ axis as a downstream effector pathway in the iNAMPT overexpression–induced macrophage reprogramming towards an M2 phenotype.

**Hematopoietic human iNAMPT overexpression modulates lipid homeostasis**
The tight interplay between iNAMPT and PPARγ, a master regulator of lipid handling in macrophages[16] and the iNAMPT–associated increase in intracellular levels of $NAD^+$, which may impact lipid metabolism as well[17], hinted towards an effect of iNAMPT overexpression on macrophage lipid handling. In line with the aforementioned iNAMPT–induced up-regulation of PPARγ in BMDMs, LysMCre–PPARγ$^{flox/flox}$ BMDMs showed intrinsically reduced PPARγ mRNA expression, which was not affected by iNAMPT overexpression (Supplemental Figure VIIIA). Of note, iNAMPT augmented oxLDL–induced PPARγ up-regulation in BMDMs. ABCA1 (Supplemental Figure VIIIB) and ABCG1 (Supplemental Figure VIIIC) gene expression patterns essentially mirrored that of PPARγ. In contrast to its profound effect in BMDMs, iNAMPT had no effect on ABCA1, ABCG1, PPARγ or LXRα gene expression in the liver of animals (Supplemental Figures VIIID-G), again underpinning the leukocyte specific nature of the intervention. As a result, cholesterol ester accumulation at baseline and in oxLDL exposed BMDMs of iNAMPT$^{hi}$ chimera was attenuated, as compared to that in control chimera (Supplemental Figure VIIIH). Our findings thus suggest that iNAMPT is involved in the PPARγ–dependent modulation of macrophage lipid homeostasis.

**NAMPT and PPARγ expression are tightly correlated in human atherosclerosis**
Finally, we set out to verify whether the disclosed mutual interaction between NAMPT and PPARγ is also relevant in the context of human atherosclerosis. As an extension of a previous study showing increased expression of NAMPT in carotid lesions of patients with symptomatic disease (i.e., stroke or TIA)[8], we found that these patients also exhibited a profound up-regulation of NAMPT gene expression in PBMCs (Figure 6A). PPARγ and NAMPT expression levels were also assessed in atherosclerotic and iliac artery specimens (Oslo cohort), and in specimens from a carotid artery cohort containing early, advanced, and ruptured carotid plaques (the Maastricht Human Plaque study). Relative NAMPT mRNA (RT–PCR) (Figure 6B) and protein (immunohistochemistry) (Figure 6C) expression both increased progressively from early stable lesions to advanced and ruptured plaques (the Maastricht Human Plaque study). Plaque NAMPT (red) co-localized with the macrophage marker CD68 (green) and virtually no NAMPT staining was observed in CD68 negative cells, but not all CD68 positive cells were positive for NAMPT. This co-localization could be confirmed by confocal microscopy assessment (Oslo cohort, Figure 6D). Moreover, we observed a significant correlation between NAMPT and PPARγ expression in atherosclerotic (Figure 6E) but not healthy (Figure 6F) arteries. This correlation changed with progressive atherosclerotic disease (Figure 6G). Finally, as NAMPT was mainly expressed by plaque macrophages, the increased NAMPT expression in advanced lesions could mirror an enhanced inflammatory nature of ruptured plaques, rather than an intrinsic up-regulation in plaque macrophages. In support of this hypothesis we found that NAMPT and CD68 were strongly correlated within carotid artery plaques (Figure

6H).

## Discussion

eNAMPT and iNAMPT have been suggested to exert divergent functions. In humans, circulating eNAMPT has already been associated with atherosclerosis, both in experimental and epidemiological studies[18]. The causal involvement of iNAMPT in this disease is, however, still unclear. This study is the first to address the role of leukocyte iNAMPT in atherogenesis. Using gain/loss-of-function approaches *in vitro* and *in vivo*, we show that the activity spectrum of iNAMPT differs profoundly from that of eNAMPT, and that LV iNAMPT gene transfer does not change eNAMPT or extracellular $NAD^+$ levels. We demonstrate that hematopoietic iNAMPT overexpression confers protection against atherosclerosis by impacting on three hallmark processes: (1) improving the resistance of macrophages to apoptosis; (2) attenuating monocyte intravasation and migration in response to chemotactic signals; and (3) skewing monocyte differentiation and macrophage polarization towards an anti-inflammatory M2 phenotype. The $iNAMPT^{hi}$ phenotype was almost completely reversed by treatment with the enzyme inhibitor FK866, indicating that iNAMPT catalytic activity is instrumental in the atheroprotection. Interestingly, Li et al. have recently shown that increased hepatic NAMPT expression promotes atherosclerosis, which may seem in conflict with the present study[10]. However, this study differs in several aspects from our study. In Li's study adenoviral overexpression of NAMPT resulted in increased expression by liver parenchymal cells, which are critical in glucose and lipid metabolism, whereas we have investigated NAMPT overexpression in leukocytes. Indeed, enhanced hepatic NAMPT expression will have pronounced effects on glucose metabolism[19] and tolerance, on plasma lipoprotein levels, and on lipogenic key genes (increased PPARα, LXRα, ABCA1, and ABCG1 expressions). In our model, none of these effects were observed, underpinning the leukocyte specificity of our approach. Another major discrepancy is related to the differential functions of iNAMPT vs eNAMPT. Adenoviral gene transfer markedly elevated NAMPT levels in plasma (eNAMPT)[10], whereas our approach only affected iNAMPT expression as judged from (i) unchanged eNAMPT plasma levels *in vivo* in $iNAMPT^{hi}$ chimeras; and (ii) unchanged eNAMPT secretion by $iNAMPT^{hi}$ BMDMs *in vitro.* Importantly, we and others have previously reported that while eNAMPT exerts inflammatory and potentially pro-atherogenic activities[7], the effects of iNAMPT may in fact be opposite. For instance, we show that iNAMPT down-regulates CCR2 expression and suppresses monocyte chemotaxis, whereas exposure of $iNAMPT^{hi}$ BMDMs to eNAMPT was completely ineffective. The functional dichotomy of iNAMPT vs eNAMPT is partly related to the differences in intra- and extracellular $NAD^+$ produced by NAMPT. Intracellular $NAD^+$ will activate sirtuin proteins, which through histone deacetylation lead to e.g., modulation of gene transcription[2]. Extracellular $NAD^+$, on the other hand, can bind and activate extracellular receptors such as P2X7R triggering inflammatory pathways (e.g., NLRP3 inflammasome activation)[20]. Importantly, we show that leukocyte iNAMPT overexpression promotes increased intra- but not extracellular $NAD^+$ levels, ensuing activation of SIRT1 pathways. Again no such effects were seen when exposing $iNAMPT^{hi}$ BMDMs to eNAMPT. The contrasting results of our current study and the inverse, anti-inflammatory phenotype of systemic or cellular NAMPT inhibition by FK866 reported by Nencioni et al.[11] and Halvorsen et al.[21] may also be viewed from this perspective, in that they reflect different functions of iNAMPT and eNAMPT. While FK866 inhibits both iNAMPT and eNAMPT, Yano et al. have recently shown that FK866 also inhibit the release of

eNAMPT from monocytes[22], potentially leading to eNAMPT–skewed inhibition. In our present study, however, there was a selective up-regulation of iNAMPT and, accordingly, the effect of FK688 in this setting will only reflect iNAMPT inhibition. Finally, we cannot exclude that effects of NAMPT inhibition [FK866[11, 21] and siRNA[10]] are not completely reciprocal to that of NAMPT overexpression (present study).

Taken together, the seemingly paradoxical results of our study compared to that of previous work on systemic or hepatic inhibition could be attributed to the diverging activities of iNAMPT and eNAMPT, as well as the diverging roles of NAMPT in hepatocytes versus leukocytes. Our approach thus provides unique insights into the anti-inflammatory activity of leukocyte iNAMPT expression and in that sense our study adds to a better understanding and specification of NAMPTs complex functions in atherosclerosis (see Graphical Abstract).

Another striking finding in the present study was that plaques of iNAMPT$^{hi}$ chimeras displayed reduced macrophage accumulation. We propose two major pathways for this phenomenon. First, iNAMPT overexpression in hematopoietic cells was associated with a reduced abundance of Ly6C$^{hi}$ monocytes in circulation, which are less prone to home to atherosclerotic plaque[13]. The reduction was not due to diminished stromal Ly6C$^{hi}$ production, as we even observed augmented myelocyte CFU levels in bone marrow of iNAMPT$^{hi}$ chimeras, pointing to increased myeloproliferation. As circulating monocyte numbers, in particular of the Ly6C$^{hi}$ subset, were lowered in iNAMPT$^{hi}$ mice, this could reflect compromised Ly6C$^{hi}$ monocyte egress the traffic and their recruitment to plaque. In fact, we demonstrate reduced CCR2 expression and CCL2 chemotaxis in iNAMPT$^{hi}$ macrophages, a proper function of which is critical for monocyte intravasation[13, 23]. We were able to attribute these chemotactic effects to iNAMPT dependent activation of the PPARγ pathway. The latter is known to down-regulate CCR2 expression[24], and our findings suggest that down-regulation of CCR2 expression is linked to SIRT1–dependent PPARγ activation. Indeed, both SIRT1 and PPARγ inhibition counteracted the lowered CCR2 expression and activity in iNAMPT$^{hi}$ BMDMs. The potential molecular crosstalk of NAMPT and PPARγ and the capacity of iNAMPT derived $NAD^+$ to activate SIRT1[25], all concur with an involvement of the iNAMPT/$NAD^+$ salvage pathway on SIRT1–dependent PPARγ activity, and appears to be of major importance in mediating the atheroprotective effects of iNAMPT in our model (see Graphical abstract).

In conclusion, this study is the first to demonstrate a protective role for leukocyte iNAMPT in western type diet induced atherosclerosis in LDLr$^{-/-}$ mice, with reduced macrophage accumulation and augmented lesion stability as most prominent features. iNAMPT was shown to compromise extravasation and mobilization of monocytes out of the bone marrow, via its downstream effector PPARγ, leading to reduced accumulation of macrophages in plaque. Moreover, it led to reduced macrophage apoptosis, possibly in a PPARγ/SIRT1–dependent fashion, and induced an anti-inflammatory gene program in macrophages (see Graphical Abstract). Finally our study suggests that the increased NAMPT expression in human atherosclerotic plaques could involve a counteracting and beneficial response. Further data (e.g., prognostic data on NAMPT expression within human carotid plaques), are, however, needed to prove this hypothesis.

## Supplemental material

Supplemental material is available at ATVB.

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## Author Contribution
BB and EB designed the study. BB, TBD, IM, VH, and JO were involved in animal experiments. BB, MG, MR, SMP, AOG, RA, and LV carried out the animal *in vitro* studies. BB and IM executed the animal tissue processing and immunohistochemical analyses. LN supplied LysM–Cre$^{flox}$ bone marrow. TBD, SH, TR, BH, and PA performed human *in vitro* assays and human immunohistochemistry. BB, EB, TBD, BH, FJGM, and PA analysed and interpreted the data. BB, EB, AY, PA, TBD, and FJGM wrote the paper. All authors discussed the results and implications and commented on the manuscript at all stages.

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## Conflict of interest
None declared.

## Disclosure
Authors have nothing to disclose.

## Highlights
- Hematopoietic iNAMPT overexpression attenuates and stabilizes diet–induced atherosclerosis
- iNAMPT mediates sequestration of pro-inflammatory monocytes in bone marrow
- Macrophages are skewed toward alternative polarization by iNAMPT via PPARγ
- iNAMPT overexpression did not induce eNAMPT, and eNAMPT had no effect on CCR2 expression and promoted an inflammatory M1 phenotype in macrophages
- Gene NAMPT and PPARγ expression tightly correlate with progression of atherosclerotic lesions in humans

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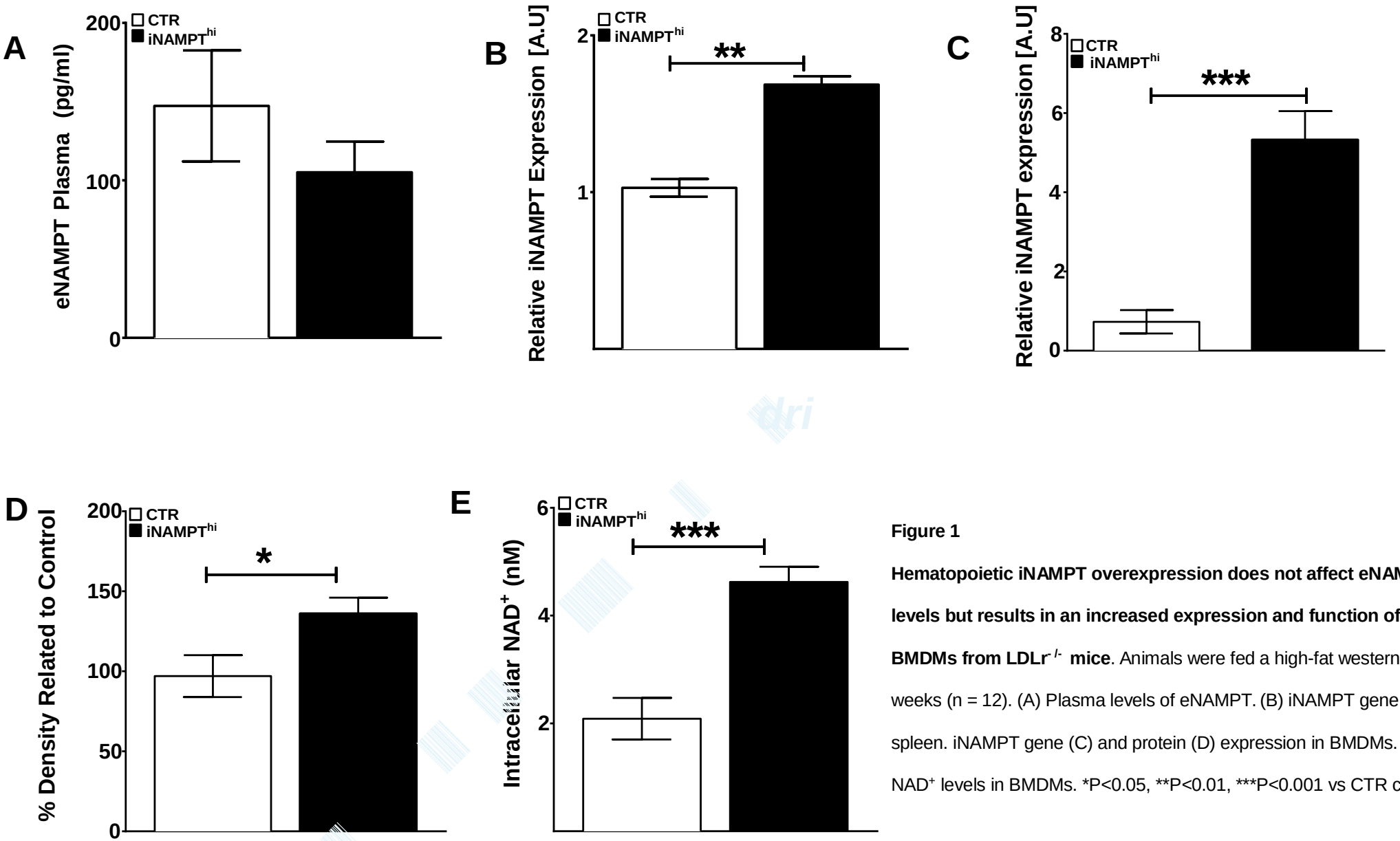


**Figure 1**

**Hematopoietic iNAMPT overexpression does not affect eNAMPT plasma levels but results in an increased expression and function of iNAMPT in BMDMs from LDLr$^{-/-}$ mice**. Animals were fed a high-fat western type diet for 12 weeks (n = 12). (A) Plasma levels of eNAMPT. (B) iNAMPT gene expression in spleen. iNAMPT gene (C) and protein (D) expression in BMDMs. (E) Intracellular $NAD^+$ levels in BMDMs. *P<0.05, **P<0.01, ***P<0.001 vs CTR chimera.

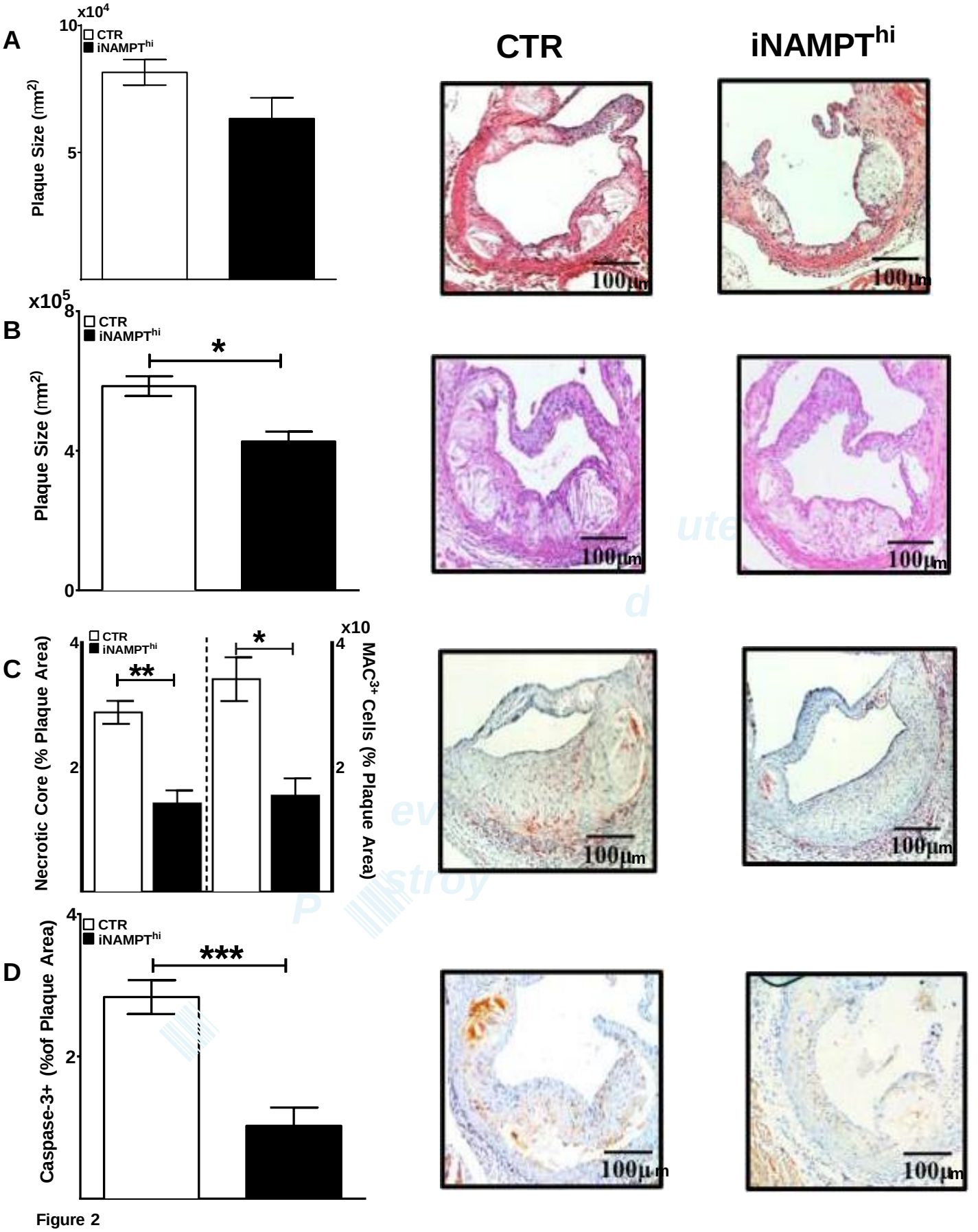


**Figure 2**

**Hematopoietic iNAMPT overexpression attenuates atherosclerotic plaque development in LDLR$^{-/-}$ mice**. (A, B) Haematoxylin-eosin staining of aortic roots with corresponding quantification of plaque size after a high-fat western type diet for 6 (A) or 12 (B) weeks. (C, D) Immunostaining for MAC3 (C) and caspase–3 (D) of aortic roots with corresponding quantification, including necrotic core area relative to intima surface (C) after a high-fat western type diet for 12 weeks (n = 12). *P<0.05, **P<0.01, ***P<0.001 vs CTR chimera.

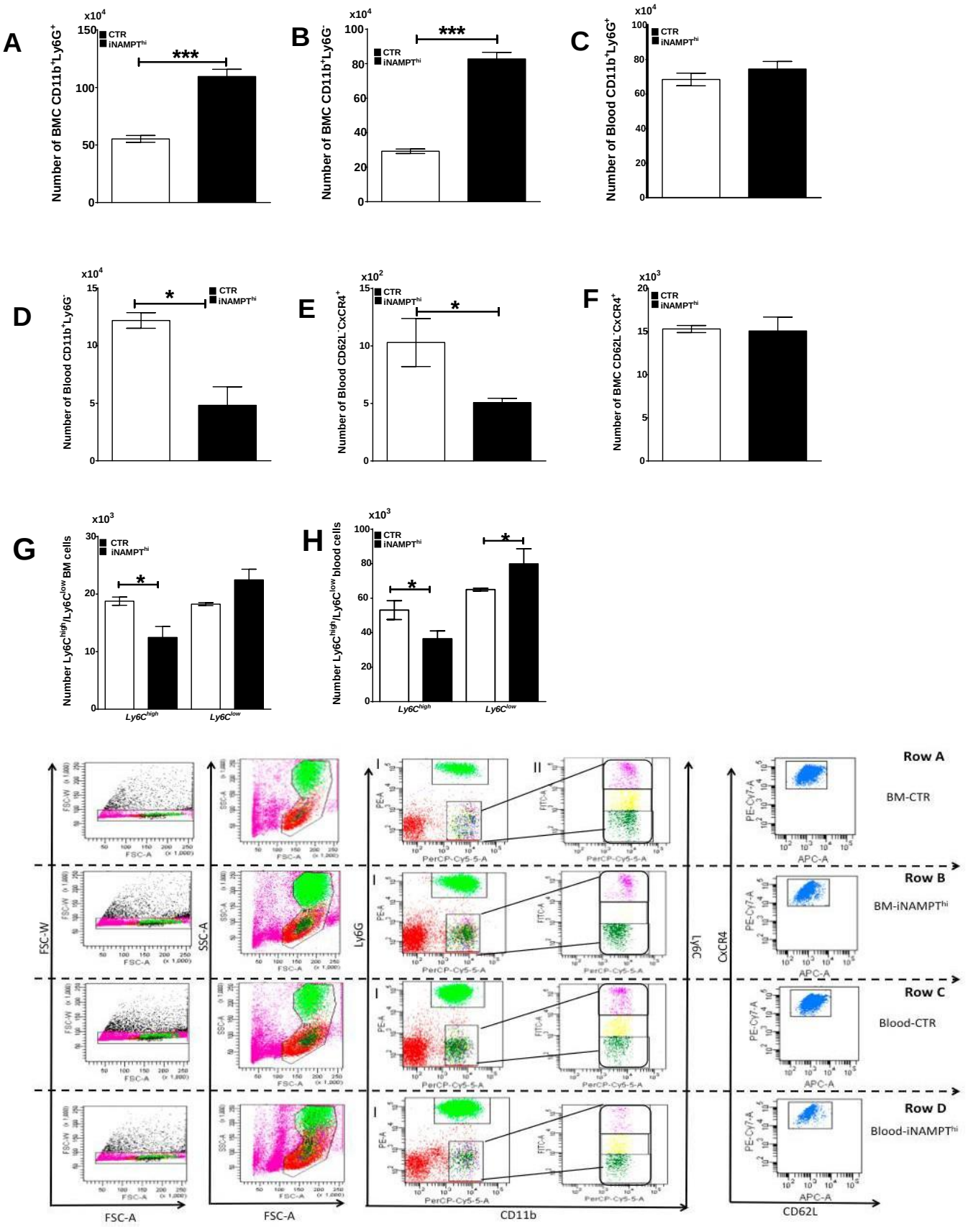


**Figure 3**

**Hematopoietic iNAMPT overexpression perturbs stromal and circulating leukocyte pools in LDLR$^{-/-}$ mic**e. Animals were fed a high-fat western type diet for 12 weeks (n = 12). (A, B) Fluorescence-activate cell sorting (FACS) analysis of absolute granulocyte ($CD11b^+Ly6G^+$) and monocyte ($CD11b^+Ly6G^-$ counts in bone marrow (A, B) and blood (C, D). Proportions of (E, F) activated, senescent granulocytes ($CD62L^{lo}CXCR4^{hi}$) and of (G, H) $Ly6C^{hi}$–to–$Ly6C^{lo}$ subsets in bone marrow (G) and blood (H). Gating strategies are demonstrated in the lower panels *P<0.05 vs CTR chimera.

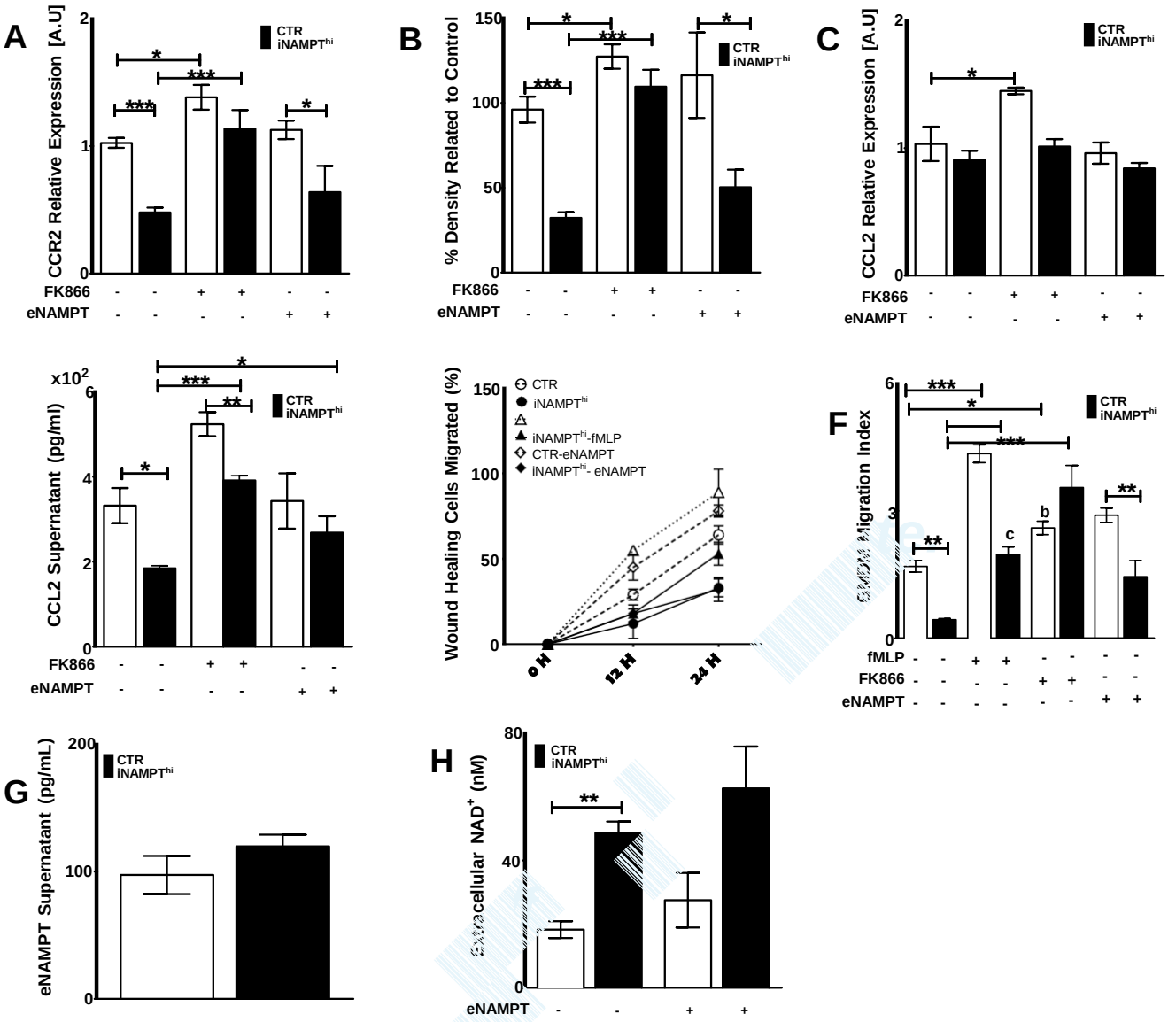


**Figure 4**

**Hematopoietic iNAMPT overexpression impairs the migratory response in BMDMs from LDLR$^{-/-}$ mice**. Animals were fed a high-fat western type diet for 12 weeks (n = 12). (A, B) Gene (A) and protein (B) expression of CCR2 with corresponding quantification in BMDMs. (C, D) Gene expression (C) and secretion (D) of CCL2 in BMDMs. (E) Percentages of migrating cells at 0, 12 and 24 h after wound-healing of BMDMs. (F) Cells migrating in response to each condition divided by cells migrating in response to medium alone. (G, H) Levels of eNAMPT (G) and $NAD^+$ (H) in the medium of BMDMs. Cells were treated with FK866 (500 nM), recombinant eNAMPT (100 ng/ml) or fMLP (1 nM) as indicated for 24 h, except where otherwise specified. *P<0.05, **P<0.01 vs CTR chimera vs untreated cells.

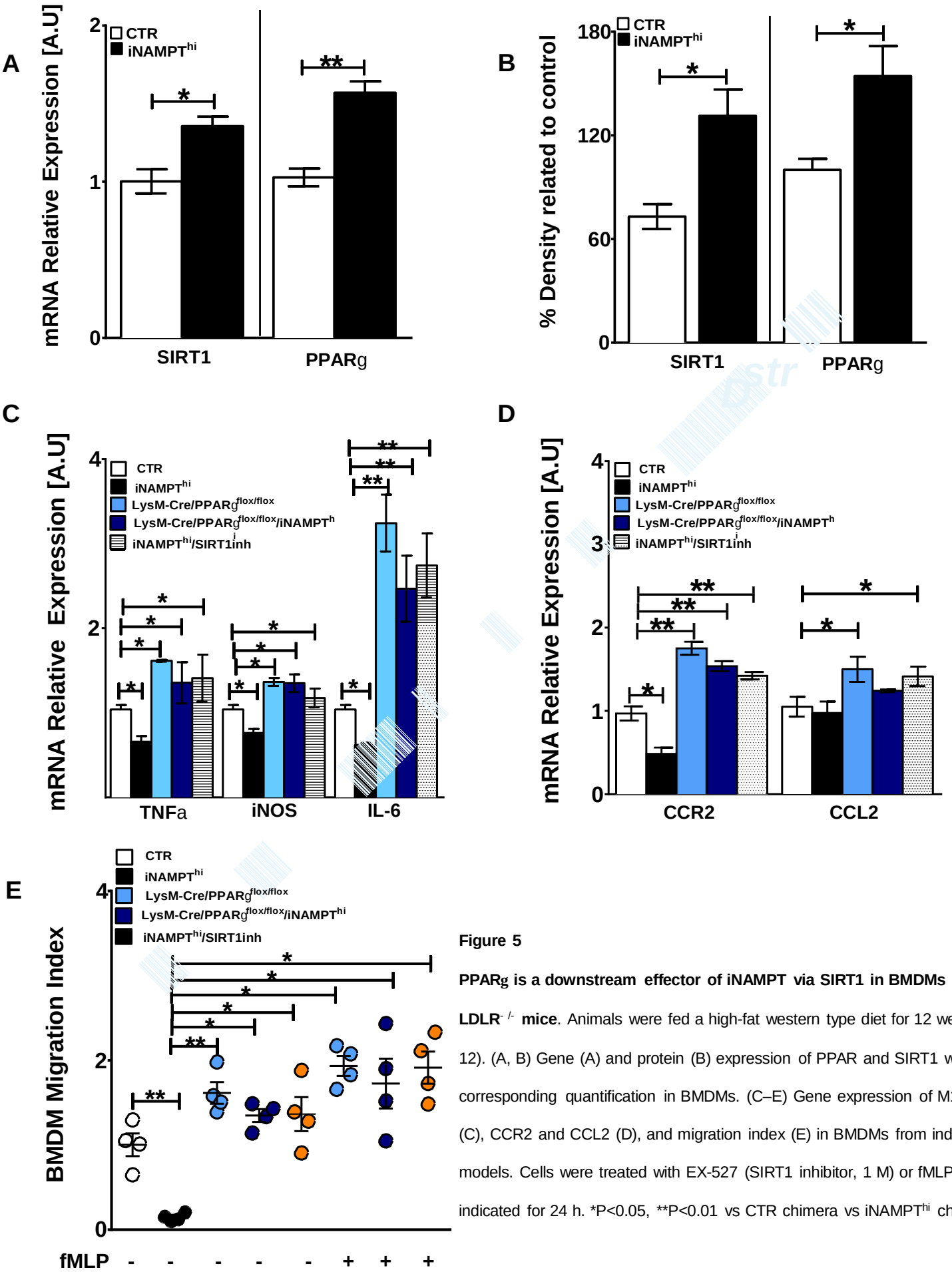


**Figure 5**

**PPARg is a downstream effector of iNAMPT via SIRT1 in BMDMs from LDLR$^{-/-}$ mice**. Animals were fed a high-fat western type diet for 12 weeks (n = 12). (A, B) Gene (A) and protein (B) expression of PPAR and SIRT1 with corresponding quantification in BMDMs. (C–E) Gene expression of M1 markers (C), CCR2 and CCL2 (D), and migration index (E) in BMDMs from indicated mice models. Cells were treated with EX-527 (SIRT1 inhibitor, 1 M) or fMLP (1 nM) as indicated for 24 h. *P<0.05, **P<0.01 vs CTR chimera vs iNAMPT$^{hi}$ chimera.

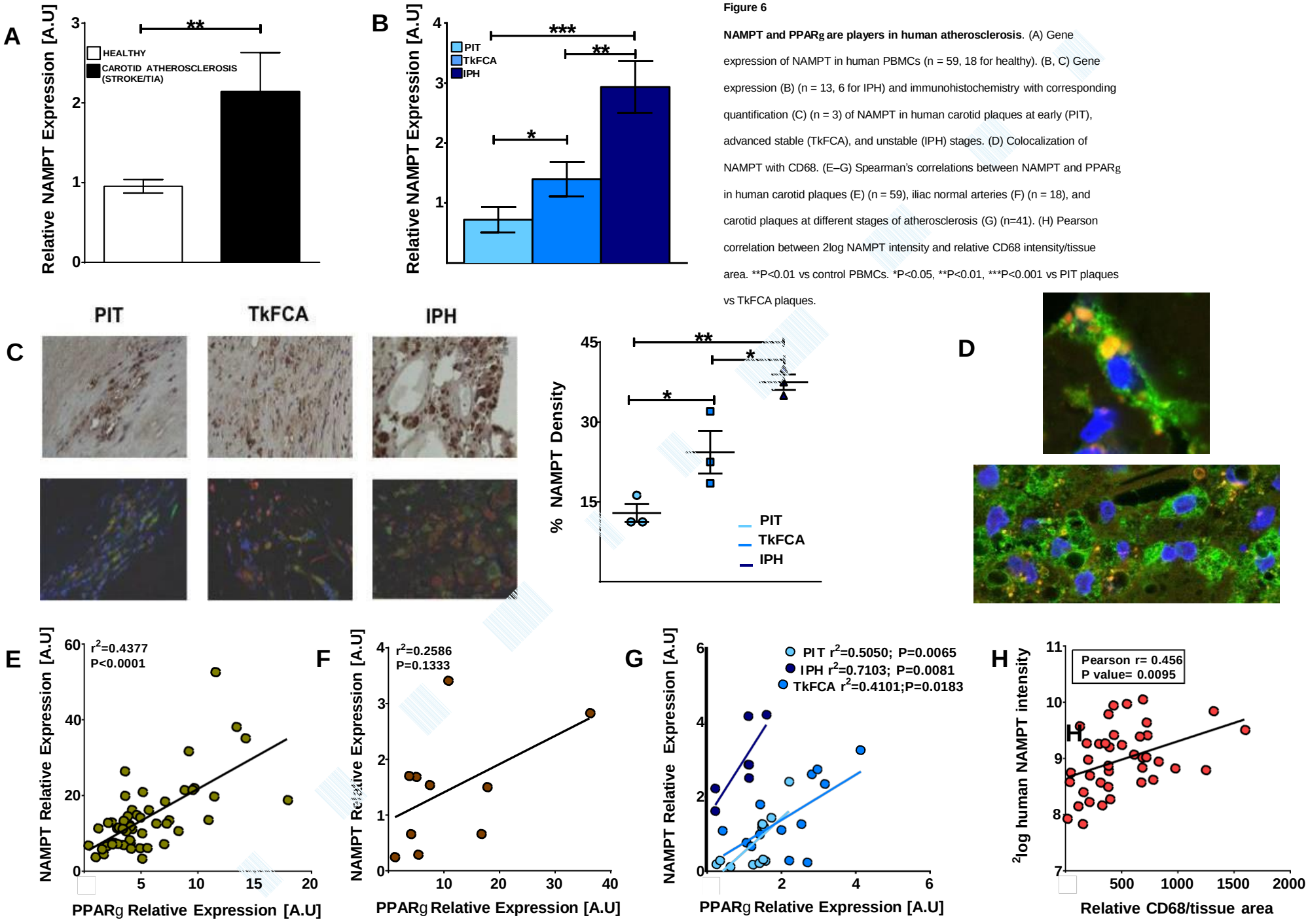


**Figure 6**

**NAMPT and PPARg are players in human atherosclerosis**. (A) Gene expression of NAMPT in human PBMCs (n = 59, 18 for healthy). (B, C) Gene expression (B) (n = 13, 6 for IPH) and immunohistochemistry with corresponding quantification (C) (n = 3) of NAMPT in human carotid plaques at early (PIT), advanced stable (TkFCA), and unstable (IPH) stages. (D) Colocalization of NAMPT with CD68. (E–G) Spearman's correlations between NAMPT and PPARg in human carotid plaques (E) (n = 59), iliac normal arteries (F) (n = 18), and carotid plaques at different stages of atherosclerosis (G) (n=41). (H) Pearson correlation between 2log NAMPT intensity and relative CD68 intensity/tissue area. **P<0.01 vs control PBMCs. *P<0.05, **P<0.01, ***P<0.001 vs PIT plaques vs TkFCA plaques.