Leukocyte Overexpression of Intracellular NAMPT Attenuates Atherosclerosis by Regulating PPARγ-Dependent Monocyte Differentiation and Function

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Objective—Extracellular nicotinamide phosphoribosyltransferase (eNAMPT) mediates inflammatory and potentially proatherogenic 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—Low-density lipoprotein 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 chemokine (C–C motif) receptor 2-dependent monocyte chemotaxis and by skewing macrophage polarization toward 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 chemokine (C–C motif) receptor 2 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 γ. Finally, iNAMPT and peroxisome proliferator–activated receptor γ showed a strong correlation in human atherosclerotic, but not healthy arteries, hinting to a relevance of iNAMPT/peroxisome proliferator–activated receptor γ pathway also in human carotid atherosclerosis.

Conclusions—This study highlights the functional dichotomy of intracellular versus extracellular NAMPT, and unveils a critical role for the iNAMPT–peroxisome proliferator–activated receptor γ axis in atherosclerosis.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2017;37:1157-1167. DOI: 10.1161/ATVBAHA.116.308187.)**

Key Words: apoptosis ■ atherosclerosis ■ diet ■ inflammation ■ phenotype

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 signaling regulator.[3] Accumulating evidence has highlighted the importance of NAMPT-mediated NAD^+ recycling, in concert with NAD^+-dependent protein deacetylases (sirtuins), 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]

Received on: June 14, 2016; final version accepted on: March 28, 2017.

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.116.308187/-/DC1.

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Nonstandard Abbreviations and Acronyms	
BMDM	bone marrow–derived macrophage
CCR2	chemokine (C–C motif) receptor 2
NAD	nicotinamide adenine dinucleotide
NAMPT	nicotinamide phosphoribosyltransferase
PPARγ	peroxisome proliferator–activated receptor γ
SIRT1	sirtuin 1

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.[7] Recently, hepatic NAMPT knockdown was seen to enhance atherosclerosis, at least partly mediated through eNAMPT,[9] while concordant with this finding, global systemic inhibition of NAMPT with FK866 dampened plaque inflammation.[10] However, the effects of iNAMPT on atherogenesis are only poorly understood.

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

Materials and Methods

Materials and Methods and Figure I 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 (Figure IIA in the online-only Data Supplement), total plasma triglyceride (Figure IIB in the online-only Data Supplement), nor cholesterol (Figure IIC in the online-only Data Supplement) levels were influenced during 12 weeks on a western type diet. At euthnization, iNAMPT chimeras had similar plasma NAMPT levels as control mice (Figure 1A). However, bone marrow cells of the $iNAMPT^{hi}$ chimera displayed upregulation of NAMPT mRNA and protein (65%, $P<0.01$; Figures 1B and 1C), an effect that was accompanied by a 2-fold increase in intracellular NAD^+ levels (Figure 1D). A similar increase in iNAMPT expression was also seen 24 hours after LV infection (Figure IIE in the online-only Data Supplement). Apparently, LV gene transfer led to overexpression of iNAMPT, but not eNAMPT, providing a unique opportunity to specifically study iNAMPT phenotypic effects.

Although 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 western type diet (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 (Figure IIIA in the online-only Data Supplement), T cell (Figure IIIB in the online-only Data Supplement), vascular smooth muscle cell (Figure IIIC in the online-only Data Supplement), and collagen content (Figure IIID in the online-only Data Supplement). 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 a 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 (Figure IIIE in the online-only Data Supplement). Likewise, FK866 treatment increased plaque macrophage (Figure IIIF in the online-only Data Supplement) and caspase-3–positive cell (Figure IIIG in the online-only Data Supplement) contents of $iNAMPT^{hi}$ mice that were similar to values in control mice. Unfortunately, however, we had no control with FK866 alone.

Thus, although we lack data on the whole aorta, we show significantly attenuated atherosclerosis in $iNAMPT^{hi}$ at two time points: both at 6 and 12 weeks, and our FK866 inhibition experiments show a similar pattern for week 8. These data strongly indicate that the effect of atherosclerosis in these animals is not by chance.

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 $iNAMPT^{hi}$ versus control chimeras (data not shown). However, bone marrow of $iNAMPT^{hi}$ chimeras was found to be enriched in $CD11b^+Ly6G^+$ granulocytes and $CD11b^+Ly6G^-$ monocytes (Figure 3A). Although circulating granulocyte levels were not affected, monocyte counts were significantly reduced in $iNAMPT^{hi}$ chimeras (Figure 3B). Analysis of bone marrow cells from $iNAMPT^{hi}$ mice demonstrated the increased levels of total colony-forming units (CFU) compared with cells from the controls (Figure IVA in the online-only Data Supplement). This effect could be almost entirely ascribed to an increased frequency of CFU–GM (granulocyte-monocyte), CFU–G, and CFU–M (Figure IVB through D in the online-only Data Supplement), 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, but not bone marrow granulocytes were reduced in $CD62L^{lo}CXCR4^{hi}$ cells, representing activated, senescent granulocytes prone to home to bone marrow (Figure 3C and 3D). This could reflect a diminished rate of neutrophil senescence or more effective stromal re-entry of senescent cells. Resident $Ly6C^{lo}$ monocytes seemed to be overrepresented in bone marrow, resulting in lower $Ly6C^{hi}/Ly6C^{lo}$ ratios (Figure 3C). This effect was paralleled to the predominance of $Ly6C^{lo}$ monocytes in circulation (Figure 3D). Consistent with an inhibitory role of iNAMPT in bone marrow $Ly6C^{hi}$ monocyte mobilization, FK866 treated $LDLr^{-/-}$ mice exhibited reduced stromal bone

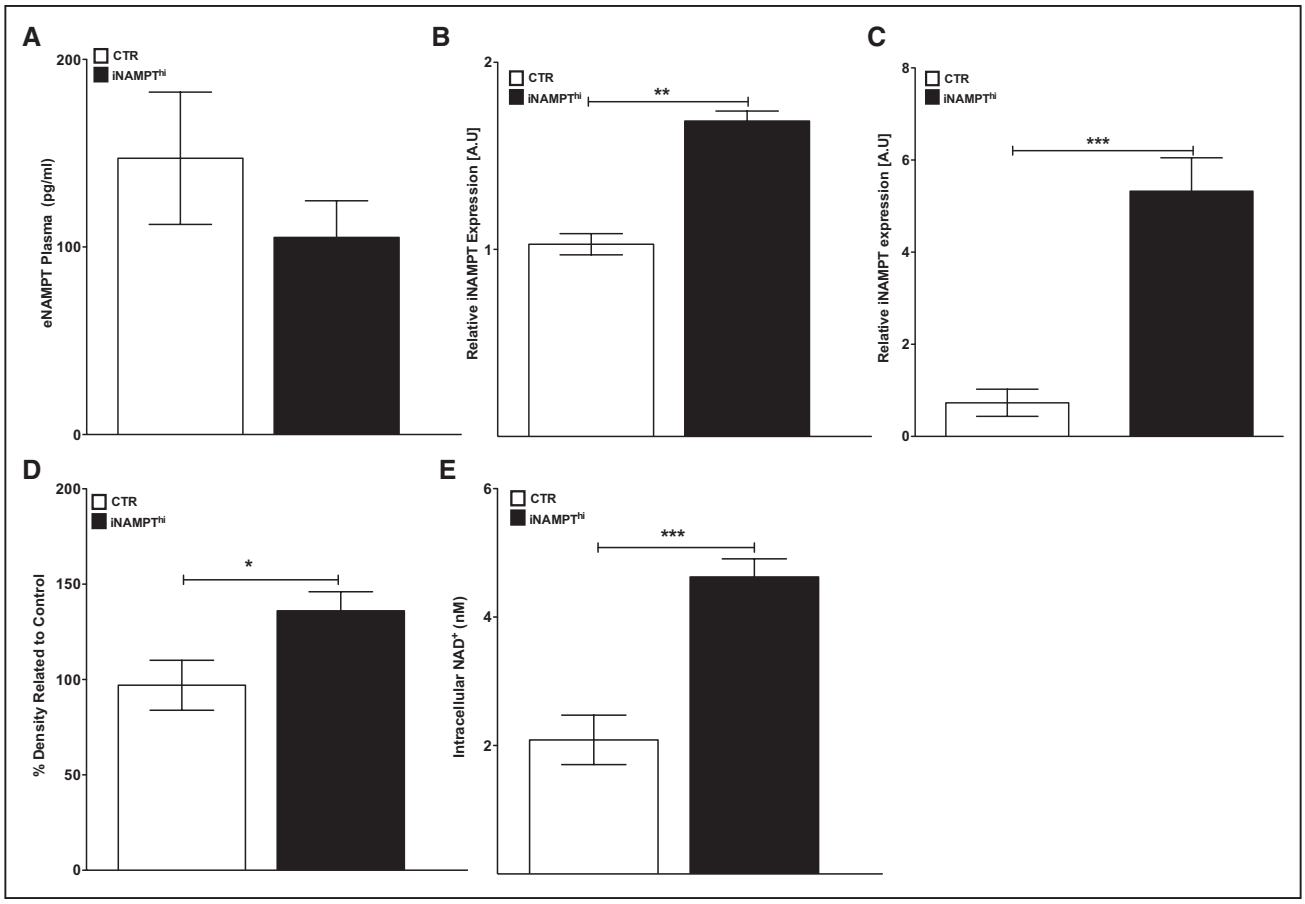

Figure 1. Hematopoietic intracellular nicotinamide phosphoribosyltransferase (iNAMPT) overexpression does not affect extracellular NAMPT (eNAMPT) plasma levels but results in an increased expression and function of iNAMPT in bone marrow–derived macrophages (BMDMs) from LDLr$^{-/-}$ mice. Animals were 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. CTR indicates control.

marrow monocyte content (Figure IVE in the online-only Data Supplement) and increased number of circulating monocytes (Figure IVF in the online-only Data Supplement).

One of the key regulatory pathways in stromal monocyte egress is the chemokine (C–C motif) receptor 2 (CCR2)–C-C chemokine ligand 2 (CCL2) axis.[11] Gene and protein analysis of CCR2 in bone marrow–derived macrophages (BMDMs) from iNAMPT^{hi} versus control chimera revealed a strong downregulation (Figure 4A and 4B and Figure VA in the online-only Data Supplement). Moreover, although CCL2 mRNA expression remained unchanged (Figure 4C), CCL2 secretion was slightly 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 4B and 4C). As baseline and fMLP (*N*-formylmethionyl-leucyl-phenylalanine) elicited cell motility (Figure 4E) and directional migration (Figure 4F) were both sharply diminished in BMDMs of iNAMPT^{hi} versus 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, which had augmented motility; while again eNAMPT treatment was ineffective (Figure 4F). Importantly, overexpressed iNAMPT was not secreted (ie, 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. Recently, Li et al[9] show that manipulating NAMPT expression within the liver influenced eNAMPT levels, and indeed, when examining NAMPT secretion after LV-NAMPT overexpression in parenchymal liver cells (HepG2 cells) in vitro we found that in contrast to BMDM, hepatocyte NAMPT is elaborated into medium (Figure VB in the online-only Data Supplement) suggesting that the release of eNAMPT at least partly is regulated in cell-specific manner.

Hematopoietic Human iNAMPT Overexpression Favors Alternatively Activated Macrophage Polarization

In inflammation, Ly6C^{hi} monocytes are recruited to the affected tissue via CCR2, among others, to give rise to classically activated M1 macrophages.[12] 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 toward alternatively activated M2 macrophages, known for their wound healing and resolution of inflammation activities.[13]

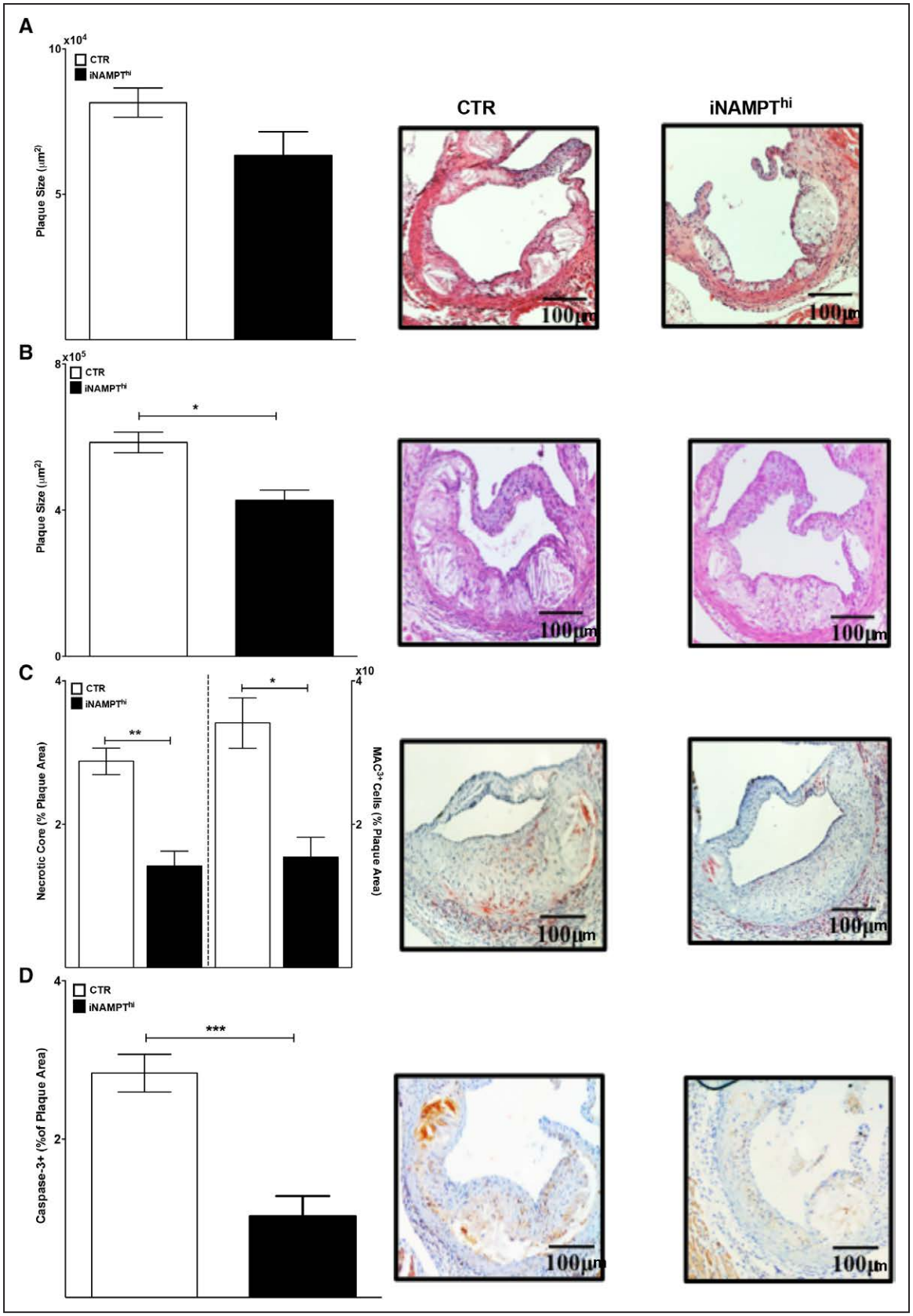

Figure 2. Hematopoietic intracellular nicotinamide phosphoribosyltransferase (iNAMPT) overexpression attenuates atherosclerotic plaque development in LDLR$^{-/-}$ mice. **A** and **B**, Hematoxylin–eosin staining of aortic roots with corresponding quantification of plaque size after a high-fat western type diet for 6 (**A**) or 12 (**B**) weeks. **C** and **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. CTR indicates control.

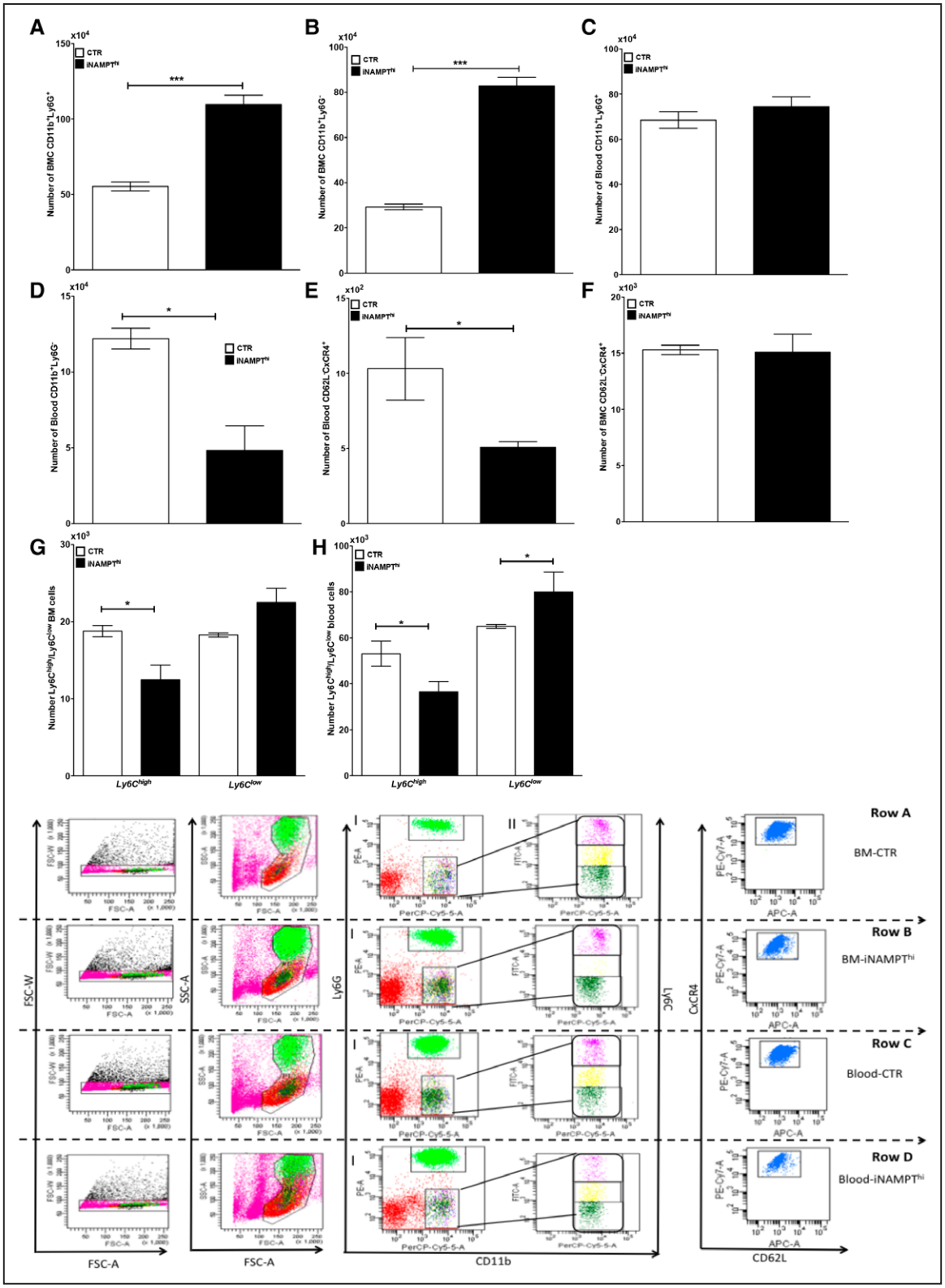

Figure 3. Hematopoietic intracellular nicotinamide phosphoribosyltransferase (iNAMPT) overexpression perturbs stromal and circulating leukocyte pools in LDLR$^{-/-}$ mice. Animals were fed a high-fat western type diet for 12 weeks (n=12). Fluorescence-activated cell sorting (FACS) analysis of absolute granulocyte (CD11b^{+}Ly6G^{+}) and monocyte (CD11b^{+}Ly6G^{-}) counts in bone marrow (**A** and **B**) and blood (**C** and **D**). Proportions of (**E** and **F**) activated, senescent granulocytes (CD62L^{lo}CXCR4^{hi}) and of (**G** and **H**) Ly6C^{hi}–to–Ly6C^{lo} subsets in bone marrow (BM; **G**) and blood (**H**). Gating strategies are demonstrated in the lower panels *P<0.05 vs CTR chimera. CTR indicates control.

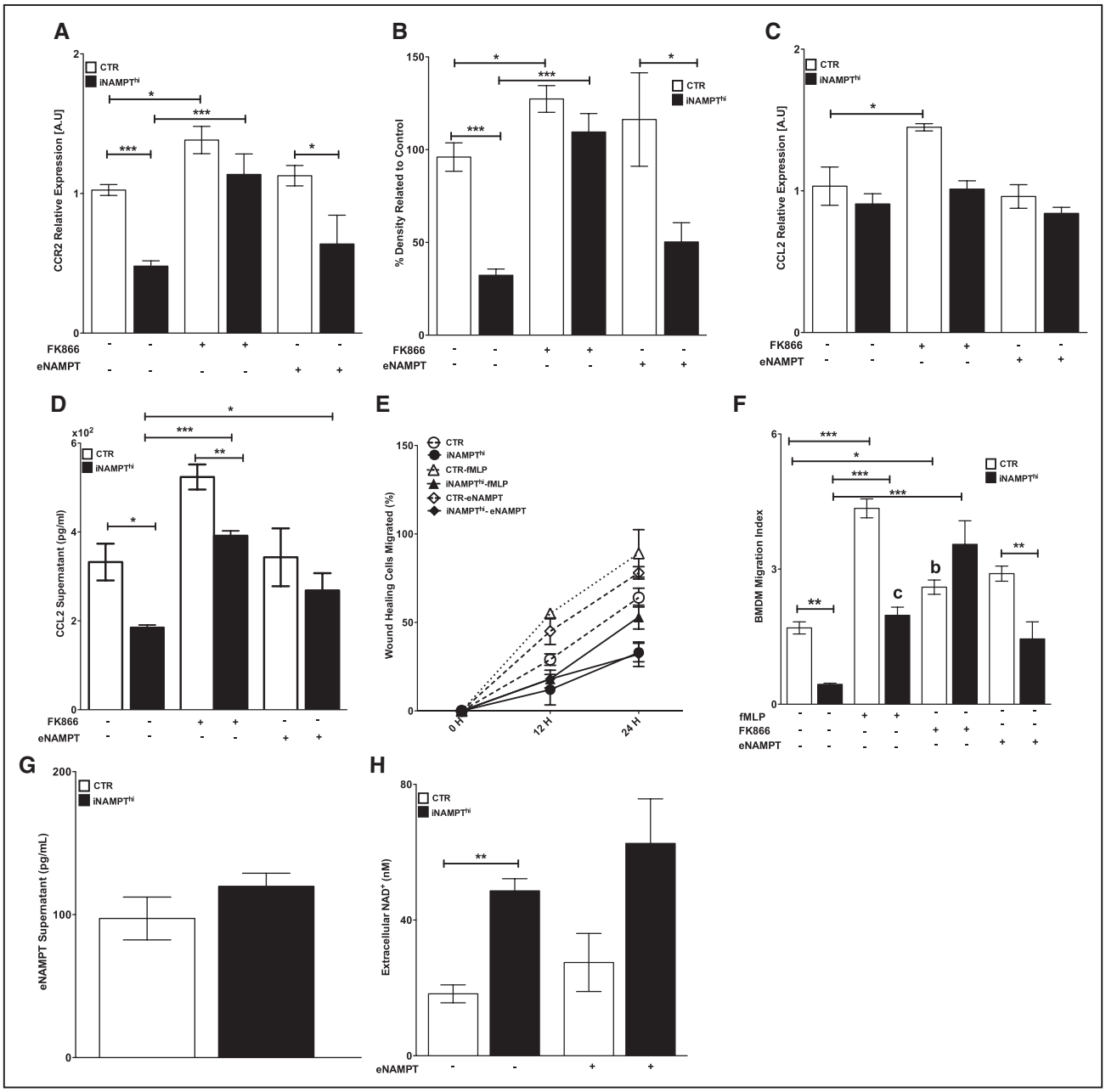

Figure 4. Hematopoietic intracellular nicotinamide phosphoribosyltransferase (iNAMPT) overexpression impairs the migratory response in bone marrow–derived macrophages (BMDMs) from LDLR$^{-/-}$ mice. Animals were fed a high-fat western type diet for 12 weeks (n=12). Gene (**A**) and protein (**B**) expression of chemokine (C–C motif) receptor 2 (CCR2) with corresponding quantification in BMDMs. Gene expression (**C**) and secretion (**D**) of C-C chemokine ligand 2 (CCL2) in BMDMs. **E**, Percentages of migrating cells at 0, 12 and 24 hours after wound healing of BMDMs. **F**, Cells migrating in response to each condition divided by cells migrating in response to medium alone. Levels of eNAMPT (**G**) and NAD$^+$ (**H**) in the medium of BMDMs. Cells were treated with FK866 (500 nmol/L), recombinant eNAMPT (100 ng/mL), or fMLP (*N*-formylmethionyl-leucyl-phenylalanine; 1 nmol/L) as indicated for 24 hours, except where otherwise specified. *P<0.05, **P<0.01 vs CTR chimera vs untreated cells. CTR indicates control.

In agreement with this hypothesis, iNAMPT^{hi} BMDMs, polarized with interferon-γ and lipopolysaccharide (M1) or interleukin-4 (IL-4) (M2), showed reduced or increased expression, respectively, of established M1 (tumor necrosis factor, IL-6 and inducible nitric oxide synthase) and M2 (IL-10, arginase 1 and to a lesser extent mannose receptor, C-type 1) markers relative to control BMDMs (Figure VIA in the online-only Data Supplement). In contrast, exposure of BMDMs from control mice to eNAMPT led to upregulated tumor necrosis factor and IL-6 expression at mRNA level and protein secretion. eNAMPT was also able to induce these inflammatory cytokines in iNAMPT^{hi} BMDMs, albeit less pronounced than in control BMDMs (Figure VIB and C in the online-only Data Supplement). 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 with control chimeras (Figure VID in the online-only Data Supplement).

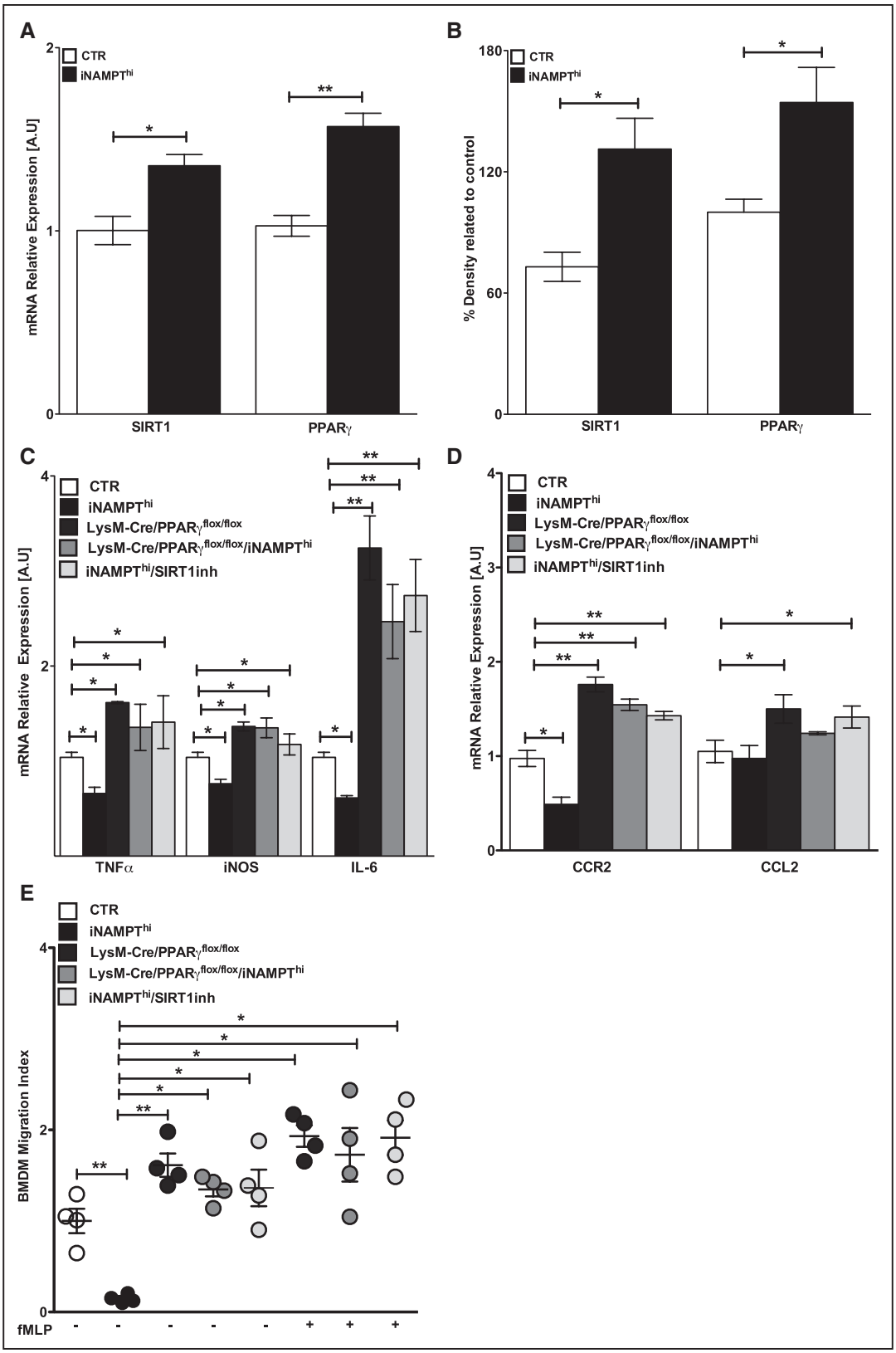

Figure 5. PPARγ is a downstream effector of intracellular nicotinamide phosphoribosyltransferase (iNAMPT) via sirtuin 1 (SIRT1) in bone marrow–derived macrophages (BMDMs) from LDLR$^{-/-}$ mice. Animals were fed a high-fat western type diet for 12 weeks (n=12). Gene (**A**) and protein (**B**) expression of PPAR and SIRT1 with corresponding quantification in BMDMs. Gene expression of M1 markers (**C**), chemokine (C–C motif) receptor 2 (CCR2) and C-C chemokine ligand 2 (CCL2; **D**), and migration index (**E**) in BMDMs from indicated mice models. Cells were treated with EX-527 (SIRT1 inhibitor, 1 M) or fMLP (*N*-formylmethionyl-leucyl-phenylalanine; 1 nmol/L) as indicated for 24 hours. *P<0.05, **P<0.01 vs CTR chimera vs iNAMPT^{hi} chimera. iNOS indicates inducible nitric oxide synthase; IL, interleukin; and TNF, tumor necrosis factor. CTR indicates control.

Hematopoietic Human iNAMPT Overexpression Promotes PPARγ Activation in an SIRT1-Dependent Manner

PPARγ has been proposed as a key regulator of M2 polarization.[14] Interestingly, in silico pathway analysis (string-db.org) linked PPARγ to NAMPT (Figure VII in the online-only Data Supplement), 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, sirtuin 1 (SIRT1), which is reported to regulate PPARγ activity.[13] BMDMs from iNAMPT^{hi} chimeras featured upregulated expression of PPARγ and its regulator SIRT1 when compared with control (Figures 5A and 5B). However, conditional deletion of PPARγ (ie, 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 wild-type versus 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 upregulated 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/PPARg$^{flox/flox}$ BMDMs, whereas it reverted CCL2 expression to levels observed in control and iNAMPT^{hi} BMDMs from wild-type 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^{hi}-induced macrophage reprogramming toward 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[15] and the iNAMPT-associated increase in intracellular levels of NAD^+, which may affect lipid metabolism as well,[16] hinted toward an effect of iNAMPT overexpression on macrophage lipid handling. In line with the aforementioned iNAMPT-induced upregulation of PPARγ in BMDMs, LysMCre-PPARγ$^{flox/flox}$ BMDMs showed intrinsically reduced PPARγ mRNA expression, which was not affected by iNAMPT overexpression, but iNAMPT augmented oxidized low-density lipoprotein-induced PPARγ upregulation in BMDMs (Figure VIIIA in the online-only Data Supplement). ABCA1 (ATP-binding cassette transporter 1) and ABCG1 (ATP-binding cassette sub-family G member 1) expression patterns essentially mirrored that of PPARγ (Figure VIIIB through D in the online-only Data Supplement). In contrast to its profound effect in BMDMs, iNAMPT had no effect on ABCA1 and ABCG1 and LXRα (liver X receptor alpha) expression in the liver, again underpinning the leukocyte-specific nature of the intervention (Figure VIIID through F in the online-only Data Supplement). As a result, cholesterol ester accumulation at baseline and in oxidized low-density lipoprotein-exposed BMDMs of iNAMPT^{hi} chimera was attenuated, when compared with that in control chimera (Figure VIIIG in the online-only Data Supplement). 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 (ie, stroke or TIA),[8] we found that these patients also exhibited a profound upregulation of NAMPT gene expression in peripheral blood mononuclear cells (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 expression (immunohistochemistry; Figure 6C) both increased progressively from early stable lesions to advanced and ruptured plaques (the Maastricht Human Plaque study). Plaque NAMPT (red) colocalized 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 colocalization could be confirmed by confocal microscopical 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; at which NAMPT and PPARγ expression correlation changes 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 upregulation 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.[17] 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

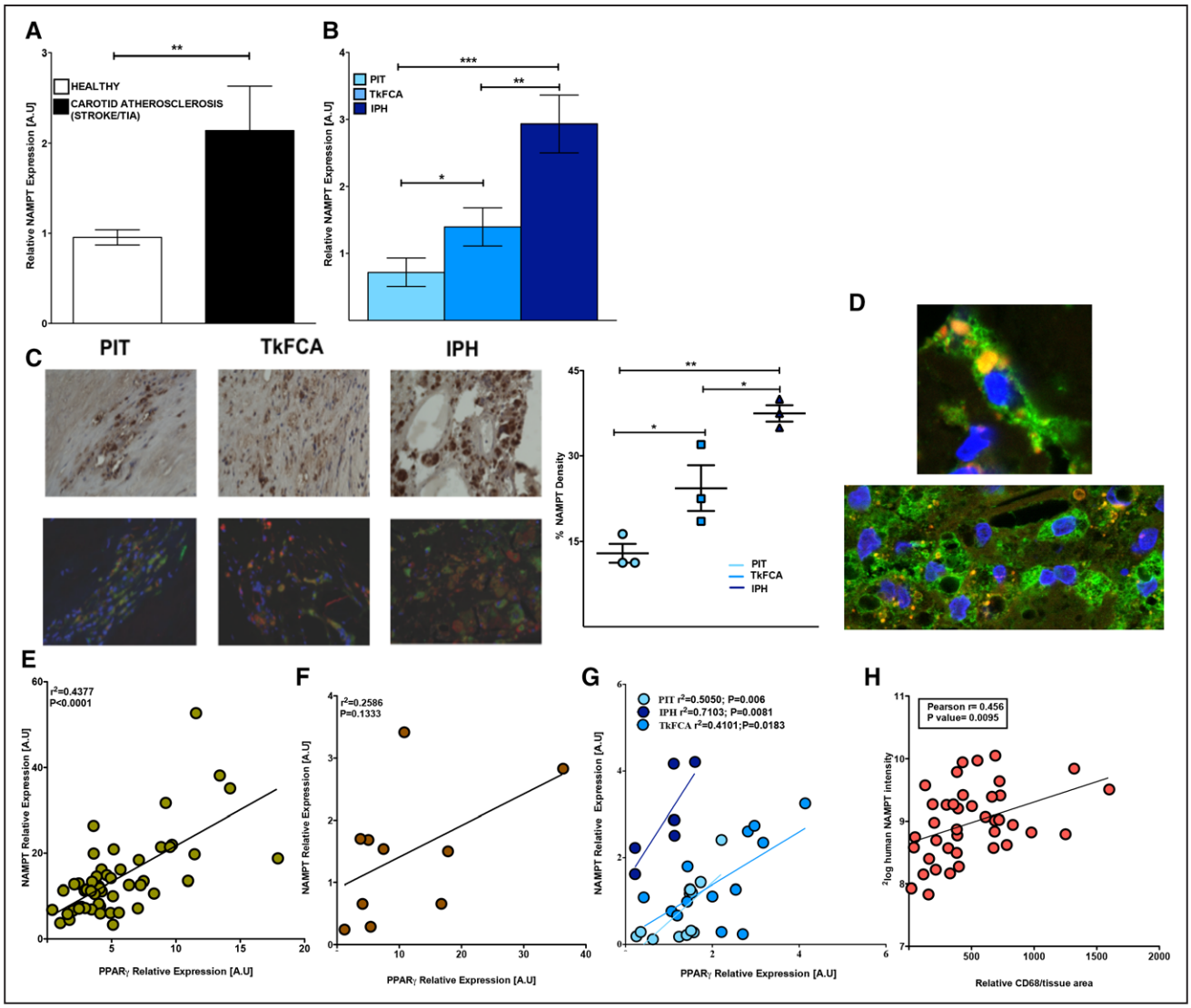

Figure 6. Nicotinamide phosphoribosyltransferase (NAMPT) and PPARγ are players in human atherosclerosis. **A**, Gene expression of NAMPT in human peripheral blood mononuclear cells (PBMCs; n=59, 18 for healthy). Gene expression (**B**; n=13, 6 for IPH) and immunohistochemistry with corresponding quantification (**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. Spearman correlations between NAMPT and PPARγ 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.

gene transfer does not change eNAMPT or extracellular NAD^+ levels. We demonstrate that hematopoietic iNAMPT overexpression confers protection against atherosclerosis by affecting on 3 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 toward 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[9] have recently shown that increased hepatic NAMPT expression promotes atherosclerosis, which may seem in conflict with the present study. However, this study differs in several aspects from our study. In Li et al's[9] 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[18] 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 versus eNAMPT. Adenoviral gene transfer markedly elevated NAMPT levels in plasma (eNAMPT),[9] whereas our approach only affected iNAMPT expression as judged from (1) unchanged eNAMPT plasma levels in vivo in iNAMPT^{hi} chimeras and (2) unchanged eNAMPT secretion by iNAMPT^{hi} BMDM in vitro. Importantly, we and others have

previously reported that although eNAMPT exerts inflammatory and potentially proatherogenic activities,[7] the effects of iNAMPT may, in fact, be opposite. For instance, we show that iNAMPT downregulate CCR2 expression and suppress monocyte chemotaxis, whereas exposure of cells to eNAMPT was completely ineffective. The functional dichotomy of iNAMPT versus eNAMPT is partly related to the differences in extracellular and intracellular NAD^+ produced by NAMPT. Intracellular NAD^+ will activate sirtuin proteins, which through histone deacetylation lead to, for example, modulation of gene transcription.[2] Extracellular NAD^+, however, can bind and activate extracellular receptors, such as P2X7R triggering inflammatory pathways (eg, NLRP3 inflammasome activation).[19] Importantly, we show that leukocyte iNAMPT overexpression promotes increased intracellular, but not extracellular, NAD^+ levels, and ensuing activation of SIRT1 pathways. Again no such effects were seen when exposing cells 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[10] and Halvorsen et al,[20] may also be viewed from this perspective, in that they reflect different functions of intracellular and extracellular NAMPT. Although FK866 inhibits both intracellular and extracellular NAMPT, Yano et al[21] have recently shown that FK866 also inhibit the release of extracellular NAMPT from monocytes, potentially leading to eNAMPT skewed inhibition. In our present study, however, there was a selective upregulation 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[10,20] and siRNA[9]) are not completely reciprocal to that of NAMPT overexpression (this study).

Taken together, the seemingly paradoxical results of our study compared with that of previous work on systemic or hepatic inhibition can be attributed to the diverging activities of intracellular and extracellular NAMPT, as well as the diverging roles of NAMPT in hepatocytes versus leukocytes. It could also reflect that regulation of eNAMPT at least partly is cell specific. In contrast to our understanding of iNAMPT, the regulation of eNAMPT is poorly understood.[22] Possibly, the release of eNAMPT is regulated at post-transcriptional or even post-translational level, in a cell-specific manner. Indeed, herein we show after LV-NAMPT overexpression in parenchymal liver cells (HepG2 cells) in vitro hepatocyte NAMPT is elaborated into medium. Our approach, thus, provides insights into the anti-inflammatory activity of leukocyte iNAMPT expression and the different regulation eNAMPT and iNAMPT and in that sense our study adds to a better understanding and specification of NAMPTs complex functions in atherosclerosis (Graphical Abstract in the online-only Data Supplement).

Another striking finding in this study was that plaques of $iNAMPT^{hi}$ chimeras displayed reduced macrophage accumulation. We propose 2 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.[12] The reduction was not because of 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.[12,23] We were able to attribute these chemotactic effects to iNAMPT-dependent activation of the PPARγ pathway. The latter is known to downregulate CCR2 expression,[24] and our findings suggest that downregulation of CCR2 expression is linked to SIRT1-dependent PPARγ activation. Indeed, both SIRT1 and PPARγ inhibition counteracted the lowered CCR2 expression and activity in $iNAMPT^{hi}$ BMDM. 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 seems to be of major importance in mediating the atheroprotective effects of iNAMPT in our model (see Graphical Abstract).

One could argue that our BM transduction protocol will target not only hematopoietic precursor cells but also other cell types, such as endothelial and mesenchymal progenitor cells. First, we show the significant upregulation of iNAMPT in BMDM from $iNAMPT^{hi}$ chimera and in the spleen being a reliable reflection of leukocyte overexpression in these mice. Moreover, even if endothelial and mesenchymal progenitor cells are (over) expressed $iNAMPT^{hi}$, they will only represent a minute fraction of circulating $NAMPT^+$ cells (≈0.001% or 10^2 endothelial progenitor cells/mL). Even more importantly, endothelial and mesenchymal progenitor cells have been repeatedly shown to not contribute to EC or smooth muscle cell renewal in the atherosclerotic plaque,[26,27] and it is, therefore, unlikely that NAMPT expression in nonhematopoietic cells would have contributed significantly to the observed phenotype in our study.

In conclusion, this study is the first to demonstrate a protective role for leukocyte iNAMPT in western 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 (eg, prognostic data on NAMPT expression within human carotid plaques), are, however, needed to prove this hypothesis. Our data also point toward caution when attempting to target NAMPT in relevant disorders such as atherosclerosis. We suggest that therapeutic approach should preferably inhibit eNAMPT without inhibiting or even increase iNAMPT, but these issues will have to be further clarified in forthcoming studies.

Acknowledgments

We gratefully acknowledge the invaluable input of Zsuzsanna Nagy, Pap Attila, Mona Skjelland, Erik T. Askevold, and Christen P. Dahl for excellent technical assistance.

Sources of Funding

E.A.L. Biessen is recipient of the establish investigator grant of the Netherlands Heart Foundation (2003T201). B. Bermudez was supported by EU grants FP7 (FP7–PEOPLE–2007–2–1–IEF and FP7–PEOPLE–2010–RG) and the "V Own Research Plan" contract from the University of Seville. S. Montserrat-de la Paz was supported by the Spanish Ministry of Economy and Competitiveness (AGL2011–29008).

Disclosures

None.

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Highlights

- Hematopoietic intracellular nicotinamide phosphoribosyltransferase (iNAMPT) overexpression attenuates and stabilizes diet–induced atherosclerosis.
- iNAMPT mediates sequestration of proinflammatory monocytes in bone marrow.
- Macrophages are skewed toward alternative polarization by iNAMPT via PPARγ.
- iNAMPT overexpression did not induce extracellular NAMPT (eNAMPT), and eNAMPT had no effect on chemokine (C–C motif) receptor 2 expression and promoted an inflammatory M1 phenotype in macrophages.
- Gene NAMPT and PPARγ expression tightly correlate with progression of atherosclerotic lesions in humans.