

EXTENDED REPORT

Nicotinamide phosphoribosyltransferase/visfatin expression by inflammatory monocytes mediates arthritis pathogenesis

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ABSTRACT

Objectives Nicotinamide phosphoribosyltransferase (NAMPT)/pre-B-cell colony-enhancing factor/visfatin exerts multiple functions and has been implicated in the pathogenesis of rheumatoid arthritis. To gain insight into its role in arthritis and given that NAMPT is identified as a novel mediator of innate immunity, we addressed the function of monocyte-derived NAMPT in experimental arthritis by selective gene knockdown in inflammatory monocytes.

Methods siRNA uptake and NAMPT expression were determined in Ly6C^{high} and Ly6C^{low} monocyte subsets following intravenous injection of siRNA against NAMPT (siNAMPT) or non-targeting siRNA (siCT) formulated with the DMAPAP cationic liposome into mice. Mice with established collagen-induced arthritis (CIA) were treated weekly after disease onset with siNAMPT or siCT and clinical features were assessed. T-helper cell frequencies, cytokine production and percentage of IL-6-producing Ly6C^{high} monocytes were analysed. Using a co-culture system consisting of purified CD14 monocytes and autologous CD4 T cells, NAMPT and cytokine production, and the percentage of IL-17-producing CD4 T cells, were determined following transfection of CD14 monocytes with siCT or siNAMPT.

Results On intravenous injection, siRNA was preferentially engulfed by Ly6C^{high} monocytes, and siRNA-mediated silencing of NAMPT expression in Ly6C^{high} monocytes inhibited CIA progression. This effect was associated with reduced IL-6 production by Ly6C^{high} monocytes, reduced proportion of Th17 cells and autoantibody titers, and decreased activation and infiltration of monocytes/macrophages and neutrophils in arthritic joints. Moreover, NAMPT-RNAi-silenced CD14 monocytes were found to reduce the percentage of IL-17-producing CD4 T cells in vitro.

Conclusions Our results show that the expression of NAMPT in Ly6C^{high} monocytes promotes many downstream effects involved in inflammatory arthritis and demonstrate the utility of targeting disease-causing genes, such as NAMPT, in Ly6C^{high} monocytes for therapeutic intervention in arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic inflammatory disorder that leads to the progressive destruction of joints. Although the aetiology of RA

remains unknown, many cell types and molecules are implicated in the pathogenesis of RA. Monocytes/macrophages are now believed to be local and systemic amplifiers of the disease with regard to their abundance as well as their paracrine effects on other immune cells and resident fibroblasts.¹ The therapeutic efficacy of conventional antirheumatic drugs coincides with the downregulation of biological functions of monocytes/macrophages, and biotherapies blocking cytokines produced predominantly by monocytes/macrophages are effective in the treatment of RA.^{2,3} The rheumatoid synovium contains activated monocytes/macrophages that produce large amounts of inflammatory mediators, including interleukin (IL)-1 β , IL-6 and tumour necrosis factor alpha (TNF α), which contribute to synovial cell proliferation, the maintenance of an inflammatory environment and structural joint damage. Notably, this cytokine milieu promotes the development and maintenance of T-helper (Th) type 17 cells⁴ that play a pathogenic role in RA by linking T-cell activation and bone resorption.^{5–7} It has recently been demonstrated that activated monocytes continuously migrate into the inflamed joints of patients with active RA⁸ where they acquire an activated phenotype and specifically promote Th17 cell differentiation, but not Th1 or Th2 cell responses.⁹ Therefore, excessive activity of CD14 monocytes participates in inflammatory disease progression, including RA, but the molecular pathways involved in their dysregulated function remain elusive.

Nicotinamide phosphoribosyltransferase (NAMPT) is an ubiquitously expressed protein that displays multiple functions.^{10,11} It was originally identified as pre-B-cell colony enhancing factor, a secreted factor that synergises with IL-7 and stem cell factor to promote the growth of B-cell precursors.¹² It is also proposed as visfatin, an adipokine produced by visceral fat that stimulates insulin signalling.¹³ It may also function as an essential enzyme in the NAD biosynthetic pathway that enhances cellular resistance to genotoxic/oxidative stress and may confer to cells of the immune system the ability to survive during stressful situations such as inflammation.¹⁴ Of note, NAMPT has recently been identified as a novel mediator of innate immunity with pro-inflammatory properties involved in the persistence of inflammation.^{15,16}

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NAMPT is upregulated in monocytes on activation and induces the production of various pro-inflammatory cytokines including IL-6.¹⁷⁻¹⁹

NAMPT has been implicated in the pathogenesis of several human diseases including RA,¹¹ and its increased expression in peripheral blood mononuclear cells is proposed as a marker of chronic inflammation.²⁰ Elevated expression of NAMPT is reported within the synovial tissue and blood of patients with RA and correlates with disease activity.^{17 21 22} Increased expression of NAMPT is also confirmed in mice with collagen-induced arthritis (CIA), both in serum and in the inflamed paws, and early in-vivo blockade of NAMPT with the small molecule inhibitor APO866 ameliorates CIA severity.^{21 23} These findings support a critical role of NAMPT in the pathogenesis of arthritis. Nevertheless, how NAMPT expression in specific cell populations impacts the inflammatory response of arthritis remains unknown.

At least two main subsets of circulating monocytes are described in humans and rodents. In mice, Ly6C^{low} monocytes survey endothelial cells and surrounding tissues for damage or viral infection while Ly6C^{high} monocytes are highly phagocytic, secrete inflammatory mediators, give rise to macrophages and dendritic cells and are involved in inflammatory disease progression.^{24 25} Given that NAMPT is expressed by both monocyte subsets and the importance of targeting the Ly6C^{high} monocytes subset for therapeutic benefit in a broad range of inflammatory disorders,²⁶ we undertook in-vivo experiments to explore the impact of the RNAi-mediated silencing of NAMPT in Ly6C^{high} monocytes in mouse CIA.

MATERIALS AND METHODS

DMAPAP cationic lipid was synthesised as previously described²⁷ and mixed with DOPE and nucleic acids as previously described.²⁸

DBA/1 mice were used in these studies, which were approved by the Ethics Committee on Animal Research of the Languedoc-Roussillon region (CE-LR-0505), and CIA was induced as previously described.²⁹ Arthritis severity was graded and analysed as previously described.³⁰ Detailed procedures are available in the supplementary files (available online only).

RESULTS

In-vivo silencing of NAMPT in Ly6C^{high} monocytes

We previously reported that the cationic liposome DMAPAP efficiently delivers small interfering RNA to CD11b⁺ monocytes and knockdown genes on systemic administration.³¹ We first examined the respective capacity of both Ly6C^{low} and Ly6C^{high} monocyte subsets to engulf siRNA lipoplexes on intravenous injection (figure 1A). Mice were injected with a single dose of 0.5 mg/kg of Cy3-labelled siRNA and the percentage of siRNA-containing cells was monitored after 24 h according to the gating strategy described in online supplementary figure S1. While less than 10% of circulating Ly6C^{low} monocytes engulfed the labelled siRNA (8.5%±1.3%), the presence of the siRNA was detected in 63%±7% of the Ly6C^{high} monocytes. Similarly, Ly6C^{high} monocytes from the splenic reservoir engulfed the siRNA more efficiently than Ly6C^{low} monocytes (30.6%±6.2% and 5.4%±1.6%, respectively). To a lesser extent, the presence of siRNA was also detected in macrophages and conventional

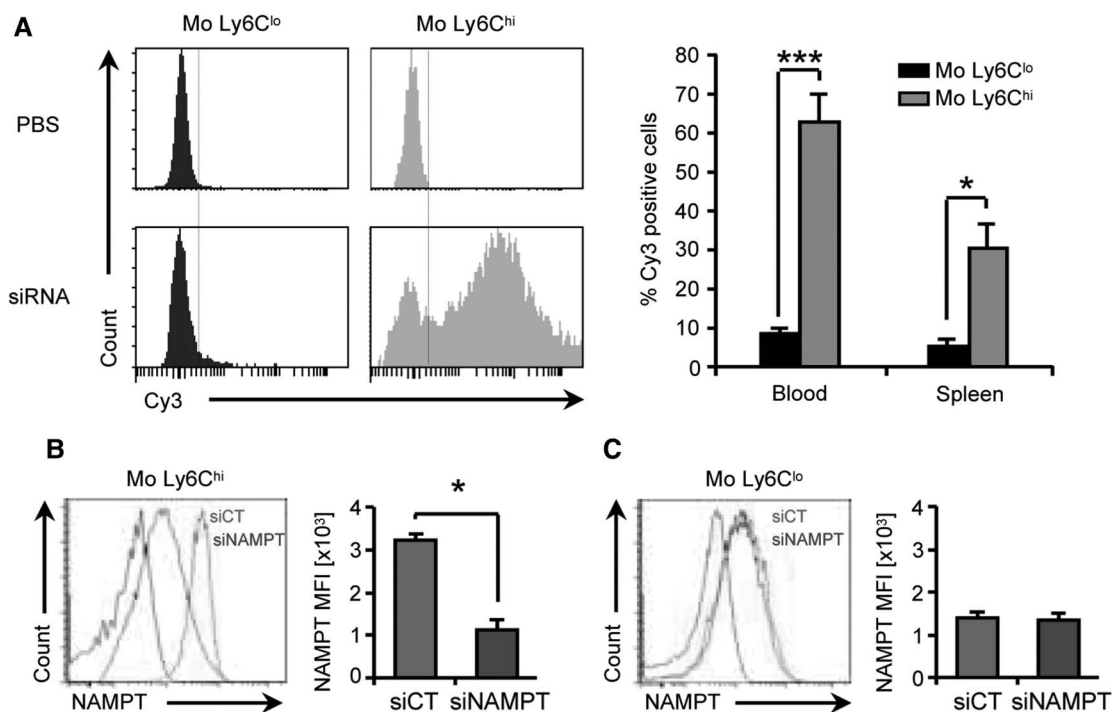


Figure 1 Efficient in-vivo delivery of nicotinamide phosphoribosyltransferase (NAMPT) siRNA in Ly6C^{high} monocytes. Mice received a single intravenous injection of siRNA (0.5 mg/kg) formulated as lipoplex as described in the Methods section. (A) Engulfment of Cy3-labelled siRNA by monocyte (Mo) subsets was quantified by flow cytometry 24 h later. As a control, mice were injected with phosphate-buffered saline (PBS). Representative histogram plots for the indicated monocyte subsets are shown for PBS (upper panels) and Cy3-siRNA-injected mice (lower panels). Graphs show percentages of Cy3 siRNA uptake within Ly6C^{low} and Ly6C^{high} monocytes from blood and spleen tissue. (B and C) Mice were injected either with anti-NAMPT siRNA (siNAMPT, black curve) or non-targeting control siRNA (siCT, white curve) formulated as lipoplexes. Cells from blood were harvested 48 h later. Expression of intracellular NAMPT protein was determined in Ly6C^{high} Mo (B) and Ly6C^{low} Mo (C) by flow cytometry. The black curve represents the isotypic control. NAMPT expression levels were quantified by mean fluorescence intensities (MFI). The data represent the mean±SEM of three mice and refer to one representative experiment out of two. *p<0.05, ***p<0.001, versus control. Access the article online to view this figure in colour.

dendritic cells from the spleen of injected animals (see online supplementary figure S2A). Of note, the siRNA was not incorporated into the CD45⁻ mononuclear cells (see online supplementary figure S2B) and was taken up by less than 1% of the circulating T and B cells (see online supplementary figure S2C).

We then evaluated the feasibility of silencing the NAMPT gene in Ly6C^{high} monocytes (figure 1B). Systemic injection of mice with DMAPAP-formulated siRNA sequences specific for mouse NAMPT (siNAMPT) resulted in a 66% reduction of NAMPT protein expression in Ly6C^{high} monocytes from blood compared to mice injected with non-targeting control siRNA (siCT). Although NAMPT was expressed at lower levels in Ly6C^{low} monocytes, its expression was not affected following the administration of siNAMPT lipoplexes. In agreement with the inefficient delivery of siRNA to neutrophils (less than 2% of the circulating or tissue Ly6G⁺ neutrophils), NAMPT levels were altered neither in the blood neutrophils (see online supplementary figure S3A) nor in the neutrophils infiltrating arthritic joints (data not shown). Together, these data demonstrate that the cationic liposome DMAPAP efficiently delivers siRNA to the inflammatory Ly6C^{high} monocytes and silences NAMPT expression, as opposed to the non-inflammatory Ly6C^{low} monocytes and other cells of the haematopoietic system, when administered intravenously in mice.

RNAi-mediated silencing of NAMPT in Ly6C^{high} monocytes impairs the development of CIA

Considering the importance of Ly6C^{high} monocytes in inflammatory disorders and inflammatory properties reported for

NAMPT, we investigated the effect of siNAMPT delivery to Ly6C^{high} monocytes in experimental arthritis. CIA mice were injected intravenously with siNAMPT lipoplexes once a week after disease onset (figure 2). Mice treated with siNAMPT lipoplexes showed a significant improvement in disease features from the first day of treatment, compared with control animals (figure 2A). Given that disease activity is associated with increased serum IL-6 levels, we determined the effect of NAMPT inhibition in Ly6C^{high} monocytes on IL-6 levels by ELISA at the time mice were killed. The results showed that the protection from disease progression in the siNAMPT-treated group was associated with a significant decrease of circulating IL-6 levels ($p=0.0485$), compared with mice administered with siCT lipoplexes (figure 2B). High levels of circulating CII-specific IgG antibodies, characterised by a high IgG2a : IgG1 ratio, are a hallmark of CIA and determine disease severity. We showed that, consistent with improved clinical features at the time of killing in the group of siNAMPT-treated mice, and opposed to the control group, a significant decrease of CII-specific IgG2a : IgG1 ratio was measured (figure 2C).

To investigate further the impact of the systemically delivered siRNA on joint inflammation, we determined the effect of repetitive siNAMPT lipoplex injections on cellular infiltrates. Consistent with the observed clinical benefit, the total number of cells infiltrating ankle joints of CIA mice was reduced in the siNAMPT-treated group compared with siCT-injected animals (figure 2D). Among these cells, decreased counts of neutrophils (see online supplementary figure S3B), resident macrophages and inflammatory monocytes (figure 2D; see gating strategy in

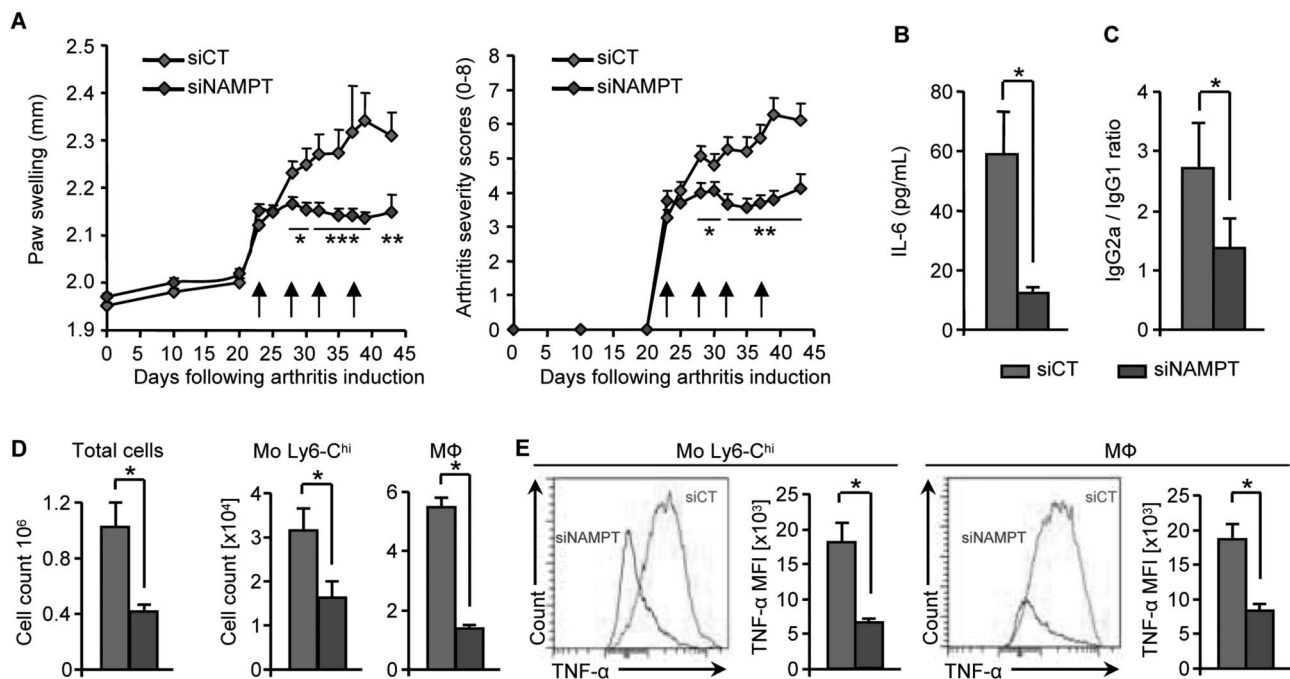


Figure 2 RNAi-mediated silencing of nicotinamide phosphoribosyltransferase (NAMPT) in Ly6C^{high} monocytes reduces the development of inflammatory arthritis. DBA/1 mice were immunised with bovine type II collagen and boosted on day 21. The siRNA (0.5 mg/kg) treatment was initiated at the time of arthritis onset on day 23 (arrows). Once a week, anti-NAMPT siRNA-containing lipoplexes were administered (siNAMPT, blue). The control group was injected with irrelevant non-targeting siRNA lipoplexes (siCT, white). Mice were killed 45 days after immunisation. (A) Paw swelling was measured for each mouse with calipers over the disease course. Arthritis severity scores were graded as described in the Methods section. (B) Levels of interleukin (IL)-6 in serum were measured by ELISA. (C) Levels of bovine type II collagen-specific IgG1 and IgG2a antibodies in sera were quantified by ELISA. (D and E) Leucocytes were isolated from ankle joints. (D) Enumeration of cells reveals significantly decreased total cells, Ly6C^{high} monocytes (Mo Ly6-C^{high}) and macrophages (MΦ) in siNAMPT-treated mice. (E) Tumour necrosis factor alpha (TNFα) expression levels, assessed by intracellular cytokine staining, were measured in Mo Ly6C^{high} and MΦ. The gates were set using isotype antibodies. Results in A, B and C are the mean±SEM of 10 mice per group and refer to one representative experiment out of three. Results in D and E are the mean±SEM of three mice per group and refer to one representative experiment out of two. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, versus control. Access the article online to view this figure in colour.

online supplementary figure S1C) were observed. Interestingly, the production of TNF α was significantly lower in the three cell types for CIA mice injected with siNAMPT lipoplexes, compared with controls (figure 2E and see online supplementary figure S3C). These results indicate that the siNAMPT systemic treatment not only led to a decrease in the number of immune cells infiltrating arthritic joints, but also in their activated status.

In-vivo inhibition of NAMPT in Ly6C^{high} monocytes induces broad immunomodulation of arthritic conditions

Considering that IL-6 is abundantly expressed in arthritis by a number of cell types, including monocytes/macrophages, and that NAMPT induces the production of IL-6 on monocyte activation, we investigated whether siRNA-mediated silencing of NAMPT in Ly6C^{high} monocytes impacts IL-6 production by this specific cell subset in vivo under inflammatory conditions. Arthritic mice were injected weekly from disease onset (arthritic score >3) with siNAMPT lipoplexes, and spleen cells were collected after 3 weeks (figure 3A). As we previously demonstrated that the CIA development is similar between phosphate-buffered saline and siCT-injected mice,^{32 33} this latter condition was used as a control in the following experiments. Flow cytometric analysis of intracellular IL-6 staining showed that in-vivo silencing of NAMPT in Ly6C^{high} monocytes during arthritis progression significantly reduced the percentage of IL-6-producing Ly6C^{high} monocytes (6.6 \pm 0.7%), as well as the production of IL-6 by these cells (mean fluorescence intensity (MFI) 21.5 \pm 0.2), compared with siCT-injected mice (12.2 \pm 0.8% and MFI 36.2 \pm 3.3, respectively). The reduction of IL-6 production on NAMPT knockdown was also confirmed by ELISA measurement of IL-6 protein levels (p=0.0221) in 24 h culture supernatant of spleen cells compared with control mice (figure 3B). Analysis of pro-inflammatory cytokine profiles of these mice showed that levels of TNF α , IL-17A and interferon (IFN)- γ were reduced in splenocytes of siNAMPT-treated mice compared with siCT-injected animals, while the production of the anti-inflammatory cytokine IL-10 was increased (figure 3B).

Considering that IL-6 is critical for Th17 differentiation and expansion in mice and its expression was reduced in Ly6C^{high} monocytes in arthritic mice treated with siNAMPT, we hypothesised that Th17 responses might be reduced in siNAMPT-treated CIA mice. The percentage of IL-17A-producing T cells and IL-17 production was measured by intracellular cytokine staining of CD4 T cells (figure 3C). The results showed a reduced percentage of IL-17A-positive T cells (0.8 \pm 0.1% vs 1.8 \pm 0.3%) and IL-17A production (MFI 24 112 \pm 1832 vs 42 881 \pm 2062) in the spleen of arthritic mice treated with siNAMPT lipoplexes relative to siCT-injected animals. Interestingly, the downregulation of the Th17 population in CIA was not due to a general effect on T-cell activation because neither the Th1 response, characterised by IFN- γ -producing CD4 T cells, nor the frequency of CD4⁺CD25⁺Foxp3⁺ T-regulatory (Treg) cells, were significantly affected (figure 3C,D). Overall, our data indicate that interfering with the NAMPT/IL-6 axis in inflammatory Ly6C^{high} monocytes enables the modulation of pathogenic Th17 cells and the reduction of inflammatory cytokines in CIA.

Silencing of NAMPT in human CD14 monocytes interferes with Th17 cell expansion

As one of the in-vivo downstream anti-inflammatory effects of NAMPT silencing in Ly6C^{high} monocytes is a decrease in Th17 cells, we explored in vitro whether NAMPT expression in human monocytes may also impact the Th17 cell responses.

First, Cy3-labelled siRNA lipoplexes (25 nM) were added to human peripheral blood mononuclear cells to determine the uptake of the siRNA (figure 4A). The CD14⁺CD16⁻ monocytes were found to incorporate the siRNA overwhelmingly (98.3% \pm 0.2%), while it was only taken up by 58.4% \pm 1.2% of the CD14^{dim}CD16⁺ monocytes. Importantly, CD14⁺CD16⁻ monocytes incorporated four times more siRNA than the CD14^{dim}CD16⁺ monocytes (MFI 16.613 \pm 185 vs 4.535 \pm 272). Consistent with what we observed following in-vivo administration to mice, human CD3 T and CD19 B lymphocytes did not reveal any positive signal for the presence of siRNA (data not shown). We next assessed the functional silencing in human monocytes using siNAMPT lipoplexes. Based on previously described procedures,^{34 35} CD14 monocytes from the blood of healthy donors were transfected with either siCT or siNAMPT in the presence of lipopolysaccharide. NAMPT silencing was quantified at messenger RNA and protein levels 2 days after transfection. More than 50% of the NAMPT transcripts and 30% of the protein levels were downregulated on siNAMPT application compared with controls, as shown by reverse transcription quantitated PCR and flow cytometry, respectively (figure 4B). The downregulation of NAMPT expression in CD14 monocytes was associated with a significant decrease in both IL-6 and TNF α expression levels (figure 4C). Next, to assess the effect of NAMPT silencing in CD14 monocytes on Th17 cell expansion, CD14 monocytes were co-cultured 48 h after transfection with autologous purified CD4 T cells, and the percentage of IL-17-producing CD4 T cells was monitored 5 days later. The addition of siNAMPT-transfected CD14 monocytes to autologous CD4 T cells resulted in a significant decrease in the percentage of IL-17A-producing CD4 T cells, as opposed to siCT-transfected monocytes (figure 4D). These data show that the inhibition of NAMPT gene expression in human CD14 monocytes reduces the expansion of Th17 cells.

DISCUSSION

NAMPT exerts multiple functions in a variety of physiological processes and its overexpression has been implicated in the pathogenesis of a number of inflammatory disorders, including cancer, atherosclerosis, diabetes and RA.¹¹ Numerous studies have shown that NAMPT regulates inflammatory mediators and apoptosis. For instance, NAMPT protects macrophages from endoplasmic reticulum stress-induced apoptosis by activating an IL-6/Stat3 signalling pathway.³⁶ We recently reported that NAMPT is overexpressed by fibroblast-like synoviocytes of RA patients (RASf) and stimulates the production of IL-6, IL-8, matrix metalloproteinase (MMP)-1 and MMP-3 in RASf, as well as IL-6 and TNF α in monocytes.¹⁷ Monocytes, a key cell type in RA pathogenesis, are themselves a source of NAMPT, which expression is upregulated under inflammatory conditions¹⁸ and able to induce IL-6 transcription in these cells,^{17-19 36 37} further substantiating the autocrine role of NAMPT in inducing IL-6 expression by monocytes. Nevertheless, the mechanisms underlying the deleterious effect of NAMPT in the inflammatory process of RA remain unclear.

In the present study, we found that systemic administration of siRNA-containing lipoplexes able to deliver and silence NAMPT in Ly6C^{high} monocytes ameliorated disease features in a mouse model of RA. Significant clinical benefit was demonstrated by reduced systemic and local inflammatory markers of arthritis, including reduced serum IL-6 levels, decreased anti-bCII antibody titres and a marked reduction in the number of Th17 cells, as well as in the number and activation status of Ly6C^{high} monocytes, macrophages and neutrophils infiltrating arthritic

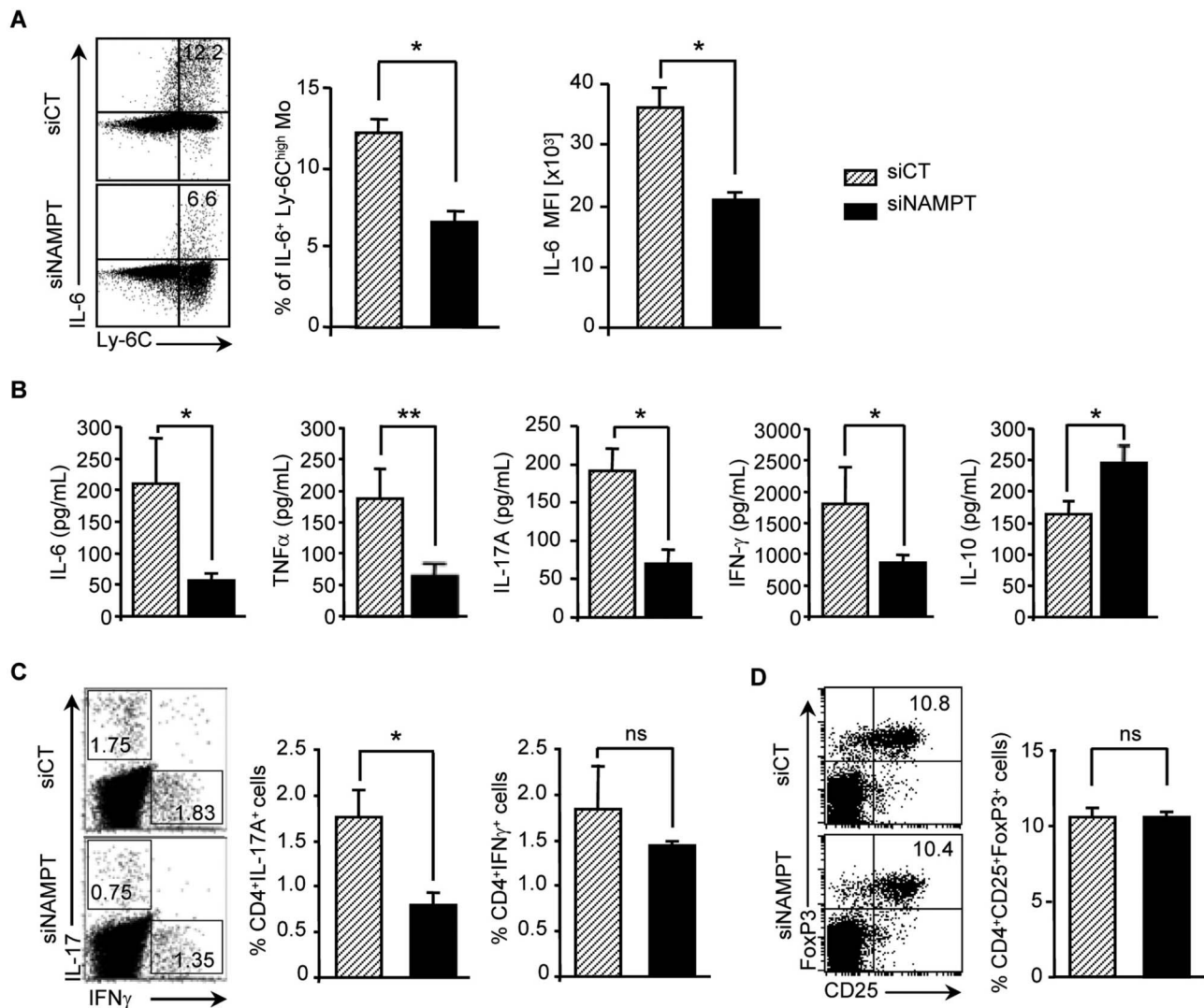


Figure 3 In-vivo inhibition of nicotinamide phosphoribosyltransferase (NAMPT)/interleukin (IL)-6 axis in Ly6C^{high} monocytes reduces inflammatory responses in collagen-induced arthritis (CIA). DBA/1 mice were immunised with bovine type II collagen and boosted on day 21. Arthritic mice (arthritis score >3) were weekly injected intravenously with 0.5 mg/kg of either anti-NAMPT siRNA (siNAMPT, black bars) or non-targeting control siRNA (siCT, hatched bars) formulated as lipoplexes. Mice were killed on day 45 and spleens were harvested. Splenocytes were cultured in 24-well format plates for 24 h with anti-CD3/CD28 antibodies. (A) IL-6 expression and production were determined by intracellular staining among Ly6C^{high} monocytes gated on CD11b^{high} F4/80^{int} cells and analysed by flow cytometry. IL-6 expression levels were quantified by mean fluorescence intensity (MFI). (B) Secretion of cytokines was measured in supernatants of cultured splenic cells of mice from the different groups using ELISA specific for mouse IL-6, IL-10, IL-17A, tumour necrosis factor alpha (TNF α) or interferon (IFN)- γ . (C) The percentage of IL-17A⁺ and IFN γ ⁺ CD4⁺ T cells was determined by intracellular staining. (D) The percentage of CD4⁺CD25⁺Foxp3⁺ T lymphocytes was determined by intracellular staining. Data in A represent the mean \pm SEM of three mice per group and are representative of one experiment out of two. Results in B–D are the mean \pm SEM of five to 10 mice per group and refer to one representative experiment out of three. * p <0.05, ** p <0.01, versus control.

joints. In arthritic joints, TNF α is one of the most deleterious cytokines implicated in inflammatory processes, and we showed that NAMPT downregulation in Ly6C^{high} monocytes leads to a significant decrease of TNF α expression by their main producers, ie, monocytes and macrophages. Our in-vitro and in-vivo studies performed on human CD14 and mouse Ly6C^{high} monocytes, respectively, showed that one of the downstream effects of knocking down NAMPT expression in this cell subset was a decreased IL-6 expression by these cells, associated with an impaired Th17 cell expansion. We cannot exclude the possibility that NAMPT silencing may affect genes other than IL-6 and TNF α and is likely to involve other mechanisms of immune modulation accounting for the observed beneficial effects. However, we can exclude the fact that the defect of Ly6C^{high}

monocyte recruitment observed in inflamed joints from siNAMPT-treated mice was due to a reduced expression of the CCR2/MCP1 axis (data not shown). Interestingly, although NAMPT gene silencing in Ly6C^{high} monocytes was associated with a markedly reduced frequency of IL-17A-producing CD4⁺ T cells, the numbers and percentage of Th1 and Treg cells were unchanged. Consistent with these findings, it has been reported that the administration of an anti-IL-6 receptor suppressed arthritis and inhibited Th17 differentiation but did not alter Th1, Th2 or Treg cell levels.^{36–38} Importantly, clinical benefits demonstrated in the trials in which RA patients were treated with the neutralising anti-IL-6R monoclonal antibody tocilizumab showed reduced IL-6 serum levels, dampened T and B-lymphocyte-mediated inflammatory responses, and suppressed

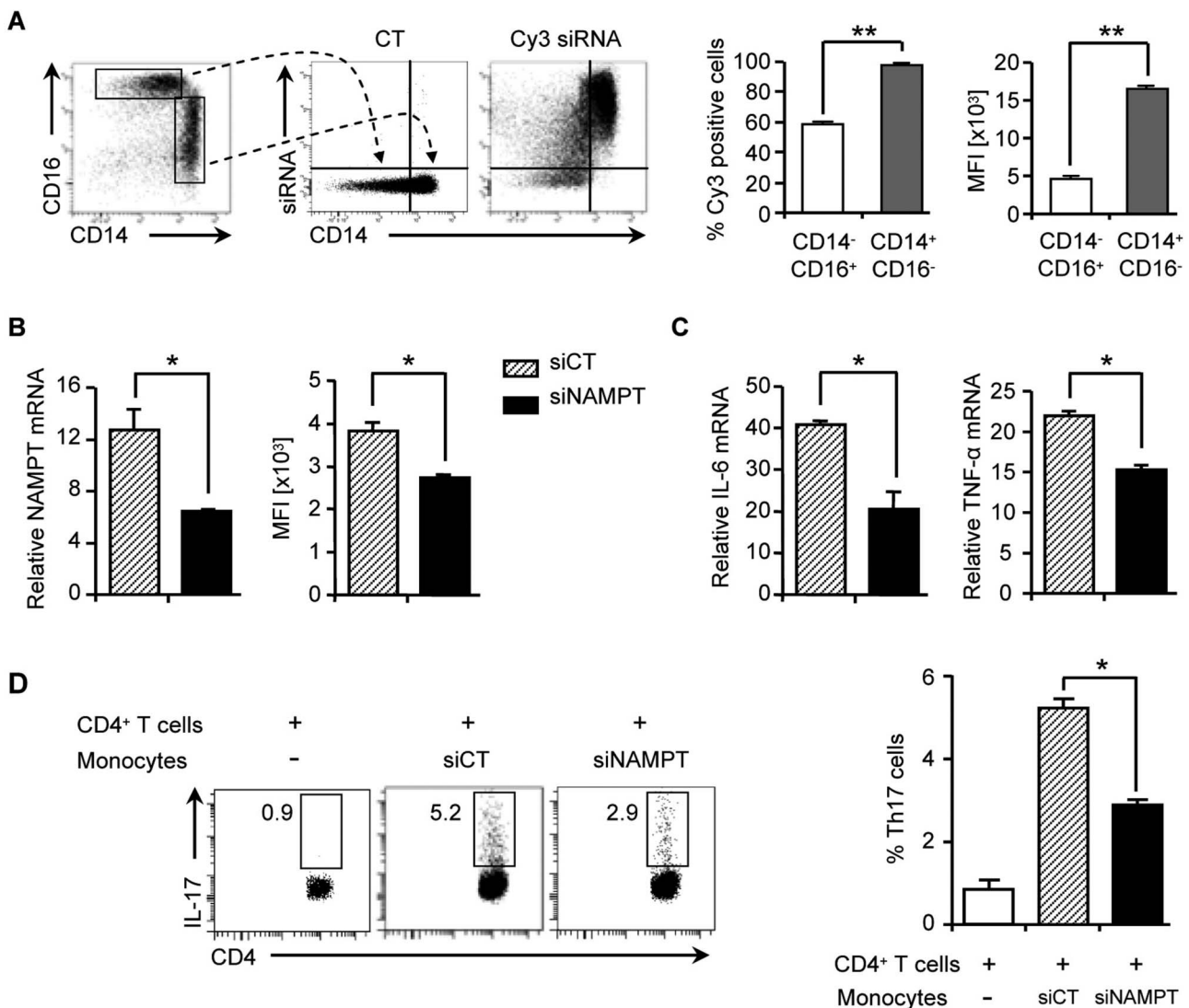


Figure 4 In-vitro silencing of nicotinamide phosphoribosyltransferase (NAMPT) in human CD14 monocytes interferes with T-helper (Th) type 17 cell expansion. Total human peripheral blood mononuclear cells (PBMC) (A) or purified CD14 cells (B–D) were transfected with siRNA lipoplexes as described in the Methods section. (A) The uptake of the formulation by monocytes was assessed by flow cytometry using Cy3-labelled siRNA. Monocytes were defined as CD3⁻, CD56⁻, CD19⁻, HLA-DR⁺ and on the basis on CD16/CD14 surface markers. The lipofection efficiency was quantified using the percentage of Cy3-positive cells and the mean fluorescence intensity (MFI) in the two human monocyte subsets. (B–D) Isolated human CD14⁺ monocytes were transfected with either non-targeting control siRNA (siCT, hatched bars) or anti-NAMPT siRNA (siNAMPT, black bars) for 48 h and stimulated with 100 ng/ml lipopolysaccharide during six additional hours. (B) Inhibition of NAMPT expression at mRNA and protein levels was quantified by reverse transcription quantitated (RT-q)PCR and fluorescence-activated cell sorter analyses, respectively. (C) Levels of downstream pro-inflammatory cytokines tumour necrosis factor alpha (TNF α) and interleukin (IL)-6 were measured by RT-qPCR. (D) Autologous CD4 T cells from healthy donors were co-cultured or not with lipopolysaccharide-stimulated monocytes, transfected with siRNA lipoplexes as indicated. After 5 days, cells were stimulated for 3 h with PMA/ionomycin and IL-17-producing CD4 T cells were analysed by flow cytometry. The mean \pm SEM of the percentage of Th17 cells is shown for triplicates per group and refers to one representative experiment out of three. * p <0.05, ** p <0.001, versus control.

x-ray progression of disease. In conclusion, we demonstrate that NAMPT expression in Ly6C^{high} monocytes participates in arthritis progression, not only confirming that NAMPT plays an important role in inflammatory responses, but also suggesting that Ly6C^{high} monocytes are among the key cellular mediators of these pro-inflammatory actions.

RA is a chronic inflammatory disorder in which CD14 monocytes continuously migrate into the injured joint tissue^{8 39 40} and act as local and systemic amplifiers of disease through the multitude and abundance of their paracrine/autocrine acting mediators.¹ Their numbers are increased in clinically affected joints and correlate with clinical signs,⁴¹ and thus strategies

aimed at interfering with this specific cell subset to provide therapeutic benefit are intensely challenged. To date, two main approaches have been used to impact on monocyte survival or recruitment, either by their depleting or by antagonising chemokine receptors important in their trafficking.⁴² Although these strategies showed promising results in animal models of RA, none of them was effective in RA patients.⁴³ The present work suggests that alternative strategies might be envisioned such as silencing the expression of a master gene implicated in pro-inflammatory functions of Ly6C^{high} monocytes to modify their activation status, their capacity to prime T and B-cell responses, to activate neutrophils and to alter overall disease

progression. The present study thus indicates that strategies based on the 'Trojan monocyte' for drug delivery to inflamed sites are of particular interest in arthritis, and may be applied to a broad range of inflammatory disorders.

As NAMPT is expressed in many cell types and exerts multiple functions, the latter strategy, however, requires overcoming the major challenges in achieving efficient in-vivo RNAi, ie, being able to deliver therapeutic siRNA to those cells. Previously, we have shown that, on intravenous administration, the cationic liposome DMAPAP formulation distributes siRNA to the myeloid compartment.³³ Here, we further refined the siRNA distribution to the respective monocyte subsets and demonstrated that siRNA is preferentially taken up by the Ly6C^{high} monocyte subset, as opposed to Ly6C^{low} monocytes, in the blood, spleen, liver and arthritic joints (data not shown), resulting in efficient and specific silencing of NAMPT in Ly6C^{high} monocytes. Although to a lesser extent, siRNA uptake was also detected in tissue resident macrophages and conventional dendritic cells, thought to arise from Ly6C^{high} monocytes under inflammatory conditions.⁴⁴ Importantly, we show here that siRNA was not efficiently taken up by non-haematopoietic cells and only very weakly incorporated into T and B lymphocytes or neutrophils (<2% of positive cells) and that no variation in NAMPT expression was evidenced in Ly6C^{low} monocytes. Our in-vitro data on human peripheral blood mononuclear cells show that the liposome formulation enables the efficient delivery of siRNA to classic CD14⁺CD16⁻ monocytes. To a lesser extent, siRNA was also taken up by non-classic CD14^{dim}CD16⁺ monocytes. However the amount of siRNA engulfed by this subset (showed by MFI) is very low compared to CD14⁺CD16⁻ monocytes. Although comparing efficiencies between in-vitro and in-vivo experiments is not relevant, these data suggest that different mechanisms might be involved in cationic liposome uptake by monocyte subsets, and perhaps between human and mouse monocytes, that need to be further characterised.

Several studies have demonstrated the importance of Ly6C^{high} monocytes in the pathogenesis of inflammatory disorders. In experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis, Ly6C^{high} monocytes accumulate in the blood and migrate to the central nervous system before disease onset, where they differentiate into dendritic cells and macrophages in experimental autoimmune encephalomyelitis lesions.⁴⁵ Moreover, increased numbers of circulating Ly6C^{high} monocytes are associated with enhanced disease severity. In a mouse model of myocardial infarct, Ly6C^{high} monocytes were found first to migrate to the site of inflammation during the acute phase.⁴⁶ Finally, when the accumulation of Ly6C^{high} monocytes in sites of inflammation was prevented by silencing CCR2 expression, a reduction in atherosclerotic lesions in apoE^{-/-} mice and of myocardial ischaemia injury was reported, as well as a prolonged islet graft survival.²⁶ Altogether, these studies underscore the contribution of Ly6C^{high} monocytes to pathological events in a broad range of inflammatory and autoimmune disorders. The specific implication of Ly6C^{high} monocytes was not, however, determined in arthritis. Here, using the experimental mouse CIA model, we showed that Ly6C^{high} monocytes also play a key role in arthritis pathogenesis.

Our findings identify NAMPT as a critical gene that can be targeted to modulate pro-inflammatory cytokines produced by Ly6C^{high} monocytes, to impact on their crosstalk with other immune cells and to interfere with inflammatory responses in arthritis. The downstream molecular mechanisms responsible

for the anti-inflammatory effects of NAMPT-mediated silencing in Ly6C^{high} monocytes, however, remain to be elucidated.

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