Supplemental Materials and Methods

Patient samples

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (GE Healthcare, Milan, Italy) centrifugation, and cultured in RPMI-1640 with 5% or 10% FCS, 50 μg/mL gentamicin, 100 U/mL penicillin, 100 μg/mL streptomycin (all from Sigma, Milan, Italy).

Sample analyses included CD38 and ZAP-70 stainings (cutoff positivity values of 20%), IgHV mutational status (unmutated if ≥98% homology to the germline gene) and fluorescence in situ hybridization for chromosomes 11, 12, 13 and 17. The clinic and biologic characteristics of the patients are summarized in Supplemental Table 1.

Antibodies and reagents

For immunoprecipitation and western blot antibodies used were: mouse monoclonal Ab anti-NAMPT (clone OMNI379, Adipogen International, Vinci-Biochem, Vinci, Italy), rabbit Ab Affinity Purified anti-NAMPT (Bethyl Laboratories, Montgomery, TX), anti-Phospho-BTK (Tyr223) and anti-total BTK (both from Cell Signaling Technologies, Danvers, MA), anti-actin horseradish peroxidase (HRP)–conjugated (Abcam, Cambridge, UK). Secondary reagents were: goat anti-mouse IgG-HRP conjugated (GαMIgG-HRP; Perkin Elmer, Monza, Italy), goat anti-rabbit HRP-conjugated (GαR-HRP; Santa Cruz Biotechnology, Segrate (MI), Italy). For confocal immunofluorescence studies primary antibodies were: anti-phospho-STAT3 (Tyr705, Cell Signaling Technologies), anti-phospho-ERK1/2 and anti-p65/NF-kB (both from BD Biosciences), anti-CD68 AlexaFluor 488-conjugated and anti-CD163 AlexaFluor 647 (both from BioLegend, Milan, Italy), anti-CD11b (Sigma), Phalloidin-AlexaFluor 568 (Invitrogen Life Technologies) and anti-caveolin-1 (BD Pharmigen). AlexaFluor 488 goat anti-mouse IgG (1:50), AlexaFluor 488 goat anti-rabbit IgG (1:100) and AlexaFluor 633 goat anti-mouse IgG (1:100) all from Invitrogen Life Technologies and Dylight 594 donkey anti-rabbit IgG (1:300 Jackson
ImmunoResearch, Cernusco Sul Naviglio, Italy) were used as secondary reagents. 4',6-Diamidino-2-phenylindole (DAPI, Life Technologies) was used for nuclear staining.

For FACS analyses antibodies used were anti-CD19-APC-Vio770 (Miltenyi Biotech, Calderara di Reno (BO), Italy), anti-CD19-PE, anti-CD4-PerCP anti-CD5-FITC, anti-CD3-PE, anti-CD14-PE, anti-CD4-PE-Cy5, anti-CD8-FITC and-PE, anti-CD11b-APC, anti-CD25-PE anti-CD80-PE-Cy5, anti-CD127-AlexaFluor 647, anti-CD206 AlexaFluor 488, and anti-PD-L1-PE (all from eBioscience), anti-CD68 AlexaFluor 488, anti-CD163 AlexaFluor 647 and anti-PD-1-PE-Cy7 (all from BioLegend). The anti-isotype control antibodies were purchased from the same companies.

For immunocytochemistry (ICC) antibodies used were: anti-CD11b (Sigma), anti-CD68 (Abcam), anti-mouse and anti-rabbit HRP-conjugated (EnVision™ System, Dako). For immunohistochemistry (IHC) and tissue immunofluorescence mouse anti-NAMPT (clone OMNI379, Adipogen), rabbit anti-CD11b (Sigma), goat anti-CD23 (R&D Systems, Milan, Italy), rabbit anti-CD163 (NOVUS Biologicals, Milan, Italy), rabbit anti-Ki-67 (Abcam) antibodies were employed.

Functional grade purified antibodies used to treat cells were: agonistic anti-CD38 (10 μg/ml, clone IB4, kind gift of Prof F. Malavasi, University of Turin, Italy), anti-CD40 (10 μg/ml, BioLegend) and polyclonal anti-IgM (10 μg/ml, Southern Biothec, Birmingham, AL).

Polyclonal NAMPT neutralizing antibody was generated as described. It was used at a final concentration of 50 μg/ml. Pre-immune goat serum at the same concentration was used as control.

Blocking mAb to human mannose receptor (CD206) was from InvivoGen (San Diego, CA), used at 5 μg/ml.

Commercial purified recombinant human NAMPT (rNAMPT, 200 ng/ml, Adipogen), was derived from HEK 293 cells, with endotoxin level <0.1EU/μg of protein (<0.01 ng/μg of protein) as determined by LAL test (product data sheet).
Other reagents were: nicotinamide mononucleotide (NMN), FK866, polymyxin B solution, FITC-DEXTRAN, Bay 11-7082 (10 µM) and S3I-201 (10 µM) all from Sigma, recombinant human IL-2 (100 IU/ml), IL-4 (50 ng/ml) and CXCL12 (100 ng/ml) all from R&D Systems, CpG oligonucleotide (1 µg/ml, InvivoGen), carboxyfluorescein succinimidyl ester (CFSE 5 µM, Invitrogen Life Technologies), Ibrutinib (10 µM, Selleckchem, Aurogene, Rome, Italy) and Giemsa (MERCK S.p.A., Vimodrone, Italy). Lenalidomide was from Celgene (San Diego, CA) and was dissolved in DMSO. A wild-type recombinant NAMPT protein and enzymatic mutant variant H247E were produced in Prof. Wolberger’s lab as detailed in 2.

**Immunocytochemistry**

After staining with primary antibodies, coverslips were fixed in methanol. Endogenous peroxidase activity was blocked with hydrogen peroxide (6% solution, Sigma, Milan, Italy) before staining with secondary antibodies. 3,3’-diaminobenzidine (EnVision™ System, Dako) was used to visualize the reaction before counterstaining with hemalum (MERCK-Millipore, Vimodrone, Italy).

**Light microscopy**

Immunocytochemistry and immunohistochemistry images were acquired using a CANON EOS 600D camera fitted to AXIO Lab. A1 ZEISS microscope (Arese, Italy).

**Cell culture conditions**

Purified CLL cells (3-5x10⁶/ml) were cultured for up to 5 days in complete medium. A combination of anti-CD38 monoclonal antibody (mAb, 10 µg/ml) + IL-2 (100 IU/ml), anti-CD40 mAb (10 µg/ml) + IL-4 (50 ng/ml), CpG + IL-2 (1 µg/ml and 100 IU/ml, respectively), immobilized IgM polyclonal antibody (10 µg/ml) were added. Where indicated cells were pre-treated with Ibrutinib (10 µM, 30 minutes, 37 ºC). PBMCs, sorted monocytes or NLC were treated with rNAMPT or the mutant form for indicated times. As internal control, in a set of experiments culture medium was supplemented with polymixin B (5 µg/ml) to efficiently block endotoxin-induced cytokine production.
NLCs were pre-treated with NF-kB and STAT3 inhibitors (Bay 11-7082 and S3I-201, respectively, both used at 10 μM) for 60 minutes at 37 °C, where indicated.

**NAMPT activity determination in lymphocyte extracts and CLL plasma**

The assay allows determination of NAMPT activity by converting NMN to NAD, via three consecutive reactions consisting of NMN deamidation to NaMN, NaMN adenylyation to NaAD, and NaAD conversion to NAD. The reactions are catalyzed by the bacterial recombinant enzymes NMN deamidase (PncC), NaMN adenylyltransferase (NadD) and NAD synthetase (NadE). The formed NAD is finally quantitated by the fluorometric cycling assay.³

Cells (2x10⁷) were washed twice with PBS, and cell pellets were homogenized with 200 μl of 50 mM TRIS/HCl, pH 7.5, 0.15 M NaCl, 1 mM DTT, 1 mM PMSF, 0.002 mg/ml leupeptin, antipain, chymostatin and pepstatin. Homogenates were centrifuged (20,000xg, 10 minutes) and the supernatants immediately used. Protein concentration was carried out according to ⁴. CLL plasma was collected from heparinized blood from CLL patients by centrifugation at 800 rpm (5 minutes). Alcohol dehydrogenase (ADH) was included in the multi-coupled reaction mixtures to allow reduction of interfering endogenous NAD during the incubation. The acidic treatment used to stop the reaction caused destruction of the formed NADH, which would interfere with the cycling assay. The assay mixture contained ethanol buffer (30 mM HEPES, pH 8.0, 1 % v/v ethanol, 8.4 mg/ml semicarbazide), 6 Units/ml ADH (Sigma A3263), 0.067 mg/ml BSA, 40 mM HEPES, pH 7.5, 10 mM MgCl₂, 10 mM NaF, 2.5 mM ATP, 1.0 mM PRPP, 0.05 mM Nam, 0.5 Units/ml NadD, 0.1 Units/ml PncC, and 0.2 mg/ml cell extract, or 66 % (v/v) plasma. A control mixture in the presence of 5 μM FK866 was also processed in parallel. Reaction mixtures were incubated at 37°C, and at suitable time intervals 30 μl aliquots were withdrawn and added with half their volume of 1.2 M cold HClO₄ to stop the reaction. After 5 minutes on ice, samples were centrifuged (16,000xg, 3 minutes), and supernatants were neutralized with 1 M K₂CO₃. After centrifugation, 20 μl aliquots
were transferred into a flat-bottom 96-well black plate. NaAD conversion to NAD was started by adding 50 mM HEPES, pH 7.5, 0.15 M KCl, 1.4 mM ATP, 50 mM NH₄Cl, 11 mM MgCl₂ and 0.06 U/ml NadE, to a final volume of 145 μl. After incubation (30 minutes, 37 °C) the NAD cycling reaction was started by adding 96 μl of cycling reagent to each well. The amount of NaAD present in the wells, corresponding to the amount of NMN formed by the enzyme, was calculated by interpolating on a NaAD standard curve the corresponding rate of fluorescence increase, after subtracting the respective control. All measurements were performed in duplicate.

**NAD and NMN determination**

Nucleotides were extracted from B lymphocytes, by resuspending pellets from 10⁶ cells in 0.4 M HClO₄. After 5 minutes on ice, samples were centrifuged (16,000xg, 5 minutes) and pellets resuspended in 100 μl of formic acid, incubated (5 minutes, 37 °C) and used for the determination of the protein concentration according to ⁴. Supernatants were neutralized with 1 M K₂CO₃, after 20 minutes at -80 °C they were centrifuged as above and suitable aliquots were used for NAD and NMN determination.

Extraction of NMN from plasma and media was carried out by adding 250 μl of samples to 750 μl boiling 75% ethanol in 10 mM HEPES, pH 7.5. After 1 minute at 100 °C and centrifugation at 16,000xg for 5 minutes, pellets were treated as above for protein determination, and supernatants were dried by speed-vac. The obtained pellets were resuspended in 50 μl water, and used for NMN determination.

NMN was measured upon derivatization with acetophenone and spectrofluorometric HPLC analysis, carried out as described in ⁵, with minor modifications. Briefly, up to 50 μl of the nucleotide extract were mixed with 100 μl KOH 1N and 50 μl acetophenone. The solution was incubated (15 minutes, 4 °C) before adding 100 μl of formic acid (5 minutes, 100 °C). Samples were then injected into an HPLC system, consisting of a Supelcosil C18 T column (25 cm x 4.6 mm, 5 μM)
and a fluorimetric detector with excitation and emission wavelength of 332 and 454 nm, respectively. The column was equilibrated with 0.1 M potassium phosphate buffer, pH 2.12 (buffer A), and elution was carried out with a discontinuous gradient from buffer A to buffer A, containing 40% acetonitrile. Measurements were performed by using a duplicate sample analyzed in parallel and containing a known amount of NMN spike.

Primers for qRT-PCR

Commercially available primers (TaqMan Gene Expression Assays; Life Technologies) used were: Hs00237184_m1 (NAMPT), Hs00985639_m1 (IL-6), Hs00174103_m1 (IL-8), Hs00234142_m1 (CCL3), Hs00268113_m1 (CCL18), Hs01574247_m1 (CCL22), Hs00961622_m1 (IL-10), Hs01073447_m1 (IL-12A), Hs01125301_m1 (PD-L1), Hs00984148_m1 (IDO1), Hs01056533_m1 (IRF4), Hs00998133_m1 (TGFB1) and Hs01113624_g1 (TNF). Actin (ACTB) and B2-microglobulin (B2M) were used as housekeeping genes: Hs99999903_m1 (ACTB) and Hs00984230_m1 (B2M).

Reactions were done in triplicate from the same cDNA reaction (technical replicates).

Supplemental References


Supplemental Table 1. Main clinical and molecular characteristics of the cohort of 130 patients

Analyses included CD38 and ZAP-70 stainings [cut-off positivity values of ≥20%], IgVH mutational status (unmutated UM if ≥98% homology to the germline gene) and fluorescence in situ hybridization (FISH) for chromosomes 11, 12, 13 and 17.

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Supplemental Figure Legends

Supplemental Figure 1. Ibrutinib inhibits CCL3 and NAMPT transcription induced by CLL activation. (A) Box plots showing expression of CCL3 mRNA in purified CLL lymphocytes (n=10) cultured for 24 hours in the presence of the indicated signals. Where specified, cells were pre-treated with Ibrutinib (30 minutes, 10 µM). (B) qRT-PCR analysis showing expression of NAMPT mRNA in purified CLL lymphocytes cultured with the indicated stimuli (24 hours). When indicated, cells were pre-treated with Ibrutinib. (C) Purified CLL cells activated as indicated in (B) were lysed and iNAMPT expression levels determined by western blot. Cumulative results (n=5) are shown in the box plot. (D) Representative experiments showing phosphorylation of BTK upon BCR cross-linking with or without Ibrutinib pre-treatment (n=3). (E) Box plot showing Annexin-FITC (AV) and propidium iodide (PI) staining of CD19+ cells upon BCR cross-linking with or without Ibrutinib pre-treatment (n=5). The graph on the right shows NAMPT expression and the corresponding percentage of viable cell in each condition in a representative patient.

Supplemental Figure 2. Biochemical characterization of eNAMPT and nicotinamide mononucleotide (NMN) in CLL plasma and in CLL culture supernatants. (A) The nature of the protein detected by the eNAMPT ELISA assay was confirmed by biochemical analyses. Western blot showing 10x-albumin depleted RPMI-1640 + 10% FCS alone (10x med) or added with 20 ng/ml rNAMPT (10x med + rNAMPT). The NAMPT-deprived fractions from medium alone or from medium + rNAMPT were obtained by immunoprecipitation using anti-NAMPT (OMNI379) mAb. rNAMPT was loaded as positive control. (B) Representative chromatogram of concentrated plasma, after NMN derivatization with acetophenone and spectrofluorometric HPLC analysis. No NMN is detectable in CLL plasma (blue line). In the inset an enlargement of the chromatogram section corresponding to NMN elution time. Representative chromatogram of 14 plasma samples tested.
Supplemental Figure 3. Effects of exposure of purified CLL cells to rNAMPT. (A) CFSE-labeled-CLL cells were treated for 5 days with the indicated signals. Graph shows cumulative data (n=10). (B) Box plots showing cumulative results of chemotaxis experiments performed using rNAMPT both in the upper (upper ch.) and the lower chambers (low ch.). CXCL12 was used as the positive control. (C) Dot plots showing Annexin-FITC (AV) and propidium iodide (PI) staining of CLL cells cultured with or without rNAMPT for 5 days. The graph represents cumulative data. (D-F) qRT-PCR analysis showing expression of TNF-α (D), TGF-β (E) and CCL3 (F) mRNA in purified CLL lymphocytes treated with rNAMPT (24 hours).

Supplemental Figure 4. rNAMPT induces macrophage differentiation from circulating monocytes from HD donors. (A) FACS analyses of PBMC preparations from HD individuals in untreated condition and after rNAMPT exposure for 5 days. An increased number of monocytes/macrophages (on the basis of morphological gate) was highlighted in +rNAMPT panels. (B-C) PBMC preparations from HD were plated in complete medium with or without rNAMPT. After 5 days cells were fixed and stained for CD11b. Box plot in (B) shows cumulative data showing the number of CD11b+ cells in at least 4 different ×20 fields obtained from 7 independent HD samples. (C) Differentiated cells were grown on coverslips and tested for CD11b expression by immunocytochemistry. Original magnification ×20.

Supplemental Figure 5. rNAMPT induces macrophage differentiation of circulating monocytes from CLL patients. (A) FACS analyses of PBMC preparations from CLL patients in untreated condition and after rNAMPT exposure for 5 days. An increased number of monocytes/macrophages (on the basis of morphological gate) was highlighted in +NAMPT panels. (B-C) Cellular morphology was analyzed by Giemsa staining. The graph in (B) summarizes data from the 15 patients analyzed. The number of macrophage-like cells was calculated counting cells in at least 4 different ×40 fields. (C) Giemsa staining in a representative patient. Original
magnification ×40. (D) CD68 immunofluorescence staining on the same cells described in (B-C) analyzed by confocal microscopy. Original magnification ×20. (E) CD14⁺ monocytes from CLL patients were flow sorted and cultured with rNAMPT, confirming an increase in the number of differentiated cells. Original magnification x20.

**Supplemental Figure 6.** The monocyte subset of CLL patients shows constitutive M2 skewing. (A) qRT-PCR analysis for *IRF4* and *IL-12* in purified CD14⁺ monocytes from CLL patients and HD individuals. (B) FACS analysis of CD163 and CD206 expression on CD14⁺ circulating monocytes from CLL patients (n=15) and HD (=5) individuals.

**Supplemental Figure 7.** rNAMPT induces HD monocytes to acquire M2-like features. (A) Box plots showing qRT-PCR analysis of M2 cytokines expression in purified CD14⁺ monocytes from HD individuals treated for 24 hours with rNAMPT. (B) Confocal microscopy analyses revealed increased fluorescence intensity of CD206 on purified HD CD14⁺ monocytes in the presence of rNAMPT in culture. Original magnification x63. Box plots show cumulative analyses of pixel intensity (arbitrary units) of all the fluorescent measurements after fixing a region of interest corresponding to each cell in at least 4 fields for different samples.

**Supplemental Figure 8.** rNAMPT enhances M2 macrophage polarization of NLC. (A) Histogram showing the absence of CD80 expression on NLC checked by FACS. (B-D) Confocal microscopy analyses revealed increased fluorescence intensity of CD11b and CD68 on NLC generated in the presence of rNAMPT. Original magnification x63. Box plots show cumulative analyses of pixel intensity (arbitrary units) of all the fluorescent measurements for CD11b (C) and CD68 (D) after fixing a region of interest corresponding to each cell in at least 4 fields for different samples. (E) Box plot showing *IRF4* mRNA expression levels in NLC from CLL patients obtained using conventional methods or differentiated in the presence of lenalidomide (n=6). (F) Heat map showing *NAMPT* gene expression profiling in monocyte (MO), T and B lymphocyte (Ly T and LyB
respectively) flow sorted populations from CLL and HD samples. NAMPT expression was represented as Log2 of RQ calculated as relative expression on ACTB housekeeping gene.

**Supplemental Figure 9. NAMPT is expressed in the proliferation centers of CLL LN. (A-B)** Immunohistochemical analysis of CD68 and CD11b expression on macrophages in reactive and CLL LN. Images at x20 original magnifications. **(C)** Immunofluorescence images showing partial overlap between NAMPT staining (shown in red) and CD163 (white) in CLL LN samples. **(D-E)** Within the proliferation center however, NAMPT appears to have a broader reactivity, showing partial colocalization with CD23⁺/Ki-67⁺ proliferating CLL lymphocytes. Original magnification x63. **(F)** Box plot showing cumulative analyses of NAMPT mean pixel intensity (arbitrary units) near Ki-67⁺ and in Ki-67⁻ areas.

**Supplemental Figure 10. A neutralizing pAb anti-NAMPT and specific inhibitors of NF-kB and STAT3 drastically block NAMPT-induced signaling in NLCs. (A-C)** NLCs were treated (30 minutes) with rNAMPT or with a combination of rNAMPT and anti-NAMPT blocking pAb (50 μg/ml). Cells were then stained for the presence of p-STAT3 (green fluorescence). DAPI (blue) was used to visualize nuclei. Cumulative data (n=5) are shown in **(D)**. **(E-I)** qRT-PCR analysis showing inhibition of CCL18, IL-10, IL-6, IL-8 and CCL3 gene expression in NLC pre-treated with NF-kB and STAT3 inhibitors (Bay 11-7082 and S3I-201, 10 μM respectively) for 60 minutes at 37 °C, before adding rNAMPT (24 hours). Cumulative data from 4 independent experiments are shown in box plots.

**Supplemental Figure 11. Phagocytosis of FITC-conjugated dextran particles is independent of CD206. (A)** Conventionally obtained NLC (n=6) were pre-treated for 1 hour with neutralizing mAb anti-CD206 (5 μg/ml) and then incubated (15 minutes, 37 °C) with FITC-DEXTRAN (1 mg/ml in PBS + 5% FCS) with or without rNAMPT. Phagocytosis was confirmed by confocal microscopy analysis. Original magnification x63. Box plots show cumulative analyses of pixel intensity (a. u.) in at least 4 fields for the different samples.
Supplemental Figure 12. The mutant NAMPT H247E induces NLCs differentiation as the wild-type. NLCs were generated in medium alone or in the presence of a functional rNAMPT protein or mutant H247E at the same concentration (200 ng/ml). NLCs Giemsa staining (A) and CD68 ICC staining (B) are shown. Original magnification ×20.
Supplemental Figures

SUPPLEMENTARY FIGURE 1
SUPPLEMENTARY FIGURE 2
SUPPLEMENTARY FIGURE 3
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SUPPLEMENTARY FIGURE 4
SUPPLEMENTARY FIGURE 5
SUPPLEMENTARY FIGURE 6
SUPPLEMENTARY FIGURE 7
SUPPLEMENTARY FIGURE 8
SUPPLEMENTARY FIGURE 9
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SUPPLEMENTARY FIGURE 12