BIOCHEMICAL PHARMACOLOGY 73 (2007) 831–842

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Nicotinamide inhibits B lymphocyte activation by disrupting MAPK signal transduction

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ARTICLE INFO

Article history:
Received 31 August 2006
Accepted 27 November 2006

Keywords:
B lymphocytes
Vitamines
Nicotinamide
Immunosuppression
ADP-ribosyl transferases
Meta-iodobenzylguanidine

ABSTRACT

Nicotinamide (NAm) represents both a pharmacological agent known to express cell preserving and anti-inflammatory properties, and a useful investigational tool to elucidate cellular pathways regulating a wide range of cellular functions. We demonstrate in this study that exogenous NAm, when used at pharmacological doses, inhibits activation of primary murine B lymphocytes in response to multiple ligands. NAm appears to affect a membrane proximal event leading to MAPKs activation, a transduction pathway shared by multiple receptors including the antigen-specific B cell receptor, CD38, CD40 and TLR4 receptors. NAm inhibited phospho-ERK accumulation, and only marginally affected phospho-p38 and phospho-JNK induction upon BCR stimulation of naive B lymphocytes. Accordingly, NAm also affected the expression of known targets of the MAPK ERK pathway such as CD69 and cyclin D2. Based on a comparison with well-characterized pharmacological inhibitors, we suggest in this work that NAm may inhibit a post-translational modification mediated by a yet unidentified mono(ADP-ribose)transferase. Collectively, our observations indicate that in addition to its previously described effect on cells of the innate immune system, NAm is able to modulate the activity of B lymphocytes suggesting a potential role of this vitamin in regulating antibody-mediated autoimmune disorders.

1. Introduction

Nicotinamide adenine dinucleotide (NAD) is known to play a major role as a co-enzyme in numerous oxidation–reduction reactions. Over the past decades, growing evidence has also stressed the non-redox functions of NAD in cellular physiology. In particular, NAD has been shown to serve as a substrate for reactions involved in covalent protein modification, and for the generation of small intracellular signalling molecules. Mono(ADP-ribose)transferases (ARTs) and poly(ADP-ribose) polymerases (PARPs) catalyze the transfer of the ADP-ribose moiety of NAD to acceptor proteins, a post-translational modification known to affect target protein function. Mono-ADP-ribosyltranferases (ARTs) represent a family of ectoenzymes including GPI-anchored membrane proteins with an extracellular catalytic domain (ART1 to ART4) and secreted forms (ART5 to ART7) that modify extracellular substrates by addition of an ADP-ribose to a specific residue [1]. In contrast, the enzymatic activity of PARPs (of which distinct 18 members have been identified in mammals) leads to the transfer of

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Abbreviations: NAD, nicotinamide adenine dinucleotide; NAm, nicotinamide; ART, mono(ADP-ribose)transferase; PARP, poly(ADP-ribose)polymerase

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doi:10.1016/j.bcp.2006.11.024

branched polymers of ADP-ribose to intracellular substrates (including nuclear proteins). This family of enzymes is involved in maintenance of genome integrity, cell viability, apoptosis and regulation of numerous cellular functions [2]. Recently, the important role of NAD as an essential cofactor in protein deacetylation catalyzed by selected members of a class of enzymes collectively known as ''sirtuins'' has also been uncovered. This family of enzymes is conserved from bacteria to mammals (for which seven members, SIRT1 to SIRT7 have been described) and regulates a wide range of biological processes including gene silencing, aging, cell survival in response to stress, cell differentiation and metabolism [3]. These enzymes catalyze a unique reaction in which the cleavage of NAD and the deacetylation of substrate are coupled to the formation of *O*-acetyl-ADP-ribose, a novel metabolite exhibiting biological effects when microinjected in living cells [4,5]. Moreover, the enzymatic activity of sirtuins involves an ADP-ribosyl transfer reaction, and it is noteworthy that at least one member of this family, SIRT6, displays no detectable deacetylase activity but is able to act as mono(ADP-ribose)transferase [6]. Finally, NAD also represent a precursor for the synthesis of signalling molecules such as cyclic ADP-ribose (cADPR) and nicotinic acid dinucleotide phosphate ($NAADP^+$), both known to represent Ca^{2+} mobilizing agents from intracellular stores [7,8].

All these ''NAD-consuming'' reactions result in the release of nicotinamide (NAm), which in turn serves as an endogenous end product inhibitor with distinct efficiency depending on the enzymatic activity considered. Numerous evidences indicate that NAm represent a pharmacological agent able to inhibit a broad range of ADP-ribosyl transferases. Noteworthy, nicotinamide, in conjunction with nicotinic acid, represents an essential vitamin (Vitamin B3 or niacin), acting as an exogenous precursor for NAD in most living organisms [9].

The ability of NAm to interfere with numerous enzymatic reactions involving NAD-consuming enzymes and its relatively low toxicity in vivo, has led numerous investigators to evaluate the effect of exogenous NAm on several physiological processes. Indeed, interest in NAm has recently shifted from its role as a nutrient to that as a novel pharmacological agent with possible beneficial effects on a wide range of pathologies including cancer, diabetes and infectious disease [10,11]. Despite the lack of precise knowledge on its mode of action, NAm has also been shown to display anti-inflammatory properties, including inhibition of inducible NO synthase and pro-inflammatory cytokine secretion from immune cells and suppression of MCH class II and intracellular adhesion molecule ICAM-1 expression by endothelial cells [12,13]. The reported effects of NAm on the innate immune system led us to consider the possibility that NAm may also affect the cells of the adaptive immune system, and the aim of the present study was therefore to evaluate the effect of NAm on primary murine B lymphocytes. Using a panel of in vitro and in vivo assays we demonstrate in this study that pharmacological doses of NAm inhibit B cell proliferation and activation in response to several ligands. NAm appears to selectively inhibit a membrane proximal event leading to the activation of the MAPK kinases in B lymphocytes, thus widening the possible range of physiological targets affected by this natural compound.

2. Materials and methods

2.1. Animals

Six- to eight-week-old female BALB/c mice were purchased from Harlan (Horst, The Netherlands) and housed in a pathogen-free environment. The experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by the local Committee.

2.2. Medium and reagents

The medium in all experiments was RPMI 1640 supplemented with 5% FCS (excepted for Western blotting experiment, 0.5%), penicillin, streptomycin, glutamine, non-essential amino acids, and 50 μM 2-ME.

Lipopolysaccharides from *Escherichia coli* 0111:B4 (LPS), Thapsigargin, Ionomycin, phorbol 12-myristate 13-acetate (PMA), nicotinamide (NAm), 6(5H)-phenanthridinone (Ptd), and *meta*-iodobenzylguanidine (MIBG) were obtained from Sigma–Aldrich (St. Louis, MO). Anti-IgM $F(ab')_2$ was from Jackson ImmunoResearch (Soham, Cambridgeshire, UK). Anti-CD40 (clone 1C10) and anti-CD38 (clone 90) were from eBioscience (San Diego, CA). Anti-PLCgamma2 (Q20) and anti-cyclin D2 (M20) were from SantaCruz Biotechnology (Heidelberg, Germany). U0126 and antibodies to phospho-p44/42 MAP kinase (Thr202/Tyr204), p44/42 MAP kinase, phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, phospho-p38 MAP kinase (Thr180/Tyr182), p38 MAP kinase alpha were purchased from Cell Signaling (Danvers, MA). Anti-phosphotyrosine (mouse mAb 4G10) was from Upstate Biotechnology (Dundee, UK).

2.3. B cell purification

B cells were prepared from spleen cell suspensions by positive magnetic selection. Briefly, cells were incubated with anti-CD19-conjugated magnetics beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and purified with MACS columns (Miltenyi Biotec), according to manufacturer's recommendations. In some experiments, B cells were isolated from spleen by depletion of non-B cells. Briefly, cells were labelled with biotinylated ter-119, anti-CD43 and anti-CD11b eBioscience (San Diego, CA). Cells were then incubated with streptavidin magnetic beads (Miltenyi Biotech) and passed on magnetic column (Miltenyi Biotech). The resulting cell population always contained >95% $CD45R^+$ B cells.

2.4. B cell functional assays

B cells (2.5×10^5 cells/well) were incubated for 2 days in complete medium in 96-well plates in the presence of the indicated agonists: anti-IgM (0–20 μg/ml), LPS (0–15 μg/ml), anti-CD40 (0–20 μg/ml), anti-CD38 (0–2 μg/ml) and a combination of Ionomycin (0–270 ng/ml) and PMA (10 ng/ml). Cell proliferation was revealed by $[^3H]$-thymidine (Amersham Bioscience Buckinghamshire, UK) incorporation at 48 h, or by evaluating the residual fluorescence of CSFE-labelled cells as described [14]. Briefly, B cells were incubated at 10^7 cell/ml in serum-free medium containing 10 μM of 5(6)-carboxyfluorescein diacetate

N-succinimidyl ester (CFSE from Sigma–Aldrich) for 10 min at 37 °C. The labelling was stopped by adding 5 vol. of ice-cold medium and, after two washes, CFSE-labelled cells were used for in vitro stimulation. Cells (2×10^6 cells/well) were incubated for 4 days in complete medium in 24-well plates in the presence of different agonist antibodies or stimulatory agents. Proliferation of cell was analyzed at 96 h by flow cytometry. The ability of stimulated B cells to secrete IgG1 antibodies was evaluated by standard ELISA, using the LO-MG1.2 and the LO-MG1.13 anti-mouse IgG1 monoclonal rat antibodies as, respectively, capture and revealing reagents (both purchased from Serotec, Oxford, UK). Standard curves were established using a source of normal mouse serum with known concentrations of IgG1.

2.5. Flow cytometry analysis

Cells were washed in FACS buffer (PBS containing 0.1% BSA and 0.01%, w/v, NaN3) and incubated for 30 min with FITC-labelled anti-CD69 mAb (clone H1.2F3 eBioscience, San Diego, CA), FITC-labelled anti-CD25 mAb (clone PC61, eBioscience), or PE-conjugated anti-CD45R mAb (clone RA3-6B2, eBioscience). Cells were washed and incubated for 5 min with propidium iodide (PI, Sigma-Aldrich). Cells were then washed and analyzed on a FACSort using CellQuest software (BD PharMingen, San Diego, CA).

2.6. Calcium analysis

Cells were washed in calcium/magnesium free HBSS and incubated at (5×10^6 cells/ml) with 0.25 mM sulfinpyrazone (Sigma-Aldrich), 100 μg/ml pluronic acid F-127 and 5 μM Fluo-4 (both from Molecular Probes, Leiden, The Netherlands). Loading was conducted at 37 °C for 30 min. Cells were washed twice in completed medium supplemented with 0.25 mM sulfinpyrazone and incubated 30 min in presence or in absence of NAm.

For stimulations, Fluo-4-loaded cells (400 μl, 2×10^6 cells/ml) were pre-incubated 5 min at 37 °C and analyzed for Fluo-4 emission at 525 nm with the CellQuest software (BD PharMingen), as previously described [14]. Baseline measurements were acquired for 15 s before stimulation with anti-IgM, Thapsigargin or Ionomycin during 2 min.

2.7. Western blotting and immunoprecipitation

B lymphocytes (2×10^7 cells/conditions) purified by positive selection (Figs. 4 and 5) or by negative selection (Fig. 2) were pre-incubated in medium supplemented with 0.5% FCS with different inhibitors (30 min with Ptd, 20 min with MIBG or NAm) and stimulated with anti-IgM (10 μg/ml) or a combination of Ionomycin (50 ng/ml) and PMA (10 ng/ml) for different times. The stimulation was stopped by addition of ice-cold PBS and centrifugation of cells. Proteins were extracted from B cells pellets with RIPA lysis buffer. In each experiment, equal amounts of protein for each condition (15–20 μg) were subjected to SDS-PAGE (10% polyacrylamide gels) followed by immunoblotting as previously described [15]. Immunoreactive proteins were detected by ECL (Amersham Biosciences).

For immunoprecipitation, cells were lysed in cell lysis buffer (100 mM NaCl, 20 mM Tris, pH 7.4, 10 mM NaF, 1% Brij 96) as previously described [16]. After 30 min on ice followed by centrifugation at $10{,}000 \times g$ for 15 min, cell lysates supernatant were pre-cleared with protein A-sepharose (Amersham Bioscience). The proteins were immunoprecipated with the PLCgamma2 mAb. The immunoprecipitates were washed three times with lysis buffer and analyzed by Western blotting.

2.8. In vivo treatment

Naive animals were first injected i.p. with 500 mg/kg of NAm or PBS and 1 h after with 800 μg/kg of LPS or PBS. The splenocytes were analyzed by FACS 18 h after the second injection.

2.9. Statistics

The data were expressed as the means ± S.D. Statistical analyses were performed with Graphpad Prism 3.0 (available from Graphpad Software at www.graphpad.com). The tests used for each experiment are described in corresponding legends.

3. Results

3.1. Nicotinamide inhibits both proliferation and the ability of primary murine B cells to secrete immunoglobulins in response to polyclonal stimulation

To evaluate the effect of exogenous NAm on BCR-induced cell proliferation, purified $CD19^+$ B cells from a naive mouse were stimulated in vitro with anti-IgM antibodies in the presence of graded doses of NAm. As shown in Fig. 1A and F, NAm effectively inhibited the proliferation of B cells in response to BCR stimulation. Note that both [^{3}H]-thymidine incorporation (an S-phase marker; Fig. 1A) and CFSE-based proliferation (measuring cell division and viability; Fig. 1F) assays were used to illustrate the ability of NAm to inhibit primary B cell proliferation. In marked contrast, B cell retained the ability to respond to a combination of pharmacological agents bypassing the early steps of signal transduction (Fig. 1E), suggesting that NAm had most probably interfered with a proximal event of the signalling cascade. To further evaluate the effect of this vitamin on signalling events controlling cell proliferation, B cells were stimulated with several ligands known to interact with distinct surface receptors. As shown in Fig. 1B–D (see legend for ligand identification), NAm inhibited B cell proliferation in response to anti-CD40 and anti-CD38 antibodies and in response to LPS, a TLR4 ligand. These observations suggest that NAm interferes with a membrane proximal event shared by most receptors controlling cell proliferation. Cyclin D2 controls the G1 to S phase transition in response to BCR stimulation, and is required for adequate B cell proliferation, representing therefore a useful target to validate the inhibitory role of NAm on B cell proliferation [17]. Quiescent splenic B lymphocytes were stimulated by anti-IgM antibodies for 24 h, and whole cell extracts examined by Western blot for cyclin D2 expression. As shown in Fig. 1G,

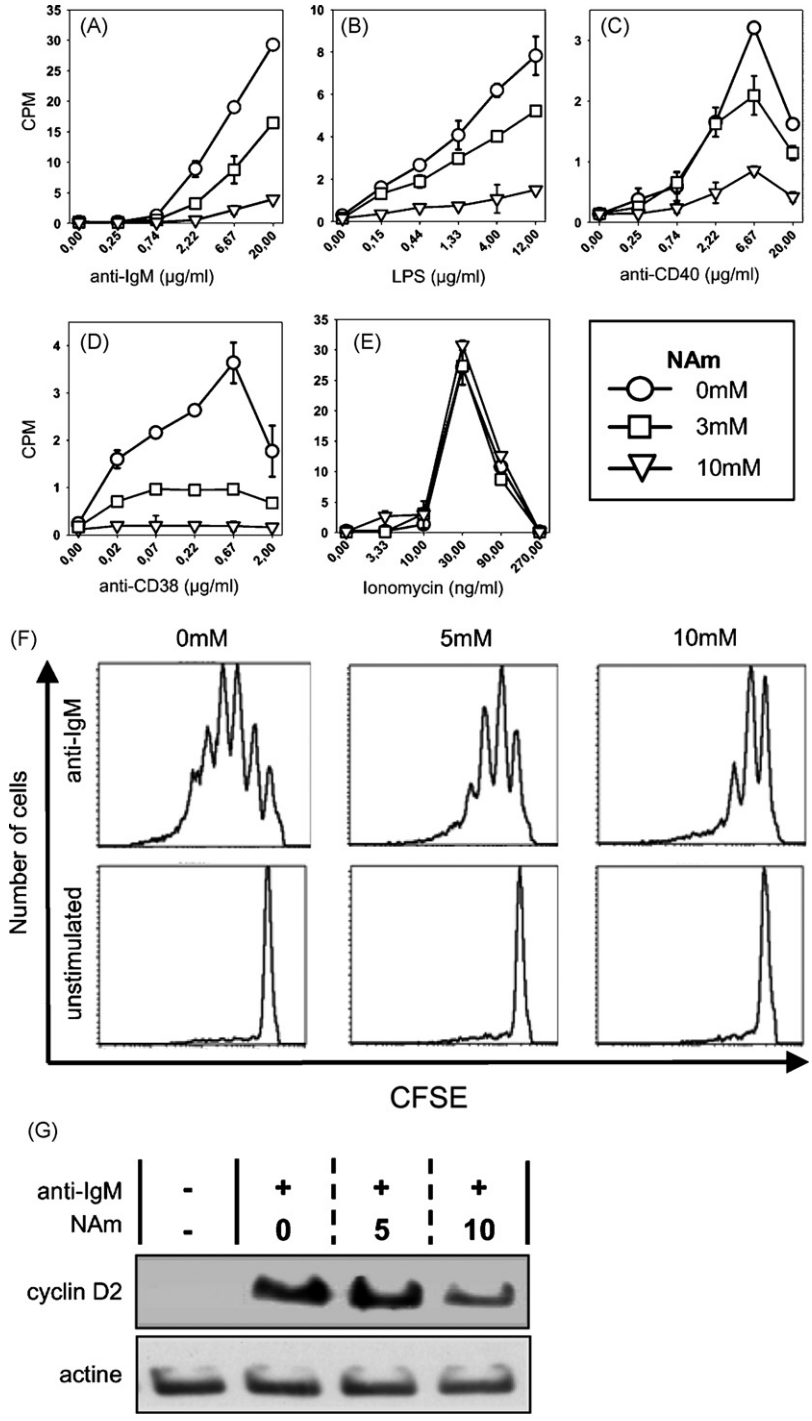

Fig. 1 – Sensitivity of mitogen-stimulated splenocyte growth to NAm. B lymphocytes were cultured for 2 days with the indicated mitogens in the presence or not of NAm (0, 3 or 10 mM) and proliferation was assessed by [^{3}H]thymidine incorporation at 42 h. (A) Anti-IgM, (B) LPS, (C) anti-CD40, (D) anti-CD38, (E) a combination of Ionomycin and PMA (10 ng/ml). This experiment has been reproduced three times with similar results. (F) Splenocytes were loaded with CFSE and stimulated with anti-IgM (10 μg/ml) in the presence of NAm (0, 5 or 10 mM) for 4 days. Spleen cells were gated on the basis of forward and side scatter, CD45R$^+$ B cells were gated and analyzed for the fluorescence of CFSE by flow cytometry. Similar results were obtained in three independent experiments. (G) B lymphocytes were stimulated with anti-IgM (3 μg/ml) in the presence or not of NAm (10 mM) for 1 day. Cells extracts were analyzed by Western blotting for the presence of cyclin D2. Actin was used to control that equal amounts of protein were loaded in each lane. Similar results were obtained in two independent experiments.

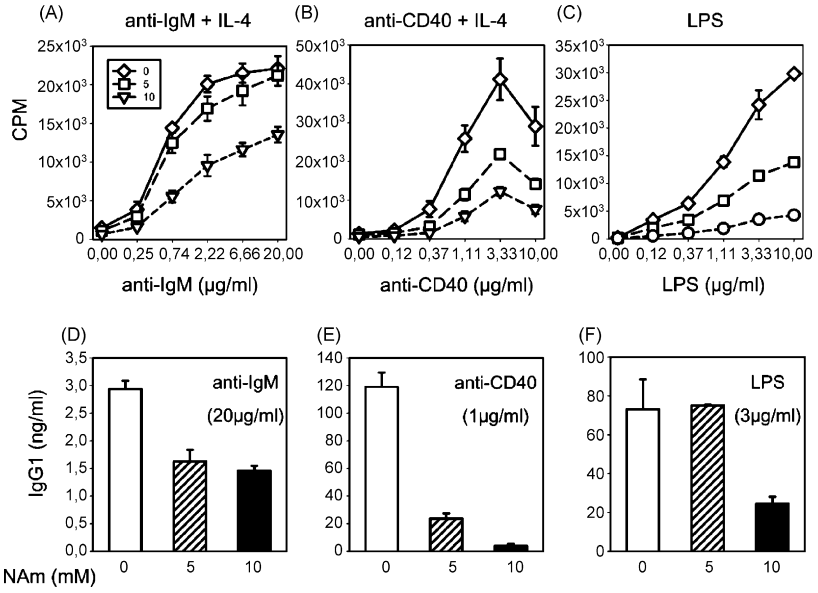

Fig. 2 – NAm affects the B cell function. (A–C) B lymphocytes were cultured for 2 days with the indicated mitogens in the presence or not of NAm (0, 5 or 10 mM) and proliferation was assessed by [^{3}H]thymidine incorporation at 42 h. (A) Anti-IgM (0–20 μg/ml) + IL-4 (20 ng/ml), (B) anti-CD40 (0–10 μg/ml) + IL-4 (20 ng/ml), (C) LPS (0–10 μg/ml). This experiment has been reproduced two times with similar results. (D–F) Splenocytes were cultured with the indicated mitogens in the presence or not of NAm (0, 5 or 10 mM) for 7 days and the IgG1 antibody titers in culture supernatants were estimated by ELISA, as described in Section 2.

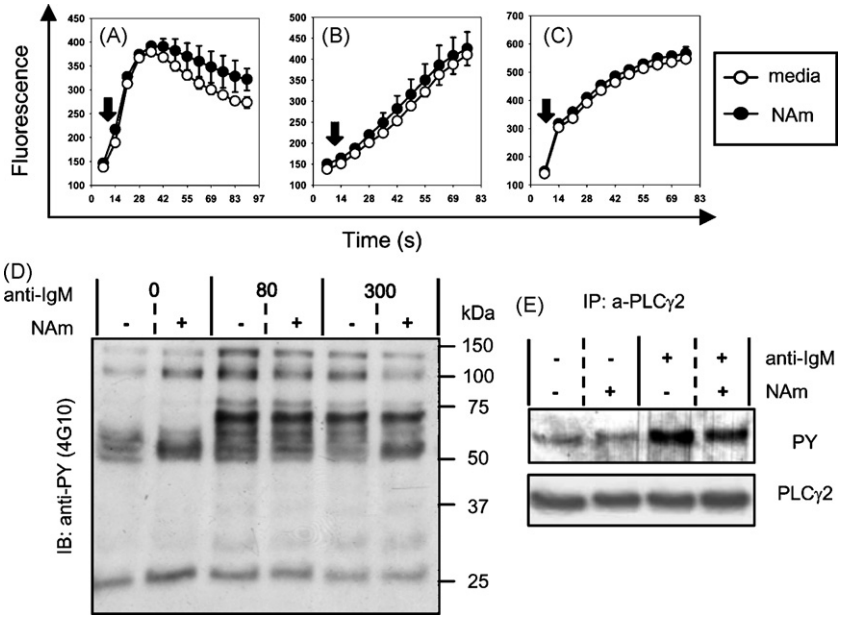

Fig. 3 – NAm does not affect early steps of BCR signalling. Splenocytes were loaded with Fluo4 and incubated with or without NAm (10 mM) for 30 min. Calcium response of control and NAm treated cells were analyzed by FACS. Spleen cells were gated on the basis of forward and side scatter, CD45R+ B cells were gated and analyzed for the fluorescence of Fluo-4. Basal fluorescence was monitored for 10 s and then cells were stimulated with (A) anti-IgM (5 μg/ml), (B) Thapsigargin (1 μg/ml) and (C) Ionomycin (100 ng/ml) (arrows). Fluorescence of cell was analyzed during 85 s. Graph represent mean of fluorescence ± S.D. Similar results were obtained in three independent experiments. (D) B lymphocytes were preincubated with or without NAm (10 mM) and stimulated with anti-IgM (10 μg/ml) for 80 or 300 s. Cell extracts were analyzed by Western blotting with an anti-phosphotyrosine antibody (4G10). (E) B lymphocytes were preincubated with or without NAm (10 mM) and stimulated with anti-IgM (10 μg/ml) for 90 s. Cell extracts were immunoprecipitated with the anti-PLCgamma2 antibody and analyzed by Western blotting with an antibody anti-phosphotyrosine (4G10) and anti-PLCgamma2 as control.

NAm significantly inhibited cyclin D2 protein expression induced by mitogenic stimuli in a dose dependent fashion. To evaluate the effect of NAm on B cell function, purified naive B cells were stimulated by polyclonal agents known to induce antibody secretion in vitro. In keeping with the previous observation, proliferation of B cells induced by anti-IgM, LPS, or anti-CD40 antibodies was inhibited by graded doses of NAm (Fig. 2A–C). Similarly, in vitro antibody production in response to the same ligands (in the presence of IL-4 as indicated in the figure legend) was sensitive to NAm (Fig. 2D–F), demonstrating thus that this vitamin was able to inhibit differentiation of naive B cells into antibody-producing cells.

3.2. *NAm does not inhibit the early BCR-induced calcium response*

BCR engagement induces a wave of phosphorylation events on tyrosine residues leading to the activation of PLCgamma2, an enzyme that ultimately leads to Ca^{2+} mobilization through the generation of second messengers such as inositol trisphosphate (IP_3). To evaluate the effect of NAm on intracellular calcium mobilization, naive B lymphocytes were incubated in the presence of NAm before stimulation by anti-IgM antibodies. As shown in Fig. 3A, NAm failed to significantly affect the anti-IgM-induced Ca^{2+} response in naive B cells. Control and treated cells displayed a similar capacitative calcium entry in response to Thapsigargin (Fig. 3B) and Ionomycin (Fig. 3C), indicating that NAm did not significantly affect calcium homeostasis and/or internal stores. In keeping with this observation, exogenous NAm did not significantly inhibit the early wave of BCR-induced tyrosine phosphorylations. Whole cell extracts of control and NAm-treated B cells were analyzed by Western blot using an antibody to phosphotyrosine, as shown in Fig. 3D. As expected, BCR stimulation led to the tyrosine phosphorylation of several substrates (compare lanes 1 and 3 in Fig. 3D), but the intensity of the observed bands was not significantly affected by NAm. Accordingly, NAm only marginally affected the tyrosine phosphorylation of PLCgamma2, an activation step required for adequate generation of soluble inositol phosphates controlling the calcium response (Fig. 3E). Collectively, these data indicate that the BCR-signalling pathway leading to PLCgamma2 activation does not represent a target for NAm.

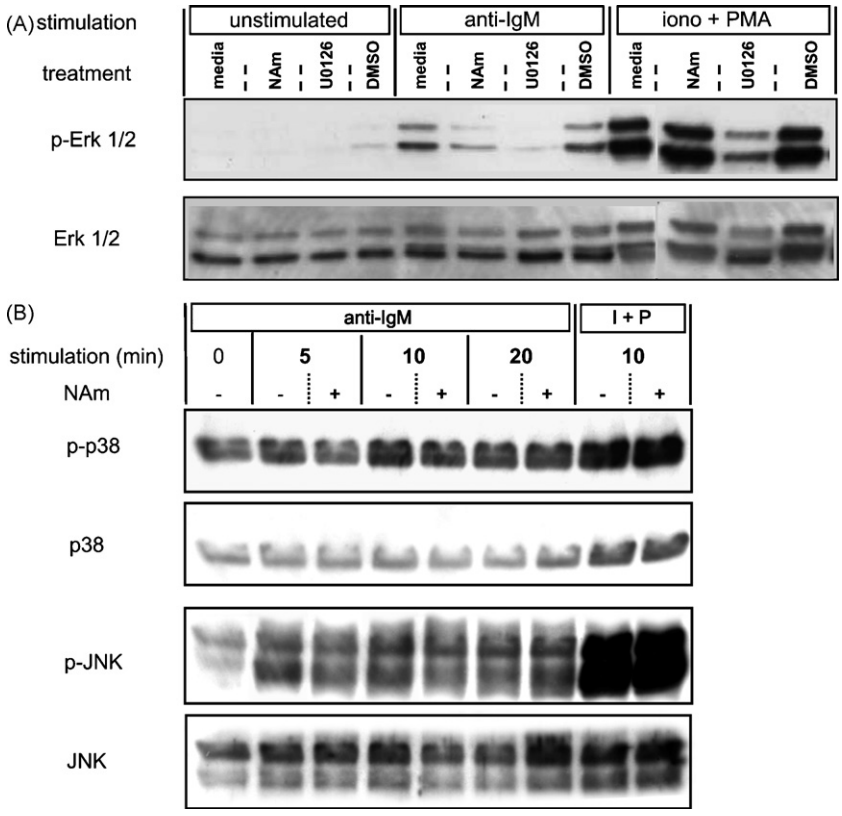

Fig. 4 – NAm inhibits ERK phosphorylation. (A) B lymphocytes were incubated 30 min in media, NAm (10 mM), U0126 (10 μM) or DMSO (U0126 solvant) and stimulated during 2 min with anti-IgM (10 μg/ml) or with a combination of Ionomycin (50 ng/ml) and PMA (10 ng/ml). Activation of ERK is analyzed with specific antibodies against the phosphorylated forms of the kinase. An antibody against total ERK proteins was used to verify that equal amounts of protein were loaded in each lane. (B) B lymphocytes were incubated 30 min in the presence or not of NAm (10 mM) and stimulated during 5, 10 or 20 min with anti-IgM (10 μg/ml) or with a combination of Ionomycin (50 ng/ml) and PMA (10 ng/ml). Activation of p38 and JNK is analyzed with specific antibodies against the phosphorylated forms of the kinases. An antibody against total MAPK proteins, (p38alpha and JNK) was used to verify that equal amounts of protein were loaded in each lane. This experiment has been reproduced three times with similar results.

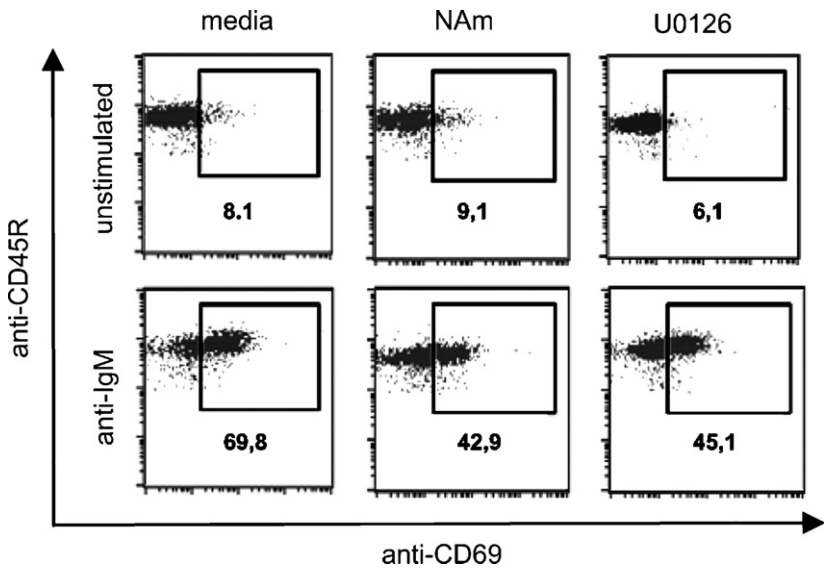

Fig. 5 – NAm and the Erk inhibitor, U0126 affects CD69 expression by activated B cells. Splenocytes were preincubated in the presence or not of NAm (10 mM) and U0126 (20 μM) for 1 h and then stimulated as indicated with anti-IgM (3 μg/ml) for 7 h. Spleen cells were gated on the basis of forward and side scatter, and expression of CD69 analyzed on viable (as judged by PI exclusion) and CD45R-expressing B cells. At least 20,000 events ($CD45R^+$ and PI^-) were collected from each sample. The proportions of $CD69^+$ and $CD25^+$ are indicated in each quadrant. This experiment has been reproduced two times with similar results.

3.3. *NAm inhibits MAPK activation in primary B lymphocytes*

Many signalling pathways implicated in B cell proliferation, including those triggered by LPS, anti-CD40, and anti-IgM, converge upon and require the activity of the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK 1/2) [18,19].

To evaluate the ability of NAm to affect MAPK activation, primary splenic B cells were stimulated with anti-IgM antibodies in the presence or absence of NAm and examined for kinases expression and phosphorylation status by Western blot. As a positive control, cells were also incubated with U0126, an inhibitor of the MEK1/2 kinases responsible for ERK phosphorylation. As shown in Fig. 4A, anti-IgM stimulation led to accumulation of both forms of phospho-ERK, which was significantly inhibited by both NAm and U0126. In keeping with our previous functional observations, NAm did not inhibit ERK phosphorylation induced by Ionomycin and PMA (a combination of pharmacological agents able to activate MEK1 in B lymphocytes, [20] and our own unpublished observations). Despite the fact that proliferation signals induced following BCR stimulation are known to be mostly mediated by ERK, we also analyzed the effect of NAm on the JNK and p38 pathways. In agreement with previous reports [21–23], BCR engagement induced a weaker JNK and p38 phosphorylation over the resting state, with a delayed kinetics (peak activity at 10 min versus 2 min for ERK, data not shown). In both instances, NAm appeared to only marginally affect BCR-induced pJNK and pp38 responses, possibly leading to a delayed but not completely inhibited response (Fig. 4B). Note that both these MAPK kinases are known to mostly regulate cell survival and cytokine production [24], and that NAm did not affect B cell survival in our experimental setting (data not shown).

To further document the ability of NAm to inhibit the ERK-mediated signalling pathway, we examined the expression of the early activation marker CD69, a well-known target of ERK in lymphocytes, in response to BCR engagement. We first confirmed previous observations indicating that CD69 was sensitive to U0126, a well-described inhibitor of the ERK pathway. As shown in Fig. 5, activation of naive B lymphocytes by anti-IgM stimulation led to the upregulation of CD69 as assayed by dual color flow cytometry using antibodies to the CD45R/B220 marker to identify B cells. Addition of both NAm and U0126 in the culture media significantly inhibited CD69 upregulation in response to anti-IgM, leaving expression of CD45R unaffected (compare the y-axis between control and drug-treated groups). Expression of the CD25 marker, whose upregulation is known to be ERK-independent [25], was used as a negative control in the next series of experiments. Naive B cells displayed low levels of CD25 and CD69 molecules which were not significantly altered by exogenous NAm at rest (Fig. 6A, first row). Upon activation, most B cells upregulated cell surface expression of the CD25 and CD69 markers. Addition of NAm in the culture media led to a marked and selective reduction in CD69 expression, with detectable effect on neither CD45R nor CD25 expression (Fig. 6A). In keeping with previous observations (see Fig. 1A), expression of both activation markers induced by Ionomycin and PMA was insensitive to NAm. IL-4 has been recently shown to affect BCR signalling by rendering BCR-induced ERK activation relatively resistant to PI3K inhibitors [26]. NAm only marginally affected CD69 expression induced by a combination of IL-4

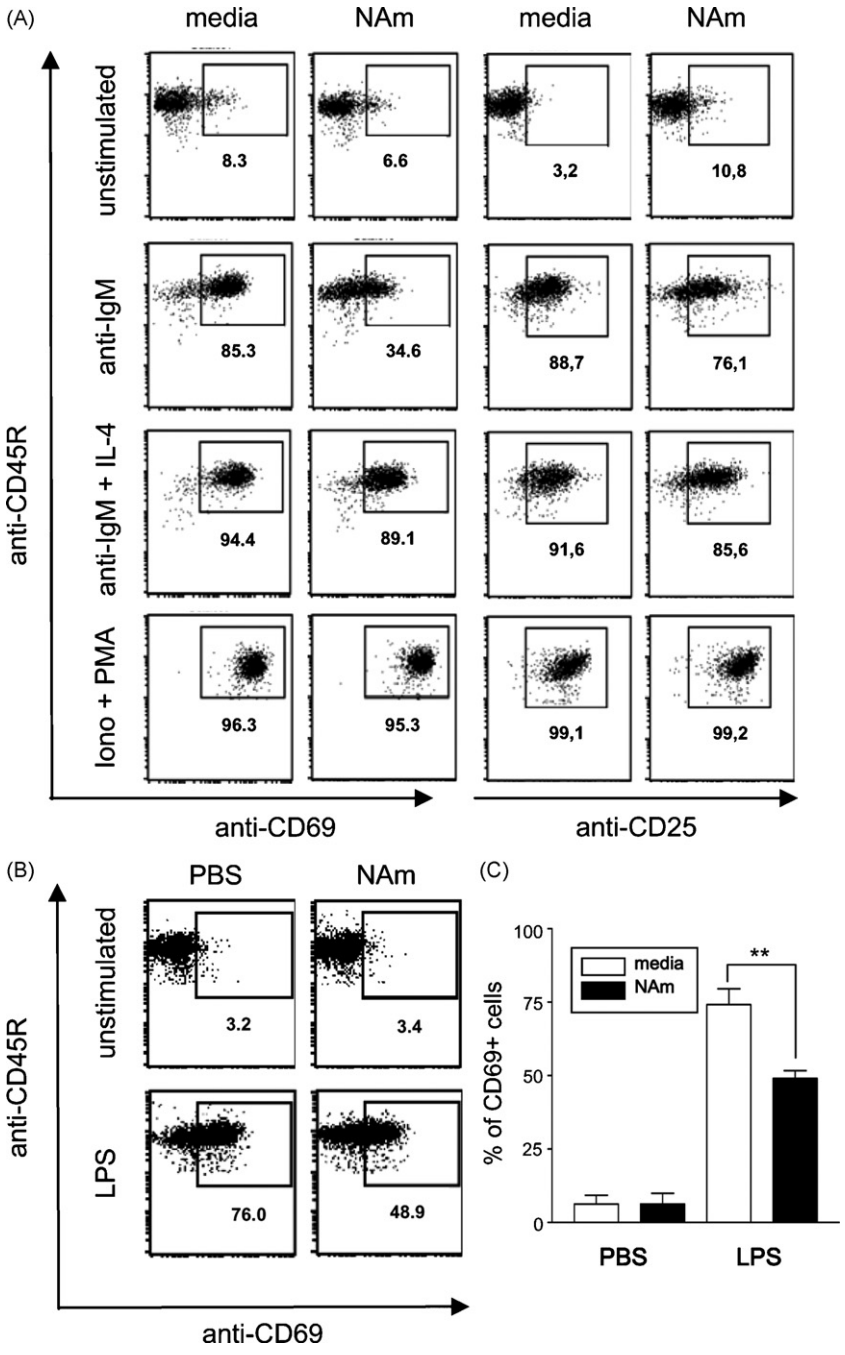

Fig. 6 – NAm inhibits CD69 but not CD25 expression by activated B cells. (A) In vitro stimulation: splenocytes were preincubated in the presence or not of NAm (10 mM) for 1 h and then stimulated as indicated with anti-IgM (7 μg/ml), IL-4 (10 ng/ml) and PMA (10 ng/ml) for 7 h. Spleen cells were gated on the basis of forward and side scatter, and expression of CD69 and CD25 analyzed on viable (as judged by PI exclusion) and CD45R-expressing B cells. At least 20,000 events ($CD45R^+$ and PI^-) were collected from each sample. The proportions of $CD69^+$ and $CD25^+$ are indicated in each quadrant. The S.D. is always minder than 1%. Similar results were obtained in three independent experiments. (B and C) In vivo stimulation: mice were treated with NAm (500 mg/kg, i.p.) and challenged 1 h later with LPS (200 μg/kg, i.p.). After 18 h of stimulation, splenocytes were analyzed by FACS as described previously. Panel B depicts a representative experiment, while panel C represents the mean (±S.D.) from three independent experiments comprising a total of 10 mice per group. Statistical analysis was realized using Wilcoxon matched *t* test: $^{**}p = 0.0078$.

and anti-IgM, suggesting that NAm inhibits the default pathway leading to ERK activation in response to BCR stimulation (Fig. 6A). Finally, to ascertain the potential in vivo relevance of this inhibition, mice were pretreated with a pharmacological dose of NAm (500 mg/kg) before LPS (200 μg/kg) injection. Animals' studies have shown that this dose of NAm leads to peak plasma levels in the 4 mM range (see ref. [27]). Splenocytes were analyzed by flow cytometry for CD45R

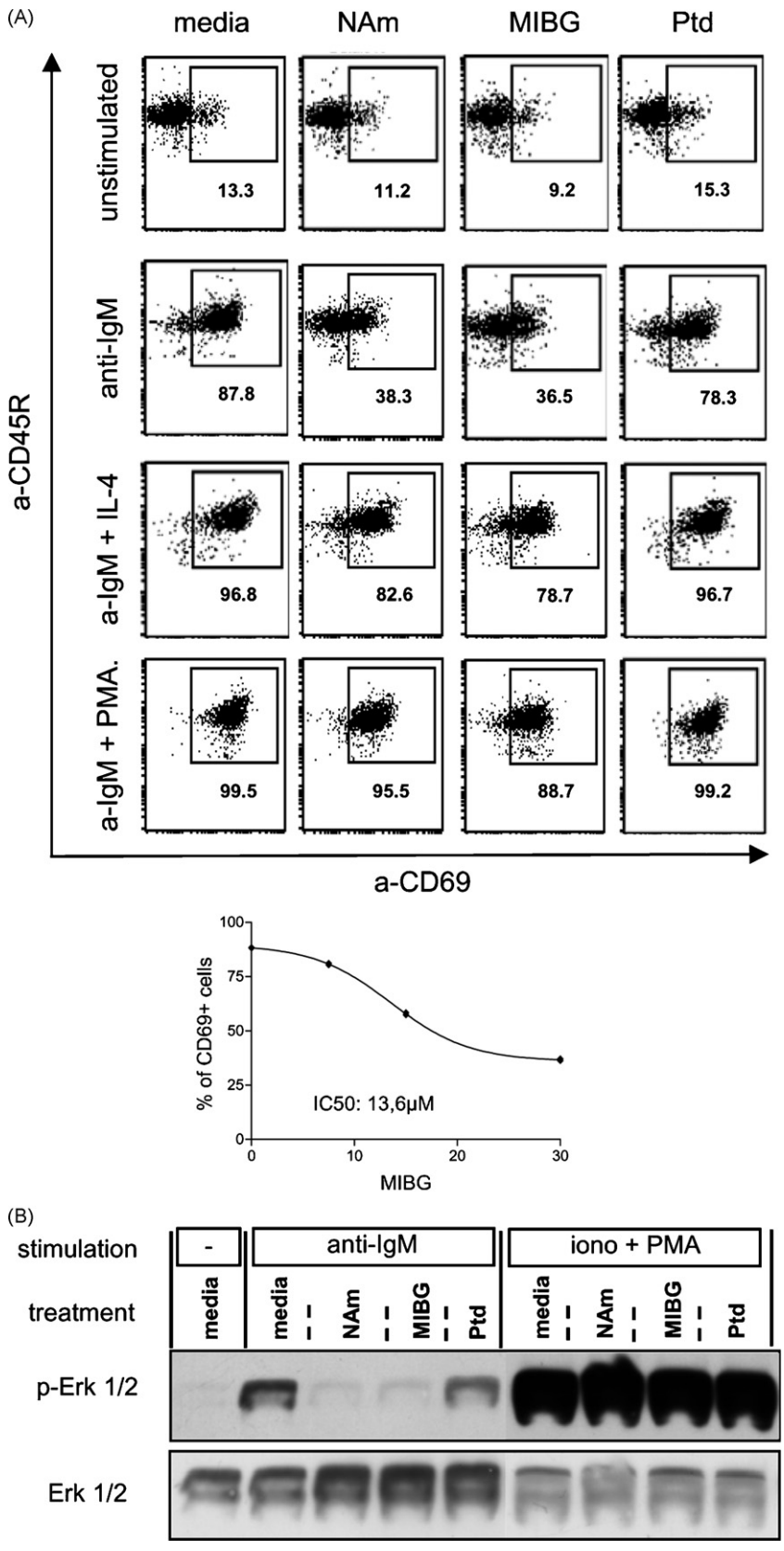

Fig. 7 – MIBG inhibit expression of CD69 and phospho-ERK accumulation in stimulated primary B cells. (A) Splenocytes were preincubated in the presence or absence of Ptd (100 μM), MIBG (30 μM) and NAm (10 mM) for 30 min and then stimulated with anti-IgM (7 μg/ml) + IL-4 (10 ng/ml) or PMA (10 ng/ml) for 7 h. Spleen cells were analyzed as described in the legend of Fig. 6 for CD69 and CD25 expression. (B) Extracts from activated B lymphocytes incubated in the presence of control media or inhibitors as indicated have been analyzed for phospho-ERK expression as indicated in the legend of Fig. 4A.

and CD69 expression 18 h later. As shown in Fig. 6B (a representative experiment) and 6C (showing the mean expression of CD69 compiled from three distinct experiments including a total of 10 mice per group), NAm significantly inhibited CD69 expression induced by LPS injection, suggesting that NAm is able to interfere with B cell activation in vivo.

3.4. *Meta-iodobenzylguanidine (MIBG), an inhibitor of mono(ADP-ribosyl)ation inhibits CD69 expression in B lymphocytes*

As previously discussed, NAm represent the end product and an inhibitor of both mono and poly(ADP-ribosyl)transferases. To gain insight into the possible target of NAm in B cells, two selective inhibitors (*meta*-iodobenzylguanidine (MIBG) and phenanthridinone (Ptd), affecting, respectively, the mono and the poly(ADP-ribosyl)transferases) have been assayed for their ability to mimic the effect of NAm on B cell activation. Expression of CD69 and activation of ERK, requiring only short-term incubation of target cells with these inhibitors, were chosen as read out assays to minimize the toxic effects of these drugs on naive B lymphocytes (our own unpublished observations). As shown in Fig. 7A, short-term incubation of B cells with MIBG, but not with Ptd led to a significant downregulation of CD69 expression on activated B cells. Notably, and as previously reported for NAm, MIBG did not affect CD69 expression when anti-IgM stimulation was performed in combination with IL-4 or PMA. MIBG affected CD69 expression in a dose-dependent manner, reaching a similar biological effect as seen with NAm when added at doses (30 μM) approaching the known IC_{50} of MIBG for mono-ADP-ribosyl tranferases. In marked contrast, Ptd, a prototypic PARP inhibitor added at supra optimal doses (100 μM, with a known IC_{50} of 10–30 μM for PARP inhibition reported in the literature [28]), only marginally affect CD69 expression in response to all agonists tested. In keeping with these observations, phospho-ERK accumulation in response to anti-IgM was relatively resistant to Ptd (Fig. 7B and data not shown), while displaying high sensitivity to both NAm and MIBG.

4. Discussion

Despite the lack of precise information on its mode of action, NAm represents a potentially valuable pharmacological agent due to its low cost, non-proprietary status and well-established safety profile in humans. NAm has been shown to protect against cell death in several in vivo models, and to inhibit cytokine production from innate immune cells, making it an attractive anti-inflammatory agent [29]. The present study extends these previous studies and demonstrates that this vitamin is able to inhibit B lymphocyte proliferation in response to a variety of stimuli, including antibodies to the antigen-specific BCR and LPS, a model ligand for the TLR class of receptors specific for microbial components. NAm did not affect B cell responses to pharmacological agents bypassing the early steps of signal transduction, suggesting that it neither affect cell viability nor the enzymatic machinery required for cell proliferation. The ability of this vitamin to inhibit the response of B cells to a wide range of ligands indicated to us that NAm may affect an enzymatic step shared by different signalling mechanisms, such as the MAPK pathway. Our observations support this hypothesis since we demonstrate in this work that NAm does not affect a specific BCR-proximal event such as calcium influx, but preferentially inhibits phosphorylation of ERK, a biological response shared by all receptors tested [18,19,26]. In keeping with this conclusion, NAm was able to affect the expression of two genes whose expression requires active ERK, such as cylinD2 (Fig. 1G and [17]) and CD69 (Fig. 5 and [30]). Moreover, these observations are in agreement with the well-described role of the MEK/ERK pathway in regulating cell proliferation. Finally, NAm did not inhibit expression of CD25, an activation-induced marker known to be insensitive to ERK inhibitors in B cells [25].

Collectively, our observations suggest that the MAPK pathway represent a molecular target of NAm in activated B lymphocytes. The precise identification of the molecular target(s) of NAm would require a detailed analysis of the enzymatic steps leading to MAPK phosphorylation in activated B cells. Despite some recent progress, the signalling pathways leading to ERK, JNK and p38 activation in B cells have not been completely elucidated. Indeed, activation of these pathways downstream of BCR and TLR receptors rests on the dynamic recruitment of several signalling components to a ''signalosome'' comprising enzymes and ''scaffold'' proteins (such as Bam32, BLNK, Grb2, Btk, Vav, and PI3K, see ref. [31] for a recent review), each of which represents a potential NAm target. Note that small GTP-binding proteins such as Ras, a well-known initiator of the ERK MAPK cascade, and several cytoskeleton-associated proteins are sensitive to microbial or endogenous mono-ADP-ribosylation [1]. Whether these proteins represent substrates for cellular ADP-ribosyl tranferases, and thus potential NAm targets, should be investigated in future work.

As previously discussed, NAm represents an end-point inhibitor of several classes of NAD-dependent enzymes. ADP-ribosyl cyclases catalyze the hydrolysis of NAD to NAm and cyclic ADP ribose, a second messenger which is capable of inducing Ca(2+) release from the sarcoplasmic reticulum (SR) via activation of ryanodine receptors (mostly type 2 and type 3, [32]) in several cell types. Collectively, our observations and those from the literature suggest that the immunomodulatory properties of NAm are not related to its ability to inhibit the generation of cADPR in primary B lymphocytes. Indeed (i) NAm did not significantly affected calcium homeostasis in response to BCR ligation (see Fig. 1); (ii) NAm was able to inhibit cell proliferation induced by ligands (such as LPS or antibodies to CD40) which do not modify intracellular calcium levels (see http://www.signaling-gateway.org/data/ for an extensive analysis of B cell signalling in response to multiple ligands); (iii) the major ryanodine receptor expressed by B cells is RyR1 [33]; and finally the signalling properties of CD38, the major ADP ribosyl cyclase expressed by B lymphocytes, is independent from its ADP-ribosyl cyclase activity [34,35].

As previously stated, the signalling function of NAD^+ is also related to its ability to donate the ADP-ribose group, which is transferred to specific amino acids in proteins (a reaction catalyzed by mono and poly(ADP-ribosyl)transferase), or to react with the acetyl group derived from acetylated proteins. The latter reaction is catalyzed by the Sir2 family of NAD^+-dependent protein deacetylases, or sirtuins. In an

attempt to identify which of these enzymatic reactions was affected by NAm in our experimental system, we tested a series of specific inhibitors affecting each of these post-translational modifications. When used at pharmacological doses (50 μM), sirtinol, a well-established Sirtuin inhibitor [3] was found to profoundly affect short-term B cell viability precluding any functional assay (our own unpublished observations). Ptd, a prototypical PARP inhibitor, was found unable to modulate expression of a well-established ERK-target (CD69, see Fig. 7). In contrast, MIBG, a compound thought to specifically affect arginine-specific mono(ADP-ribose)transferases, was able to recapitulate the effect of NAm on ERK activity (see Fig. 7). Although correlative in nature, this observation strongly suggests that NAm affects B cell activation by inhibiting a post-translational modification involving the transfer of an ADP-ribose moiety to a yet undefined target protein playing a role in the MEK–ERK pathway. This conclusion is also supported by recent experiments performed on a murine macrophage cell line, in which MIBG was found to inhibit pERK accumulation in response to LPS [36].

As previously discussed, several enzymes displaying mono-ADP-ribosyl tranferase activity and belonging to at least two distinct families in mammals have been described (ARTs and sirtuins). Indeed, transfer of an ADP-ribose to an acceptor protein appears to represent a biochemical intermediate in the NAD-dependent desacetylase activity of several sirtuins. Notably, at least one member of this family (SIRT6) displays mono(ADP-ribose)transferase activity in vitro. A detailed biochemical analysis of cellular proteins that can be labelled by [adenylate-^{32}P]-NAD in primary B cells is warranted to identify the proteins sensitive to this post-translational modification.

This study clearly identifies primary B cells as a cellular target of exogenous NAm, and could open new avenues for the treatment of auto-immune diseases mediated by antibodies. Indeed, it has been recently demonstrated that dual co-engagement of TLR receptors and BCR on cells can favour antibody production in murine models of sytemic lupus erythematosus (SLE). The ability of NAm to inhibit both TLR (see also [29]) and BCR signalling, make this compound an attractive drug candidate for treating antibody-dependent autoimmune disorders. Note however that despite the observation that NAm is able to (i) inhibit antibody production from in vitro stimulated naive B cells (see Fig. 2), and (ii) inhibit CD69 upregulation in vivo (Fig. 6), a detailed pharmacological study is warranted to evaluate the ability of exogenous NAm to affect antibody responses in vivo. These studies will require alternative methods of continuous delivery such as osmotic pumps or pellet to overcome the short in vivo half-life of this vitamin (approximately 2 h, see ref. [27]). These difficulties notwithstanding, the present studies may stimulate the search for novel synthetic compounds with more suitable in vivo pharmacokinetics and able to mimic the inhibitory effects of NAm on antibody-producing cells.

Conflict of interest

The authors declare that they have no competing financial interests.

Acknowledgements

We thank P. Veirman for animal care and D. Baup, A. Van Acker and S. Denanglaire for technical support.

This work was supported by The Belgian Program in Interuniversity Poles of Attraction Initiated by the Belgian Sate, Prime Minister's office, Science Policy Programming and by a Research Concerted Action of the Communauté française de Belgique. This work was also supported by the "Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture, FRIA", the "Fonds National de la Recherche Scientifique de Belgique", the "Fondation de MEURS-FRANCOIS", the "Fondation Rose et Jean Hoguet" and the Université Libre de Bruxelles.

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