Supporting Information to:

In vivo amelioration of endogenous anti-tumor autoantibodies via low-dose P_4N through the LTA4H/activin A/BAFF pathway

by

Yu-Ling Lin, Nu-Man Tsai, Cheng-Hao Hsieh, Shu-Yi Ho, Jung Chang, Hsin-Yi Wu, Ming-Hua Hsu, Chia-Ching Chang, Kuang-Wen Liao, Tiffany LB Jackson, David E. Mold, Ru Chih C. Huang

Contains:

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Supplementary Materials and Methods

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PBMC proliferation assay

Two hundred microliters of PBMCs ($5x10^4$ cells/ml) were seeded in each well of a 96-well culture plate and treated with increasing concentrations of P_4N (0.38, 0.75, 1.5, 3 or 6 μM) for 48 h. The number of viable cells was evaluated by MTT assay and a cell proliferation index was calculated as follows: Proliferation index = (OD_{595} of sample) /(OD_{595} of the untreated cells)×100%. PBMCs were also treated with or without P_4N (0.75, 1.5 or 3 μM) for 48 h, and probed with anti-CD20 antibodies (Roche, Mannheim, Germany) and FITC-conjugated goat anti-human IgG polyclonal antibodies (SouthernBiotech, Birmingham, AL). The percentage of B cells in treated PBMCs was determined by flow cytometry (BD Biosciences, Mountain View, California), and the indices of B cell proliferation were calculated as follows: (the number of PBMCs × the percentage of B cells in treated PBMCs)/ (the number of PBMCs × the percentage of B cells in untreated PBMCs).

The effect of P_4N on changes in B cell lineage *in vitro*

Single cell suspensions of mouse splenocytes were obtained by grinding the spleens from healthy mice. The suspended splenocytes were treated with or without 0.1 or 0.2 μM of P_4N for 48 h, and the percentage of naïve B cells, activated B cells, B1 cells or Breg cells were measured after staining with the following fluorochrome conjugates using flow cytometry (BD Biosciences): anti-CD19-FITC, anti-CD-23-APC, anti-CD-38-APC, anti-CD5-PE or anti-CD1d-Alexa 647 (BioLegend, San Diego, CA). The cell types were defined as follows: naïve B cells (CD19+CD23+), activated B cells (CD19+CD38+), B1 cells (CD19+CD5+) and Breg cells (CD19+CD5+CD1d+). The indices of different types of B cell proliferation were calculated as follows: (the number of splenocytes × the percentage of each B cell

population in treated splenocytes)/ (the number of splenocytes × the percentage of each B cell population in untreated splenocytes).

In vivo effects of P_4N on antibody production and B cell differentiation

Mice were immunized and boosted with 100 μg EGFP in 100 μl of PBS by intraperitoneal injection once every week and simultaneously treated with or without 5 mg/kg P_4N once or three times per week. Blood samples were collected from the retro-orbital plexus of the mice every week. The titers of anti-EGFP antibodies in sera were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, each well of a microtiter plate (Nunc, Wiesbaden, Germany) was coated with 100 ng of rEGFP in 100 μl of PBS. The sera were 6400X diluted by PBS and added to the antigen-coated wells, and their titers were measured as described above. The isotypes of anti-EGFP antibodies in sera were also measured as described previously.

The splenocytes of P_4N-treated or untreated mice were harvested and probed with anti-CD19-FITC, anti-CD23-APC, anti-CD38-APC, anti-CD27-APC, anti-CD138-APC, anti-CD5-PE or anti-CD1d-Alexa 647 (BioLegend) antibodies and analyzed by flow cytometry (BD Biosciences). The definitions of the cell types were as described above, except for memory B cells (CD19+CD27+) and plasma cells (CD19+CD138+). The indices of different types of B cell proliferation were calculated as follows: (the number of splenocytes × the percentage of each B cell population in treated splenocytes)/ (the number of splenocytes × the percentage of each B cell population in untreated splenocytes).

The effects of P_4N on the profile of cytokine expression

PBMCs (2×10^6 cells/ml) from seven healthy individuals were seeded in each well of a 24-well microplate and treated with 3 μM of P_4N for 24 h. Cell supernatants were collected for analysis of cytokine levels by a cytokine multiplex assay using the Bio-Plex Pro Human

Cytokine Standard Group I 27-Plex, following the manufacturer's protocol (Bio-Rad, Hercules, CA). The change levels of cytokine were calculated as follows: Change level = (value of the P_4N-treated cells) / (value of the untreated cells) × 100%.

DC isolation, culture and treatment

BALB/c mice were sacrificed by dislocation and the muscles of the feet containing the femurs and tibiae were removed. Then the bones were disinfected with 70% ethanol and washed with RPMI media. Both ends of bone were cut with scissors, and the marrow was flushed out with RPMI media. Clusters of the marrow within the cell suspension were dispersed by vigorous pipetting. After lysis of the red cells with ACK buffer, the bone marrow leukocytes at 2.5×10^6 per 10 cm-dish were cultured in 10 ml of R10 media [90% RPMI growth media, 10% RPMI completed media (50 μM β-ME, 1% P/S and 10% FCS)] containing 200 U/ml recombinant mouse GM-CSF (rmGM-CSF) for 9 days. Non-adherent cells were collected by gentle pipetting. Cell were centrifuged at 300 ×g for 5 min and cultured in 10 ml of fresh R10 media containing 100 U/ml rmGM-CSF and 0.5 μg/ml LPS for 24h. The matured DCs were obtained for further experiments.

To analyze the effects of P_4N on cell proliferation of BM-DCs, the matured DCs were seeded at 5×10^6 cells/well in 96-well cultured plate and treated with seral diluted P_4N for 24 h. Cell proliferation of BM-DC were analyzed by the MTT assay. To analyze the effects of P_4N on BAFF expression of BM-DCs, the mRNA expression of *BAFF* of BM-DCs was measured by reverse transcription PCR. Briefly, the matured DCs were seeded at 1×10^7 cells/well in 24-well cultured plate and treated with 0, 3, 6 and 12 μM of P_4N for 24 h. Then, cells were collected and total cellular RNA of treated cells was extracted with Trizol reagent (Invitrogen) and reverse-transcribed into cDNA using the Superscript RT-kit (Invitrogen). The cDNA of BAFF were then amplified by PCR. The primers for mouse BAFF were: forward primer 5'- ATGGATGAGTCTGCAAAGACC -3' and reverse primer 5'-

GGACATCGCTGTGAAACTGC -3'. All PCR reagents used to amplify the cDNA were purchased from Promega (Madison, WI, USA). The cDNA of β-actin (*Actb*) in the samples was used to normalize the loading amounts in each reaction. Finally, PCR products were resolved by electrophoresis on 2% agarose gels, stained with ethidium bromide and photographed using the Uni-photo band tool (EZ lab, Taipei, Taiwan).

Effects of P_4N on M1/M2 macrophage polarization *in vitro* and *in vivo*

THP-1 cells were seeded at 5×10^5 cells/well in 24-well cultured plate and treated w/o 6 μM of P_4N for 24 h. The *CD80* (M1 macrophage) and *CD163* (M2 macrophage) mRNA expression of human macrophage were measured by RT-PCR. The primers for human *CD80* were: forward primer 5'-AACATCACCATCCAAAGTGTC-3' and reverse primer 5'-GATGGTCCGGTTCTTGTAC-3'and the primers for human *CD163* were: forward primer 5'-ATGAGCAAACTCAGAATGGTG -3' and reverse primer 5'-TCCATGCTCCAGCCATTATTA-3'. All PCR reagents used to amplify the cDNA were purchased from Promega (Madison, WI, USA). *GAPDH* cDNA in the samples was used to normalize the loading amounts in each reaction.

To analyze the effects of P_4N on M1/M2 macrophage polarization *in* vivo, the expression of total (F4/80), M1 (CD68) and M2 (CD163) macrophages were measured by IHC staining. Briefly, CT26 tumor bearing Balb/c mice were treated with 5 mg/kg of P_4N by intra-tumoral injection for 3 and 12 days, the tumors isolated from P_4N and vehicle-treated mice were embedded in paraffin and probed with rat monoclonal anti-F4/80, anti-CD68 and CD163 (1:50 dilution; GeneTex) at 4°C overnight, and the detection antibodies were recognized using a horseradish peroxidase (HRP)-conjugated anti-rat IgG antibody (1/1,500 dilution; Santa Cruz Biotechnology). The immune complexes in the sections were visualized using the LSAB2 system (DAKO, Carpinteria, CA). The sections were counterstained with

hematoxylin, mounted, observed under a light microscope at a magnification of 400X, and photographed.

Supplementary Figures

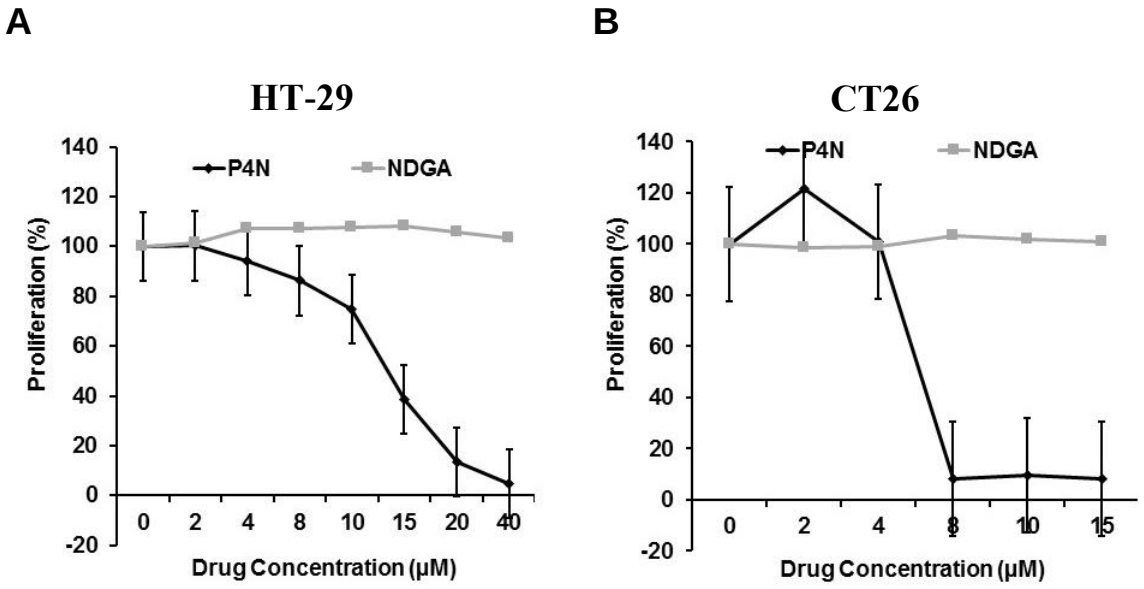

Figure S1. Cytotoxicity of P_4N and NDGA in HT-29 and CT26 colon cancer cells. HT-29 (A) and CT26 (B) cells were cultured with different concentrations of P_4N and NDGA for 48 h and assayed for cell viability using the MTT assay.

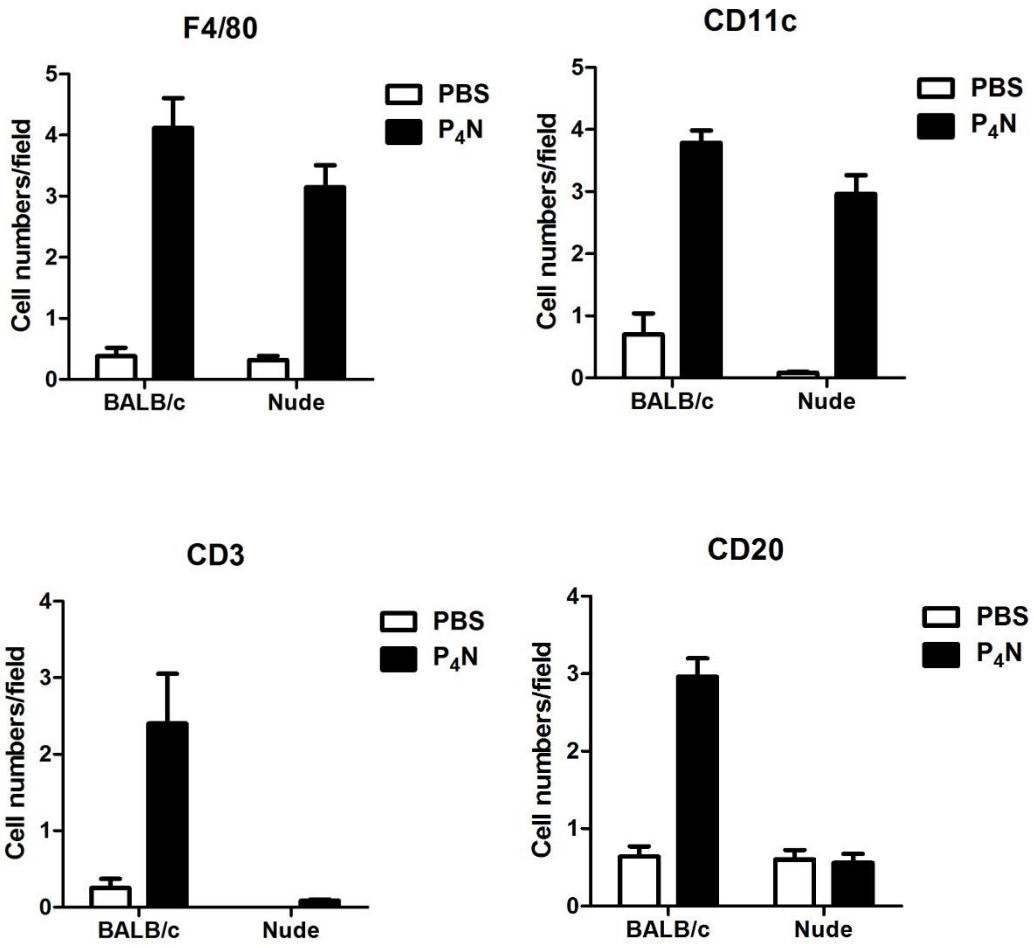

Figure S2. The infiltration of macrophages (F4/80), DC (CD11c), T (CD3) and B cells (CD20) into the tumor area after a single intratumoral injection of PBS or 5 mg/kg of P_4N. The cell numbers were counted in ten randomly chosen fields (400 X magnification) in all tumors. The results are presented as the mean ± SE of the number of cells/field of mean.

A

Protein a: 78 kDa glucose-regulated protein

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1   MMKFTVVAAA LLLLGAVRAE EEDKKEDVGT VVGIDLGTTY SCVGVFKNGR VEIIANDQGN RITPSYVAFT
80  PEGERLIGDA AKNQLTSNPE NTVFDAKRLI GRTWNDPSVQ QDIKFLPFKV VEKKTKPYIQ VDIGGGQTKT
150 FAPEEISAMV LTKMKETAEA YLGKKVTHAV VTVPAYFNDA QRQATKDAGT IAGLNVMRII NEPTAAAIAY
230 GLDKREGEKN ILVFDLGGGT FDVSLLTIDN GVFEVVATNG DTHLGGEDFD QRVMEHFIKL YKKKTGKDVR
300 KDNRAVQKLR REVEKAKRAL SSQHQARIEI ESFFEGEDFS ETLTRAKFEE LNMDLFRSTM KPVQKVLEDS
370 DLKKSDIDEI VLVGGSTRIP KIQQLVKEFF NGKEPSRGIN PDEAVAYGAA VQAGVLSGDQ DTGDLVLLDV
440 CPLTLGIETV GGVMTKLIPR NTVVPTKKSQ IFSTASDNQP TVTIKVYEGE RPLTKDNHLL GTFDLTGIPP
510 APRGVPQIEV TFEIDVNGIL RVTAEDKGTG NKNKITITND QNRLTPEEIE RMVNDAEKFA EEDKKLKERI
580 DTRNELESYA YSLKNQIGDK EKLGGKLSSE DKETMEKAVE EKIEWLESHQ DADIEDFKAK KKELEEIVQP
650 IISKLYGSGG PPPTGEEDTS EKDEL
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Protein b: ATP synthase, mitochondrial F1 complex, alpha subunit, isoform 1

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1   MLSVRVAAAV ARALPRRAGL VSKNALGSSF VGARNLHASN TRLQKTGTAE MSSILEERIL GADTSVDLEE
80  TGRVLSIGDG IARVHGLRNV QAEEMVEFSS GLKGMSLNLE PDNVGVVVFG NDKLIKEGDV VKRTGAIVDV
150 PVGEELLGRV VDALGNAIDG KGPIGSKTRR RVGLKAPGII PRISVREPMQ TGIKAVDSLV PIGRGQRELI
230 IGDRQTGKTS IAIDTIINQK RFNDGTDEKK KLYCIYVAIG QKRSTVAQLV KRLTDADAMK YTIVVSATAS
300 DAAPLQYLAP YSGCSMGEYF RDNGKHALII YDDLSKQAVA YRQMSLLLRR PPGREAYPGD VFYLHSRLLE
370 RAAKMNDSFG GGSLTALPVI ETQAGDVSAY IPTNVISITD GQIFLETELF YKGIRPAINV GLSVSRVGSA
440 AQTRAMKQVA GTMKLELAQY REVAAFAQFG SDLDAATQQL LSRGVRLTEL LKQGQYSPMA IEEQVAVIYA
510 GVRGYLDKLE PSKITKFENA FLSHVISQHQ SLLGNIRSDG KISEQSDAKL KEIVTNFLAG FEP
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B

ATPA-1: MSSILEERILGADTSVDLEETGRVLSIGDG

ATPA-2: RFNDGTDEKKKLYCIYVAIGQKRSTVAQLVKRLTDADAMK

GRP78-1: NTVFDAKRLIGRTWNDPSVQQDIKFLPFKV

GRP78-2: SSQHQARIEIESFFEGEDFSETLTRAKFEE

C

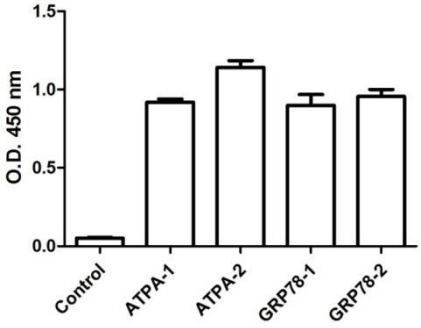
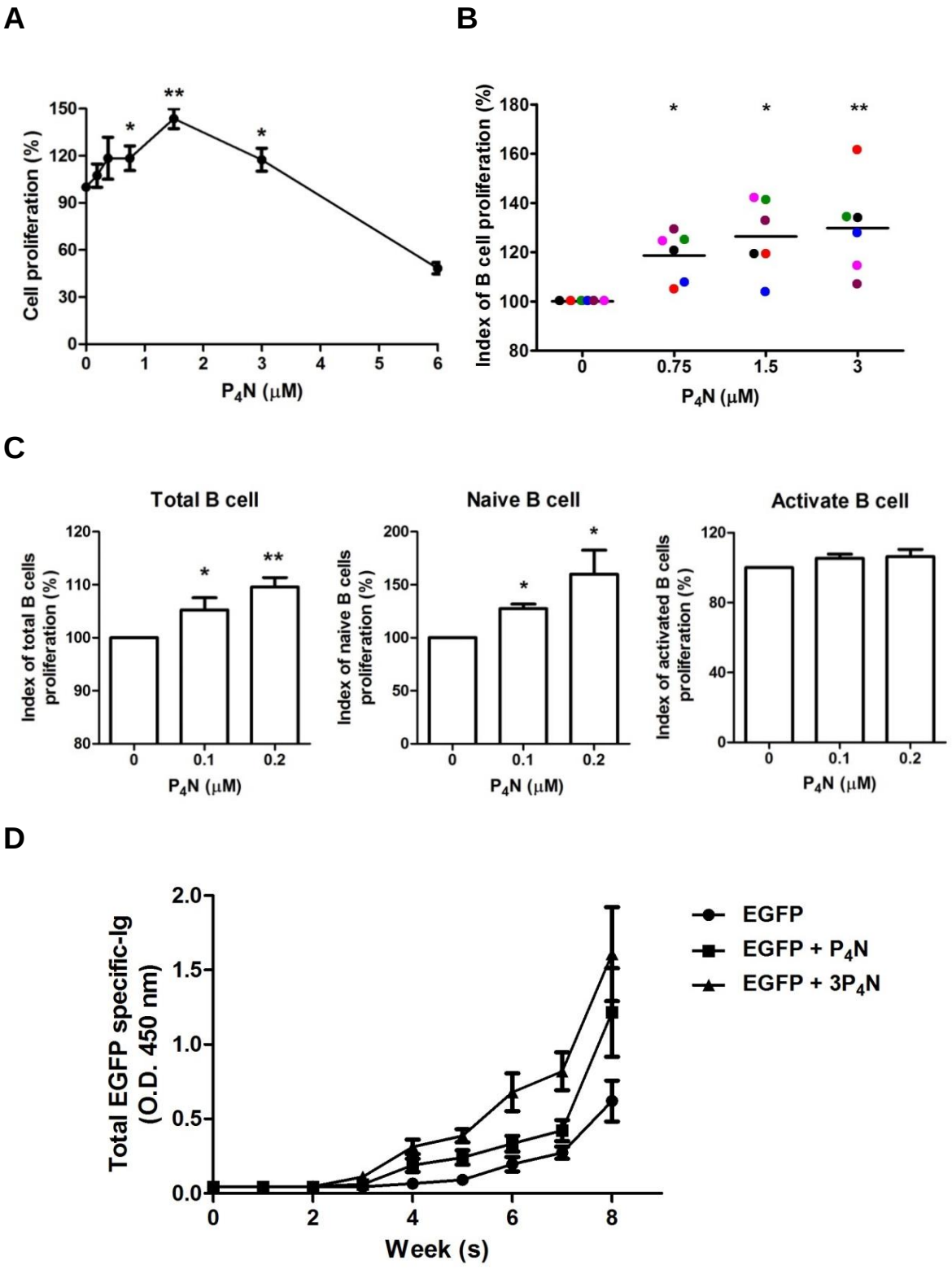
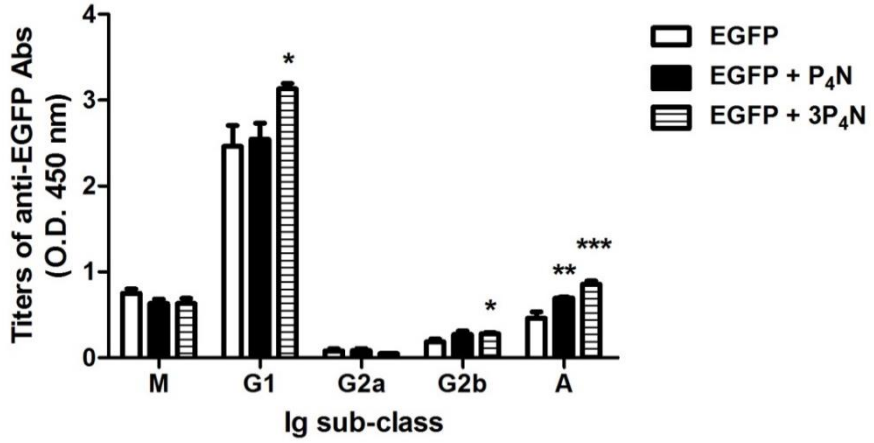


Figure S3 (A) Proteins a (78 kDa) and b (55 kDa) were analyzed by UPLC/HRMS/MS. Peptides identical to the partial protein sequences of GRP78 and ATP synthase are labeled in blue (B) The sequences of ATPA and GRP78 peptides designed to interact with the anti-ATP synthase and anti-GRP78 antibodies. Peptide sequences can be found in the protein sequences found in S3A. Location in Sequence: ATPA-1: 60-89; ATPA-2: 240-279; GRP78-1: 100-129 and GRP78-2: 310-339. (C) Binding assay of P_4N anti-sera with peptides of ATPA and GRP78.


A
Cell proliferation (%)
150
120
90
60
30
0
0 1 2 3 4 5 6
P4N (μM)
*
**
*
B
Index of B cell proliferation (%)
180
160
140
120
100
80
0 0.75 1.5 3
P4N (μM)
*
*
**
C
Total B cell
Index of total B cells proliferation (%)
120
110
100
90
80
0 0.1 0.2
P4N (μM)
*
**
Naive B cell
Index of naive B cells proliferation (%)
200
150
100
50
0
0 0.1 0.2
P4N (μM)
*
*
Activate B cell
Index of activated B cells proliferation (%)
120
80
40
0
0 0.1 0.2
P4N (μM)
D
Total EGFP specific-Ig (O.D. 450 nm)
2.0
1.5
1.0
0.5
0.0
0 2 4 6 8
Week (s)
EGFP
EGFP + P4N
EGFP + 3P4N

E



F

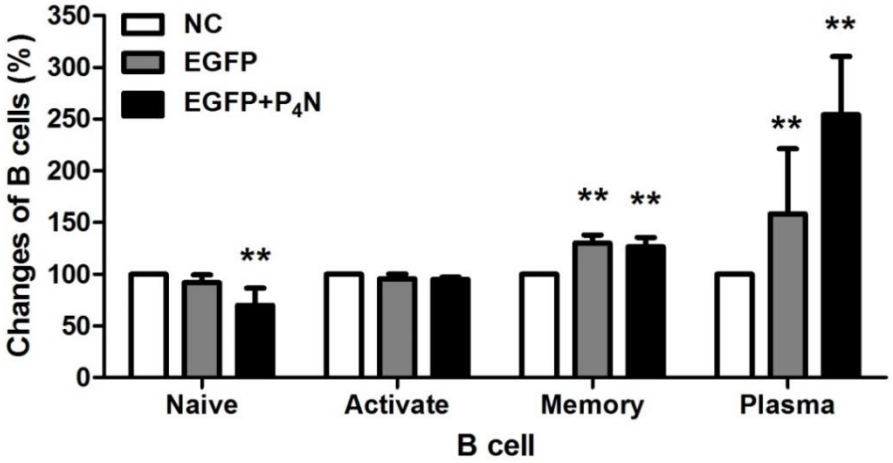


Figure S4. The effect of P_4N on B cell activation. (A) The proliferation of human PBMCs treated with different doses of P_4N was analyzed by MTT assay. The data are reported as the proliferation index. Significant differences between the P_4N-treated groups and the untreated group were indicated by * ($p<0.05$) (n=4). (B) The effect of P_4N on B cell proliferation was determined by flow cytometry. PBMCs from six healthy individuals were examined, and the results displayed with different colors. (C) The effects of P_4N on the changes in total B, naïve B or activated B cells among mouse splenocytes in vitro. Significant differences in the results of P_4N treatments compared with untreated results are indicated by ** ($p<0.01$) (n=3). (D) The effect of P_4N treatment on specific antibody production in antigen-immunized mice was monitored. The titers of total specific EGFP Igs in sera were measured by ELISA (n=5). (E) The effect of P_4N treatment on the changes in antibody classes was examined (n=5). (F) The changes in B cell types after antigen immunization with P_4N. All values (n=3) were expressed as mean ± SEM at the same dilutions. The significant differences in the results of P_4N-treated groups compared with the untreated group are indicated by *P<0.05, **P<0.01 or ***P<0.001.

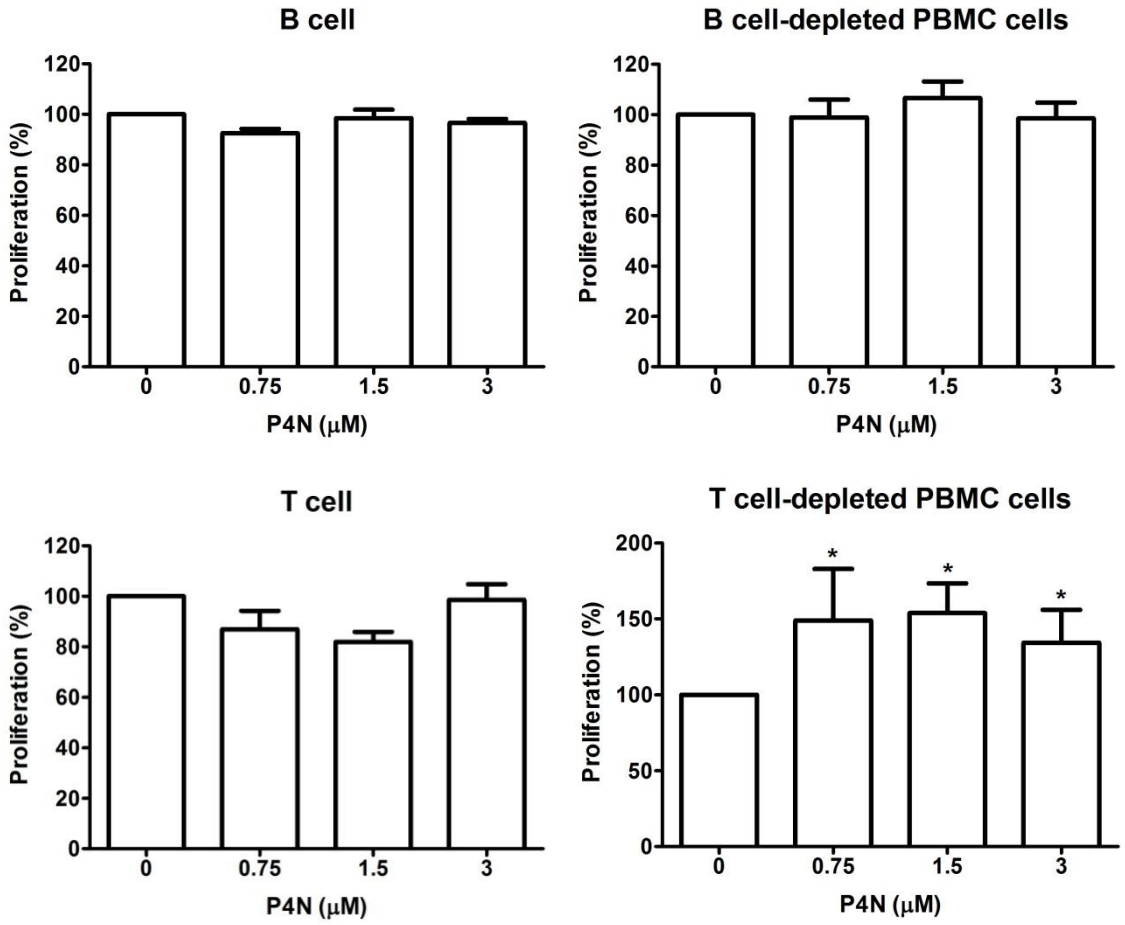


Figure S5. Effect of P_4N on cell proliferation. Human B or T cells were isolated from the PBMCs by negative selection using a magnetic sorting device (Miltenyi Biotec, Auburn, CA). Cell proliferation was determined by an MTT assay.

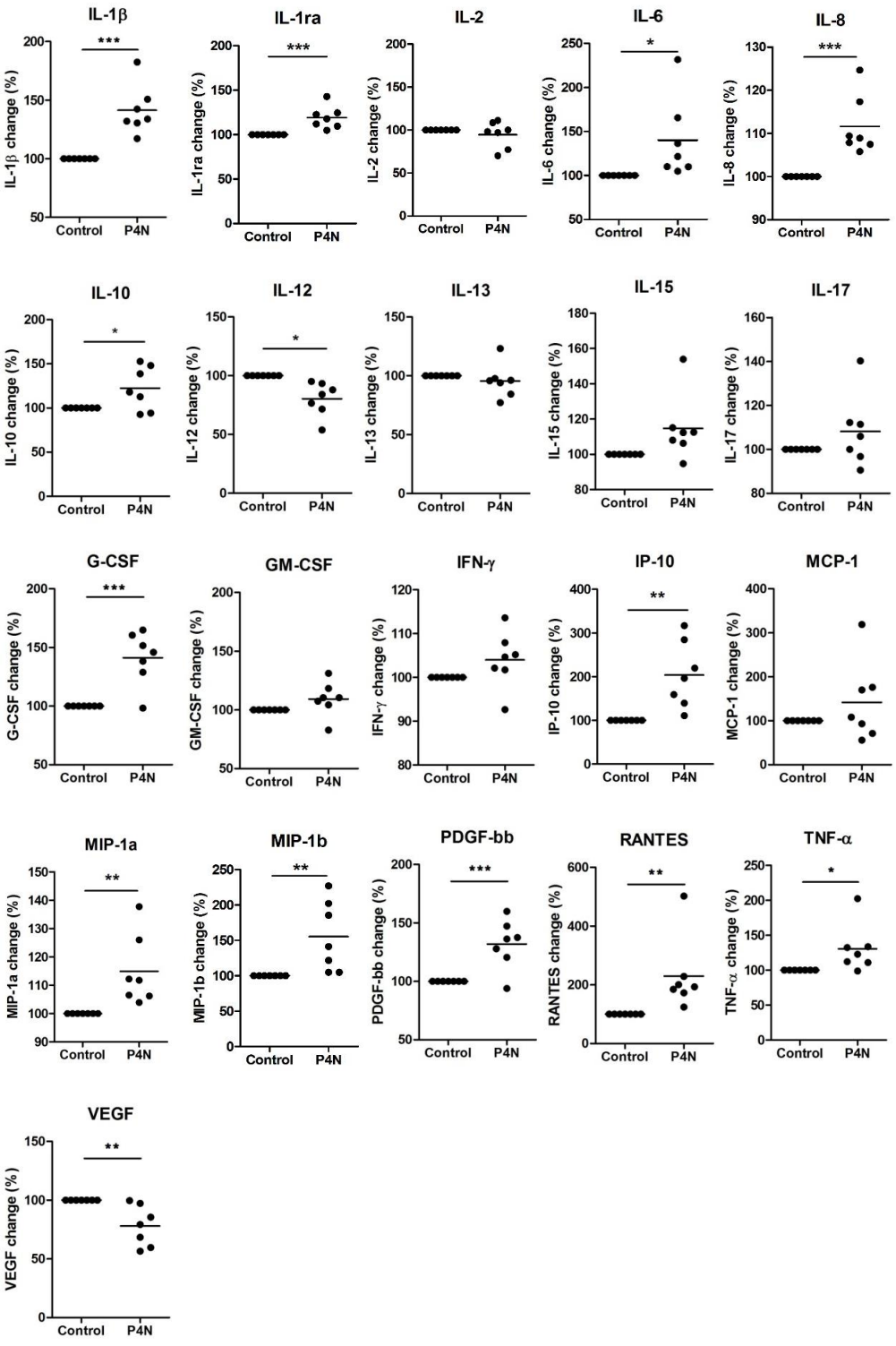


Figure S6. Cytokine and chemokine profiles of P_4N-treated PBMC. PBMCs isolated from 7 healthy donors were treated with 3 μM of P_4N for 48 h. Supernatants were collected and analyzed for human cytokines using the Bio-Plex assay. Shown are the changed results of the production of 27 human cytokines when PBMCs were treated with P_4N in three independent experiments. The levels of IL-4, IL5, IL-7, IL-9, Eotaxin and FGF were undetectable.

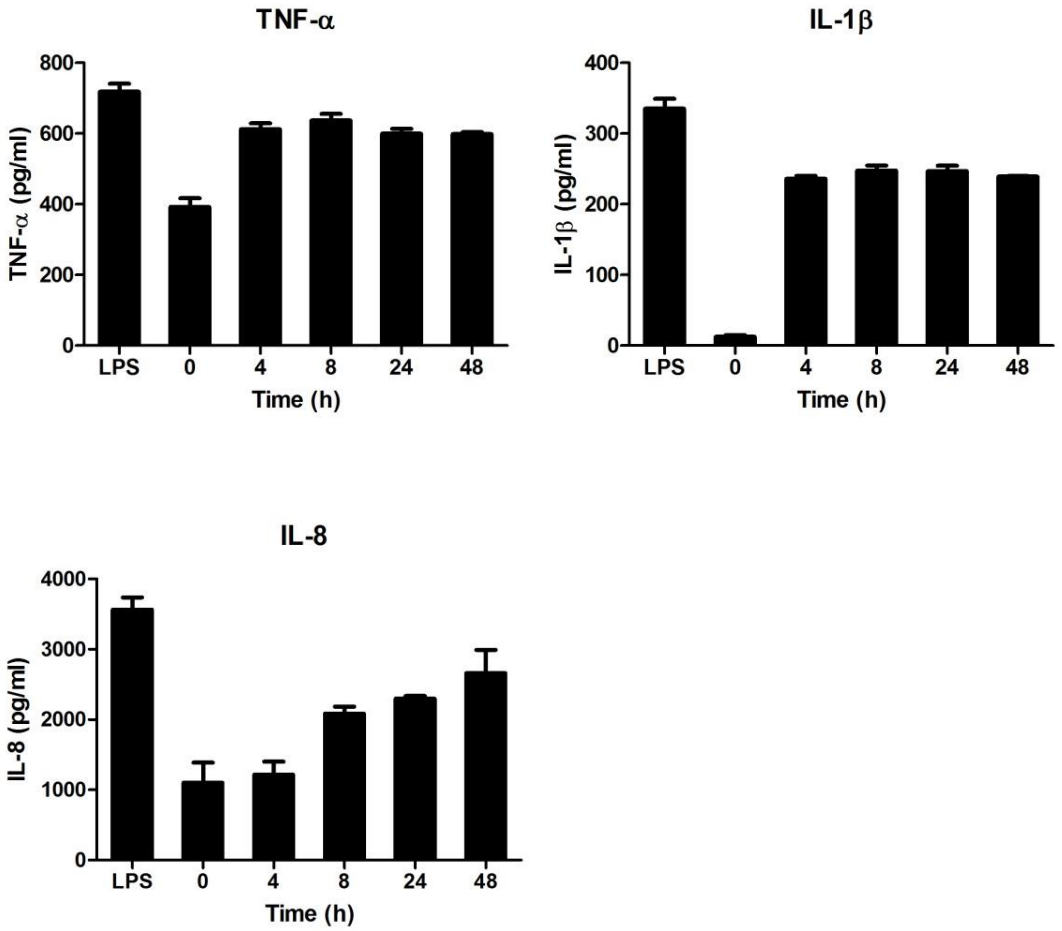


Figure S7. P_4N-induced proinflammatory cytokine production in THP-1cells. THP-1 cells were treated with 3 μM P_4N for different lengths of time. Cell supernatants were collected and TNF-α, IL-1β and IL-8 were quantitated by ELISA. All results are the mean of three independent experiments (n=6).

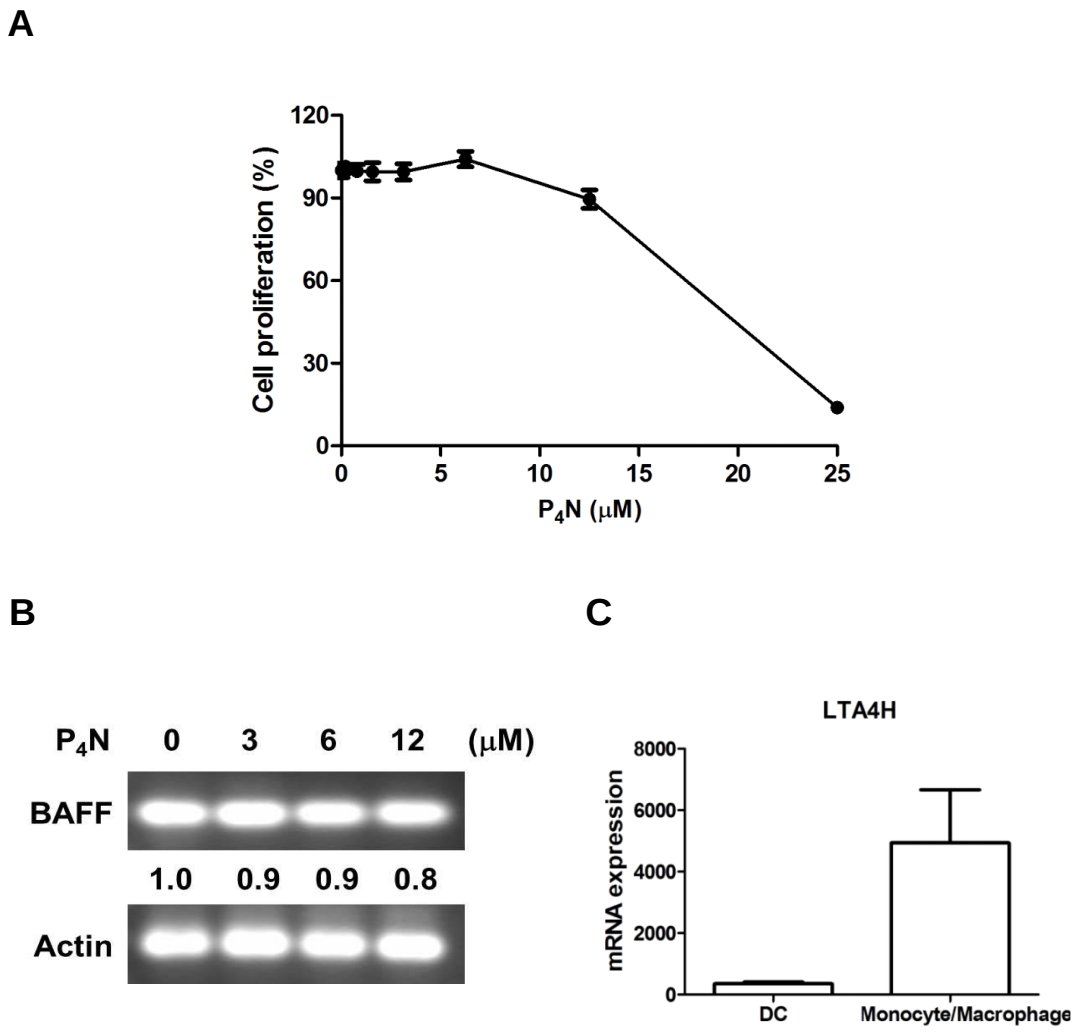


Figure S8. Effects of P_4N on mouse bone marrow-derived dendritic cells (mBM-DC). (A) Cell proliferation of P_4N-treated mBM-DC. mBM-DC isolated from three mice were individually treated with increasing concentrations of P_4N for 24 h and their proliferation was analyzed by MTT assay. The data are reported as the proliferation index. The results that differ significantly from the untreated group are indicated by *($p<0.05$) (n=6). (B) BAFF expression in P_4N-treated mBM-DC. Levels of BAFF transcripts were measured by RT-PCR. (C) LTA4H expression in mBM-DC and monocyte/macrophage. Levels of BAFF transcripts were obtained from GEO profiles data sets (GDS4421/1426807; GDS2216/208771_s; GDS1249/208771; GDS3005/208771_s; GDS2036/38081).

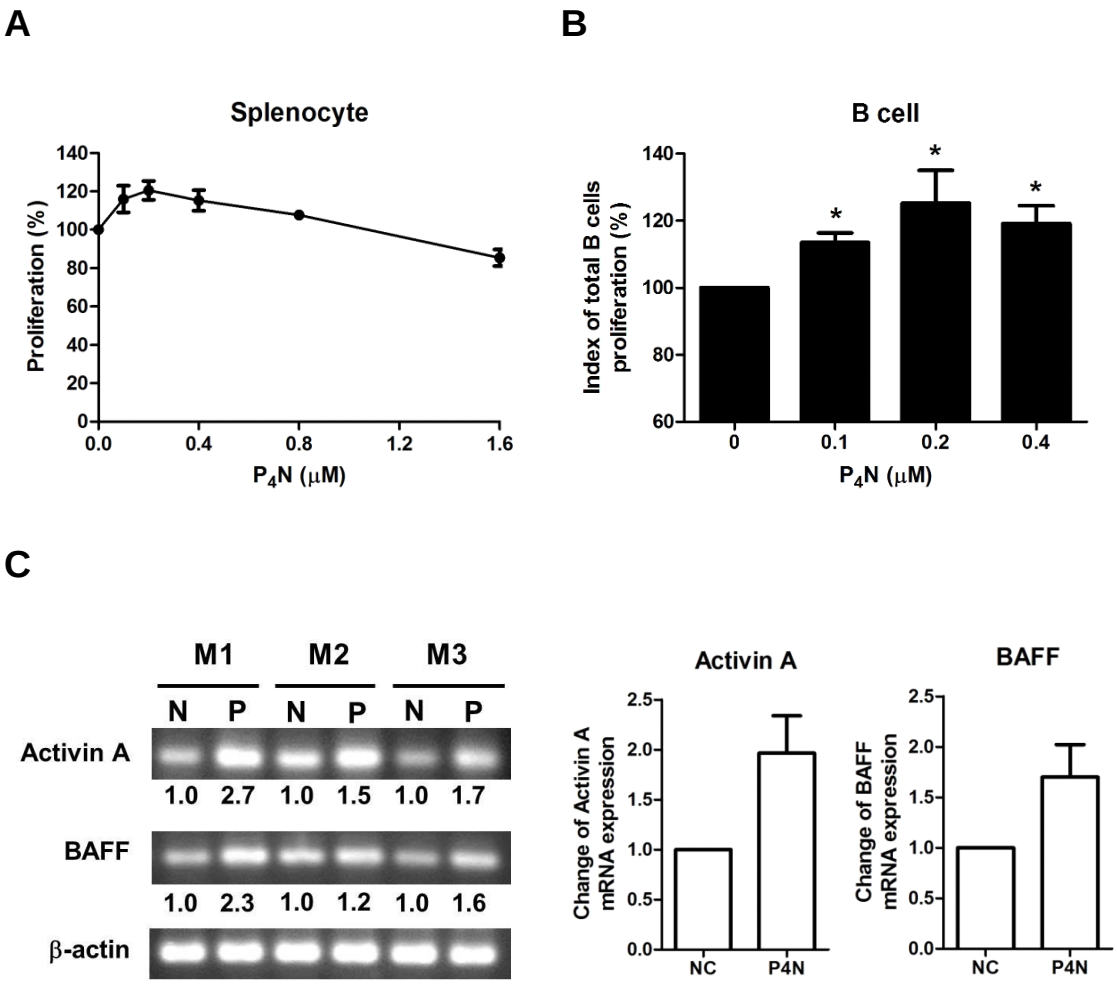


Figure S9. P_4N-induced mouse B cell proliferation via the Activin A/BAFF pathway. (A) Splenocytes isolated from three mice were individually treated with increasing concentrations of P_4N for 48h and their proliferation was analyzed by MTT assay. The data are reported as the proliferation index. The results that differ significantly from the untreated group are indicated by *($p<0.05$) (n=6). (B) After treatment with P_4N for 48h, B-cells were identified by CD19 surface marker staining and the cell number was calculated following a flow cytometer analysis. The results that differ significantly from the untreated group are indicated by *($p<0.05$) (n=6).(C) Levels of Activin A and BAFF transcripts were measured by RT-PCR., Each sample was obtained from 3 different mice; N, no treatment; P, P_4Ntreatment. The results were quantitated by densitometry, normalized to β-actin and plotted as change of Activin A or BAFF mRNA vs. treatment.

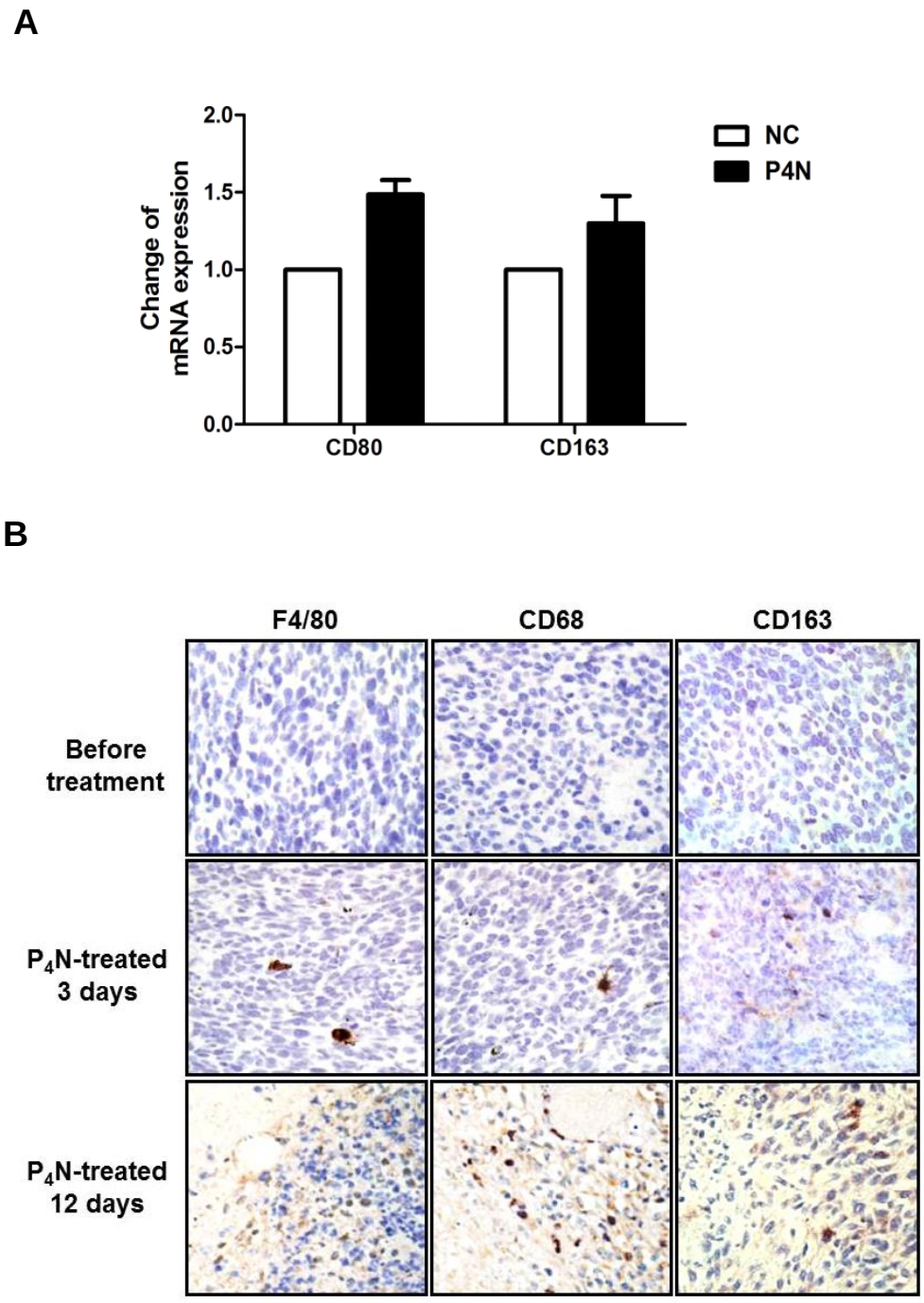


Figure S10. P_4N-induced M1/M2 macrophage polarization in vitro and in vivo. (A) CD80 (M1 macrophage) and CD163 (M2 macrophage) mRNA expression of human macrophage were measured by RT-PCR. (B) CT26 tumor bearing Balb/c mice were treated with P_4N for 3 and 12 days, the expression of total (F4/80), M1 (CD68) and M2 (CD163) macrophages were measured by IHC staining.

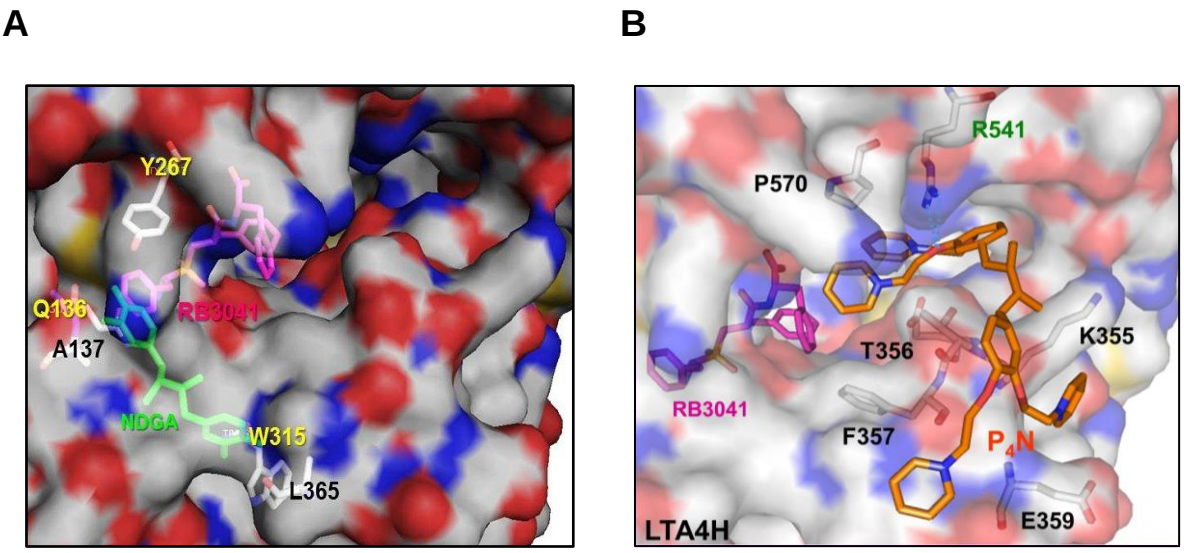


Figure S11. Docking of NDGA and P_4N with LTA4H. (A) NDGA (green) and (B) P_4N (orange) were docked with LTA4H by iGEMDOCK software (http://gemdock.life.nctu.edu.tw/dock/igemdock.php). RB3041 (Pink) is a small molecule inhibitor that identifies the active site of LTA4H. The amino acids that potentially interact with NDGA or P_4N are noted respectively.

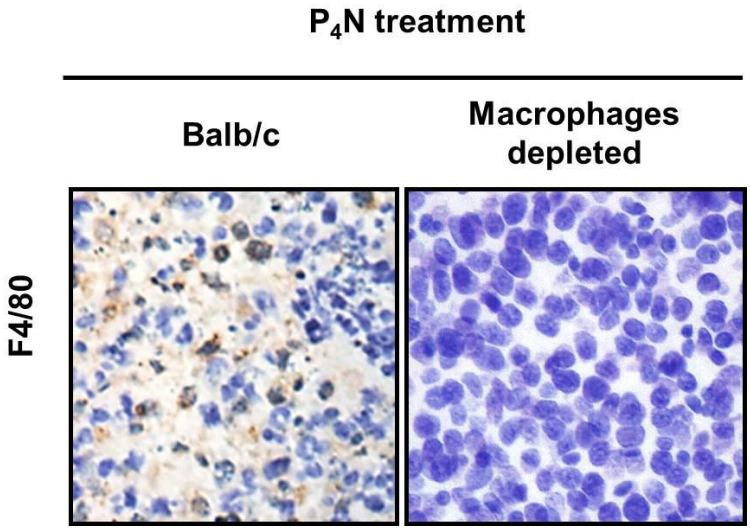


Figure S12. Macrophage depletion in P_4N-treated BALB/c mice. Macrophages were depleted with liposomal clodronate. BALB/c mice were inoculated s.c. with CT26 cells. When the average tumor mass reached ~50 mm3, the animals were treated with 5 mg/kg of P_4N by intratumoral injection every week. After treatment with P_4N, tumors were embedded in paraffin and thin sections were made and processed for IHC staining and were probed by anti-F4/80 antibodies for macrophages.

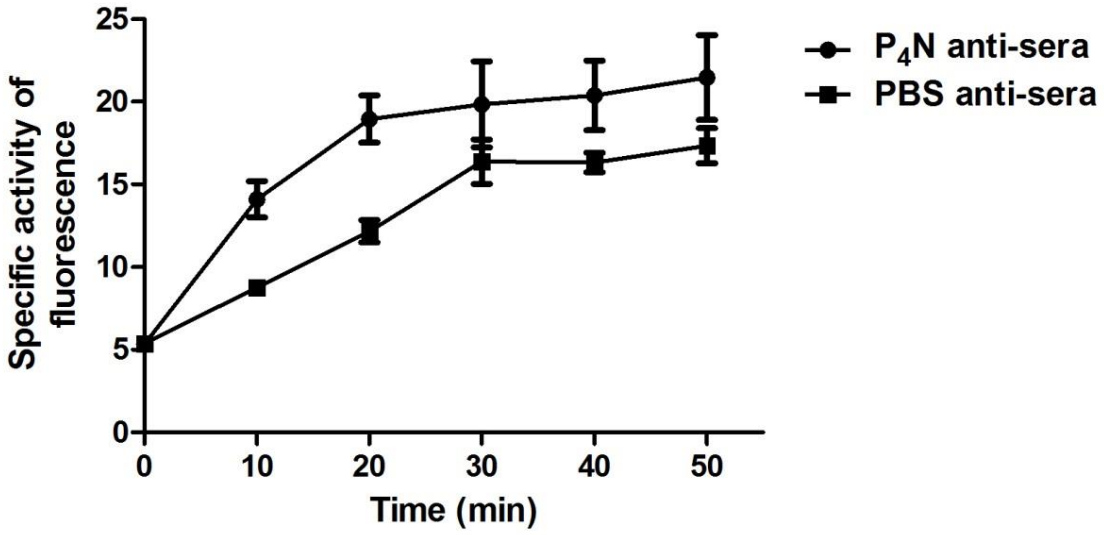


Figure S13. The affinities of anti-sera against CT26 cells after different lengths of incubation. Tumor antigens on the surface of CT26 cells were indirectly probed with P_4N and PBS anti-sera and FITC-conjugated goat anti-mouse IgG antibody. The specific fluorescent activities were measured by flow cytometry.

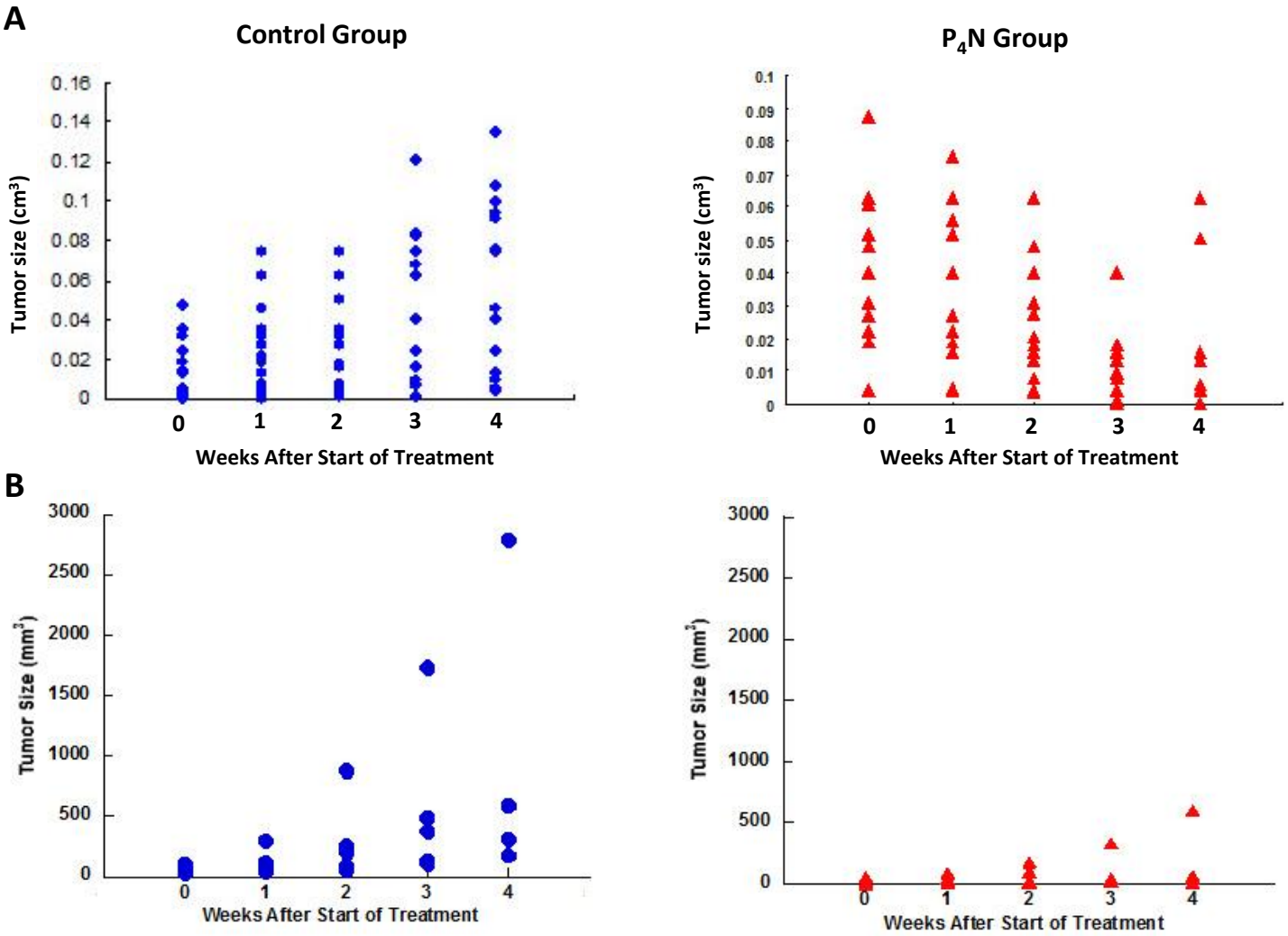


Figure S14. The effect of P_4N on the growth of tumor cell explants in nude mice. Mice bearing tumors derived from A.OVCAR-8 human ovarian carcinoma cells and B. LN229 human glioma cells were treated daily with intravenous P_4N 167 nmol/day) or PBS (vehicle). The volumes of individual tumors from control mice (blue circles) and P_4N treated mice (red triangles) were determined before treatment and weekly for 4 weeks after the start of treatment.

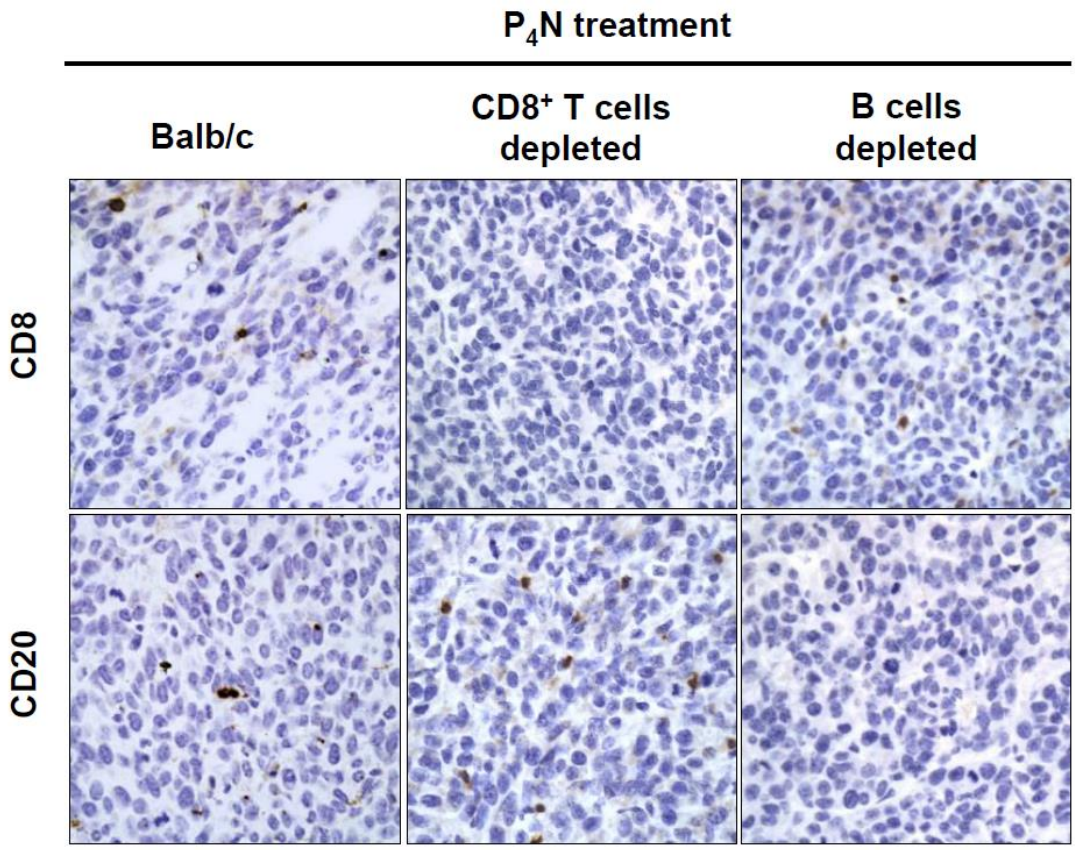


Figure S15. CD8+ T and B cell depletion in P_4N-treated BALB/c mice. CD8 T cells and B cells were depleted by monoclonal antibodies. BALB/c mice were inoculated s.c. with CT26 cells. When the average tumor mass reached ~50 mm3, the animals were treated with 5 mg/kg of P_4N by intratumoral injection every week. After treatment with P_4N, tumors were embedded in paraffin and thin sections were made and processed for IHC staining.