Ectopic ATP synthase β subunit proteins on human leukemia cell surface interact with platelets by binding glycoprotein IIb

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Supplemental Information

This document contains:
Materials and Methods
Supplementary Tables (1 in total)
Supplementary figures (8 in total)

Materials and Methods

Ethnic statement

The protocols concerning human samples and mice were approved by the ethics committee of Soochow University with issue numbers of SDFYY-2016-NSFC-074-1 and SDFYY-2016-NSFC-074-2 respectively and were performed in accordance with the Declaration of Helsinki and The Guidelines on the Humane Treatment of Laboratory Animals (Chinese Ministry of Science and Technology, 2006).

SZ22 antibodies

The monoclonal hybridoma producing SZ22 antibody was first established in this institute in 1988 [1] and has been used worldly for labeling platelets. The antibody was shown to bind to the N-terminal half of GPIIb but the exact site had not been identified [2]. Earlier experiments mainly from this institute demonstrated that SZ22 had marginal, if any, effects on various agonist-induced platelet aggregation [1].

Phage display screening for the epitope of GPIIb recognized by SZ22

Phage display screening on purified SZ22 antibodies was performed using the Ph.D.-12 peptide phage display library kit (New England BioLabs, Beijing, China) following the procedure recommended by the manufacturer. In brief, purified SZ22 (100 μg/ml) was coated onto ELISA plates (100 μl/well) and incubated with 2×10^{11} phages at room temperature for 45 min. After 3 rounds of enrichment, bound phages were eluted, titrated and amplified for the next round of screening. After three rounds of screening, bound phages were harvested and spread on host bacteria plates. Twelve isolated plaques were recovered for DNA extraction. After sequencing with the Sanger method using the primers in the kit. the corresponding 12-amino acid inserts were obtained and analyzed with Clustal 0 (1.2.1) multiple sequence alignment [3] to determine the potential epitope sequence of GPIIb recognized by SZ22.

Obtainment of to-be-studied peptides

The epitope of GPIIb recognized by SZ22 was identified as NPLKVDWG, hereon designated as N2G. Using this sequence as a core, serial peptides were chemically synthesized and HPLC-purified (Bootech Bioscience and Technology Co., Ltd, Shanghai, China). Some of them were modified at N-terminal with FITC or biotin for easy detection. “Mutant” peptides (e.g. NPWKVDLG, NsG) were used as controls. To generate N2G-guided cytotoxic peptides, a well-defined mitochondria-targeting proapoptotic sequence (KLAKLAKKLAKLAK, or KLA in short)[4] was used as cytotoxic domain. Alternative order (e.g. N2G linked to KLA at N or C terminal) and various linkers (including universal ones like GG, GA, or the de novo sequence of GPIIb) were tried using ELISA or FACS as testing methods, and finally the N2G-LP-KLAKLAKKLAKLAK (N2G-KLA) was selected for targeted cototoxicity assay.

Production of recombinant Calf2 segment of GPIIb

To determine if the epitope obtained above was meaningful in its physiological contexts, the segment around the epitope in the physiological structure of GPIIb, herein Calf2 domain in 3FCS (Figure S1) was produced by prokaryotic expression system. In brief, two DNA fragments coding the wild-type Calf2 (Calf2-N2G) or mutated form (Calf2-NsG) with a 10×His tag at C-terminal were synthesized and cloned into pET24a. Expression was induced with 0.5 mM IPTG at 27℃ and 200 rpm for 16 h. Harvested bacteria were lysed in binding buffer (10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 0.5 M NaCl, 30 mM imidazole, pH 7.4) and applied

onto Ni-IDA agarose column (DETAI BIO, Nanjing, China) column for purification. Finally, eluted proteins were dialyzed against PBS buffer and filtered with 0.22 μm membrane for sterilization.

Pull-down and mass spectrometry of N2G peptide mimetic-bound receptors or molecular partners

NB4 cells were cultured to S-phase and membrane proteins were extracted with a Membrane Protein Extraction Kit (BestBio, Shanghai, China) and quantified with a Protein Assay Kit (Takara Bio, Shiga, Japan). For preparation of affinity matrix preparation, 100 μl of 10 μM N2G-peptides were mixed with 50 μl of Streptavidin (SAV) MagBeads (Yeasen, Shanghai, China) at 4 ℃ for 3 h with shaking. After 2 rounds of washing to remove the unbound peptides with MagBeads buffer, 100 μg membrane proteins were added to the MagBeads and incubated for 2 h at 4 ℃ with shaking. Then the beads were spin washed for total of 3 times (1000 rpm for 5 min each). The final sediment was resuspended with 60 μl an appropriate volume of 1 M NaCl at 4 ℃ for 30 min. After spinning at 1000 rpm for 5 min, the supernatant containing target partners was recovered and resolved with SDS-PAGE. With help of Coomassie brilliant blue staining of the gel, the strips containing visible strands were harvested and subjected to intragel digestion, followed by mass spectrum assay with Orbitrap Elite™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Mass spectrometry data were processed with PLGS 2.4 software (Waters) and searched against the NCBInr Homo sapiens database using the MASCOT search engine (Matrix Science Inc., Boston, MA). The feedback proteins were judged by an arbitrary cutoff of score of 100 (Table S2). One of the remaining candidates that gave highest scores as well as scientifically sounding, herein ectopic ATP synthase subunit beta (ATP5B), was selected for further functional confirmation.

Production of recombinant ATP5B epitopes tandem (rATP5BesT) and its polyclonal antibodies

To maximize the chance of the intended polyclonal antibodies to block the binding of N2G-epitopes with the hypothetical ATP5B molecules, full-length human ATP5B protein was subject to B-epitopes prediction by FBCPred BCRPREDS server 1.0 [5] and Bepipred Linear Epitope [6] respectively and, assisted with the 3D structure model of ATP5B (1E79), a 247aa shortened form of artificial ATP5B construct that contains a tandem of six epitopes was designed (Supplementary Figure S1). A synthetic DNA fragment coding for this rATP5BesT with a 10 ×His Tag at C-terminal was cloned in pET24a, and expression and purification were performed as above with recombinant Calf2 segments.

Production of polyclonal antibodies against rATP5BesT

To generate polyclonal anti-rATP5BesT antibodies, New Zealand White rabbits (female, 2.8 kg of body weight) were immunized with 0.8 mg/each rATP5BesT proteins in Complete Freund's Adjuvant at hind footpads for the first dose, 0.8 mg rATP5BesT proteins in Incomplete Freund's Adjuvant in palpable popliteal lymph nodes for the second dose at day 7, and 2 mg in PBS via intravenous injection for the third dose on day 14. One week later, the rabbits were sacrificed and serum was prepared routinely. The titers of anti-rATP5BesT were measured with ELISA on rATP5BesT proteins (5 μg/ml)-coated plates. IgG antibodies were purified via saturate ammonium sulphate precipitation and dialysis against PBS. The specificity of the polyclonal anti-ATP5BesT antibodies was confirmed by staining total proteins prepared from HUVEC in western blot that was detected by a commercial anti-ATP5B antibody (not shown).

Flow cytometry and western blot for detection of peptide blocking effect

The different concentrations of synthesized N2G- or NsG-peptides were pretreated with SZ22 at room temperature for 30 min. Washed platelets (1×10^8 /ml) were added to peptide-SZ22 mixture and incubated for 30 min at room temperature. The mixture was washed with MTB (Modified Tyrode's Buffer) and labeled with PE-conjugated anti-mouse IgG (eBioscience, San Diego, CA). The blocking effect was analyzed with Cytomic FC 500 flow cytometer (Beckman Coulter Inc, Fullerton, CA). Data were obtained and analyzed using Kaluza software (Beckman Coulter, Miami, FL).

Five micrograms of denatured total protein extracted from platelets were performed by western blot. Peptides at different concentrations were incubated with SZ22 as the first antibody. The blots were incubated with peptide-SZ22 mixture overnight at 4 ℃, then with the secondary antibody (horseradish peroxidase-conjugated goat anti-mouse) (Cell Signaling Technology, Beverly, MA) for 1 h at room temperature. Blots were then detected.

Platelet aggregation and GPIIbIIIa activation

Washed platelets (2×10^8 /ml) were pretreated with 10 μM peptide at room temperature for 30 min and then were stimulated by ADP (10 μM) (Chrono-log, Havertown, PA) and Thrombin (0.05 U/ml)(Sigma, St Louis, MO). The dynamic process of platelet aggregation was continuously recorded by Chrono-Log lumi-aggregometer (Chrono-Log Corporation, Broomall, PA).

Washed platelets were pretreated with peptide and stimulated by ADP and Thrombin as above. PAC-1 antibody was added to the mixture. After incubation for 30 min at room temperature, the staining reaction was stopped by adding 500 μl MTB. GPIIbIIIa activation was analyzed by flow cytometer.

Flow cytometry for detection of anti-ATP5B antibody blocking effect on the binding of N2G peptide to cell lines.

HaCaT (CCTCC, Wuhan, China)and A431 cells (Cell Bank, Chinese Academy of Science, Shanghai, China) were grown in DMEM medium supplemented with fetal bovine serum (10%, v/v), streptomycin (100 μg/ml) and penicillin (100 U/ml). K562, NB4, HL-60, Molt4 and HMY2.CIR (Cell Bank, Chinese Academy of Science, Shanghai, China) cells were cultured in RPMI-1640 medium supplemented with fetal bovine serum (10%, v/v), streptomycin (100 μg/ml) and penicillin (100 U/ml).

Suspensions of different cells (3×10^5 cells/ tube) in 100 μl washing buffer (PBS containing 1% FBS) were preincubated with anti-ATP5B antibody (5 μg/ml) for 30 min at 4°C, respectively. Next, cells were washed twice with washing buffer to remove the excess extracellular unbound antibodies at 1200 rpm for 5 min each time and finally resuspended in 100 μl washing buffer. 100 μl FITC-labeled N2G- or NsG-peptides (20 μM) were added to the above mixture at 4°C for 30 min in the dark. Flow cytometry was performed using a Cytomic FC 500 flow cytometer. The experiment was repeated three times.

Measurement of cell surface production of ATP supposedly catalyzed by ectopic ATP synthase

To measure if pretreatment of cells would affect the hypothetical catalyzing functions of ectopic ATP synthase, a previous protocol[7] was employed with modification. In brief, harvested NB4 cells were washed with HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) for three times and resuspended in HEPES buffer with 2 mM $MgCl_2$ to 2×10^5 /ml. N2G-peptides were added to 10 μM or anti-ATP5B antibodies to 5 μg/ml and cultured for 1 h at 37 ℃. Then an equal volume of HEPES supplemented with 2 mM $MgCl_2$, 200 μM ADP, 20 mM KH_2PO_4 was added and mixed thoroughly. Supernatants were collected by centrifugation at 1200 rpm for 5 min to remove cells and, after transfer to a new tube, spun again at 4000 rpm for 3 min. ATP levels in the cleared supernatant were measured using an Enhanced ATP Assay Kit (Beyotime, Shanghai, China) following the manufacturer's protocol. The reaction plate was read in a Synergy H1 plate reader (BioTek, Winooski, VT).

Flow cytometry of human blood or marrow cell expression of ectoATP5B and aggregation with platelets

All human samples including peripheral blood and bone marrow aspirates were obtained from patients or healthy donors in this institute with informed consent and approved by the Ethical Committee of First Affiliated Hospital of Soochow University. Peripheral blood mononuclear cells (PBMCs) were purified from blood of healthy individuals using gradient Ficoll-Hypaque (TBD Science, Tianjin, China). Bone marrow mononuclear cells were separated using Lymphoprep™ (StemCell Technologies, Vancouver, Canada) according to the manufacturer's instructions. Both blood and bone marrow were subjected to cell purification in 1 hr and isolated cells were frozen with cryopreservation solution at -80 ℃ till use for assay in a same batch. Upon time for staining for ectoATP5B or binding with platelets, cell preparations were thawed and counted for viable cells. Cells (5×10^5 cells/test) were treated with 5 μg/ml rabbit anti-ATP5B antibody at 4 ℃ for 30 min, and after two washes, stained with 1 μg/ml PE-conjugated donkey anti-rabbit IgG (Biolegend, San Diego, CA) at 4 ℃ for 30 min in the dark. Expression of ectoATP5B was analyzed using a flow cytometer.

For assay of mononuclear cells binding with platelets, platelets were prepared from peripheral blood of healthy volunteers and labeled with 5 μg/ml Calcein (Yeasen, Shanghai, China) as previously described[8] and suspended in modified Tyrode buffer containing PGI2 (Abcam, San Francisco, CA) at 1.5×10^8 /ml. Human PBMCs or bone marrow mononuclear cells (5×10^6 cells/ml) and Calcein-labeled platelets were mixed at equal volumes and incubated at 37 ℃ for 30 min. After washes, the mixture was analyzed by flow cytometry.

CCK8 assays of N2G-KLA peptides effect on leukemia cells growth

To test the effect of N2G-KLA peptides on leukemia cell growth and to test if it was mediated by ectoATP5B, NB4 or HL-60 cells (3×10^5 cells/test) were pretreated with 60 μM N2G-peptides or 50 μg/ml anti-ATP5B antibodies at 37°C for 1 h and then mixed with 30μM N2G-KLA peptides. Then the cells were plated into 96-well plates and cultured at 37 ℃ for 24 h in CO_2 incubator. 10 μl Cell Counting Kit-8 subsrate (Biomake, Shanghai, China) was added and 3 h later, OD450 was measured using a Microplate Reader (BioTek Instruments, Winooski, VT).

Flow cytometry of N2G-KLA peptides effect on leukemia cells apoptosis

To test if the effects of N2G-KLA peptides on leukemia or cancer cells was mediated via apoptosis, cells were treated as above but in larger scale (e.g. 3×10^5 cells/test) and cells were plated into 6-well plates instead of 96-well ones. At the end of culture, cells were harvested and stained with Annexin V/PE and 7-AAD (2 μl of each reagent per test) according to the manufacturer's instructions (BD Biosciences). The cell apoptosis was detected by flow cytometer.

To test if N2G-KLA peptides manifested differential effect on a mixture of ectoATP5B-positive and -negative cells, NB4 cells were labelled with 20 nM 5,6- carboxyfluorescein diacetate succinimidyl ester (CFSE) as routine and mixed with same numbers of A431 cells (each 3×10^5 cells/test). N2G-peptides was added to 60 μM or anti-ATP5B antibodies to 50 μg/ml at 37°C

for 1 h, followed by addition of 30 μM N2G-KLA peptides and culture at 37 ℃ for 24 h. Cells were harvested and stained with Annexin V/PE and 7-AAD. The proportion of apoptosis in each cell type was detected by flow cytometry.

Xenograft leukemia model in NOD-SCID mice

A xenograft model of leukemia with NB4 cells was established as described[9] with modification. Briefly, specific pathogen-free male NOD-SCID mice (aged 6-8 wk) were purchased from Shanghai Laboratory Animal Center (Shanghai, China) and housed in a specific pathogen-free facility with free access to food and water. NB4 cells (4×10^6) harvested at exponential phase were injected into NOD-SCID mice (n=10 each group) via tail vein (day 1). Then 200 μg N2G-KLA peptides (1 mg/ml) were injected via tail vein on each third day, till the end of first month, thus making a total of ten dosing. Body weight and survival of the mice were monitored at the same time schedule. Mice in control groups received 200 μl PBS only or 200 μg KLA peptides.

In another experiment, mice were killed at day 36 after tumor inoculation and femurs were taken. Bone marrow cells were flushed from the femurs into RPMI-1640 medium and red blood cells were lysed. After washing and counting, the cells (1×10^6) were incubated with PE-conjugated mouse anti-human CD33 antibody (Biolegend, San Diego, CA) at 10 μg/ml and analyzed using a flow cytometer.

Structural modeling of ATP synthase–GPIIb interactions

To investigate the potential interaction between ATP5B and GPIIb, we firstly scanned the surface of ATP synthase with the N2G-epitope sequence using the PEP-SiteFinder[10]. The top 50 poses were checked to identify the most likely binding site(s). With these hypothetical interactions as restriction, the ATP5B–GPIIb complex was modeled by the protein-protein docking tool, Zdock[11].

Data Sharing Statement

For original data, please contact yiqiangwang@suda.edu.cn

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Table S1. Summary of genes identified by mass spectrum analysis of N2G-peptides-bound proteins that gave collective homologue scores over 100 *.

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	Area	# AAs	MW [kDa]
P06576	ATP synthase subunit beta, mitochondrial OS=Homo sapiens GN=ATP5B PE=1 SV=3 - [ATPB_HUMAN]	3547.21	60.87	5	24	24	126	2.152E9	529	56.5
P06576	ATP synthase subunit beta, mitochondrial OS=Homo sapiens GN=ATP5B PE=1 SV=3 - [ATPB_HUMAN]	3440.19	62.19	4	15	23	124	2.388E9	529	56.5
P25705	ATP synthase subunit alpha, mitochondrial OS=Homo sapiens GN=ATP5A1 PE=1 SV=1 - [ATPA_HUMAN]	2198.93	52.80	8	32	32	76	9.857E8	553	59.7
Q9BQE3	Tubulin alpha-1C chain OS=Homo sapiens GN=TUBA1C PE=1 SV=1 - [TBA1C_HUMAN]	1920.57	45.43	21	5	21	74	1.281E9	449	49.9
P25705	ATP synthase subunit alpha, mitochondrial OS=Homo sapiens GN=ATP5A1 PE=1 SV=1 - [ATPA_HUMAN]	1913.26	47.92	8	31	31	68	8.433E8	553	59.7
P07437	Tubulin beta chain OS=Homo sapiens GN=TUBB PE=1 SV=2 - [TBB5_HUMAN]	1836.15	60.59	20	4	22	73	9.038E8	444	49.6
P07437	Tubulin beta chain OS=Homo sapiens GN=TUBB PE=1 SV=2 - [TBB5_HUMAN]	1806.67	60.14	24	4	21	67	7.093E8	444	49.6
P68371	Tubulin beta-4B chain OS=Homo sapiens GN=TUBB4B PE=1 SV=1 - [TBB4B_HUMAN]	1644.20	56.63	25	3	20	61	5.510E8	445	49.8
P68371	Tubulin beta-4B chain OS=Homo sapiens GN=TUBB4B PE=1 SV=1 - [TBB4B_HUMAN]	1622.64	57.08	23	3	21	66	7.242E8	445	49.8
P68366	Tubulin alpha-4A chain OS=Homo sapiens GN=TUBA4A PE=1 SV=1 - [TBA4A_HUMAN]	1219.58	42.19	18	4	20	53	1.281E9	448	49.9
P68363	Tubulin alpha-1B chain OS=Homo sapiens GN=TUBA1B PE=1 SV=1 - [TBA1B_HUMAN]	1098.32	47.45	31	22	22	50	7.184E8	451	50.1
Q9BUF5	Tubulin beta-6 chain OS=Homo sapiens GN=TUBB6 PE=1 SV=1 - [TBB6_HUMAN]	731.20	14.80	3	2	8	29	4.999E8	446	49.8
P14923	Junction plakoglobin OS=Homo sapiens GN=JUP PE=1 SV=3 - [PLAK_HUMAN]	395.81	20.40	7	13	13	17	1.214E7	745	81.7
P02787	Serotransferrin OS=Homo sapiens GN=TF PE=1 SV=3 - [TRFE_HUMAN]	238.58	18.05	6	10	10	11	1.139E7	698	77.0
Q02413	Desmoglein-1 OS=Homo sapiens GN=DSG1 PE=1 SV=2 - [DSG1_HUMAN]	185.11	7.82	1	6	6	6	5.411E6	1049	113.7

P15924	Desmoplakin OS=Homo sapiens GN=DSP PE=1 SV=3 - [DESP_HUMAN]	145.19	3.62	1	9	9	9	9.734E6	2871	331.6
P01008	Antithrombin-III OS=Homo sapiens GN=SERPINC1 PE=1 SV=1 - [ANT3_HUMAN]	123.82	8.62	2	5	5	6	1.763E8	464	52.6

Note: * Three gel-slice samples were subjected to mass spectrum assay, and certain genes many showed up in more than one sample but with different peptide and collective homologue scores.

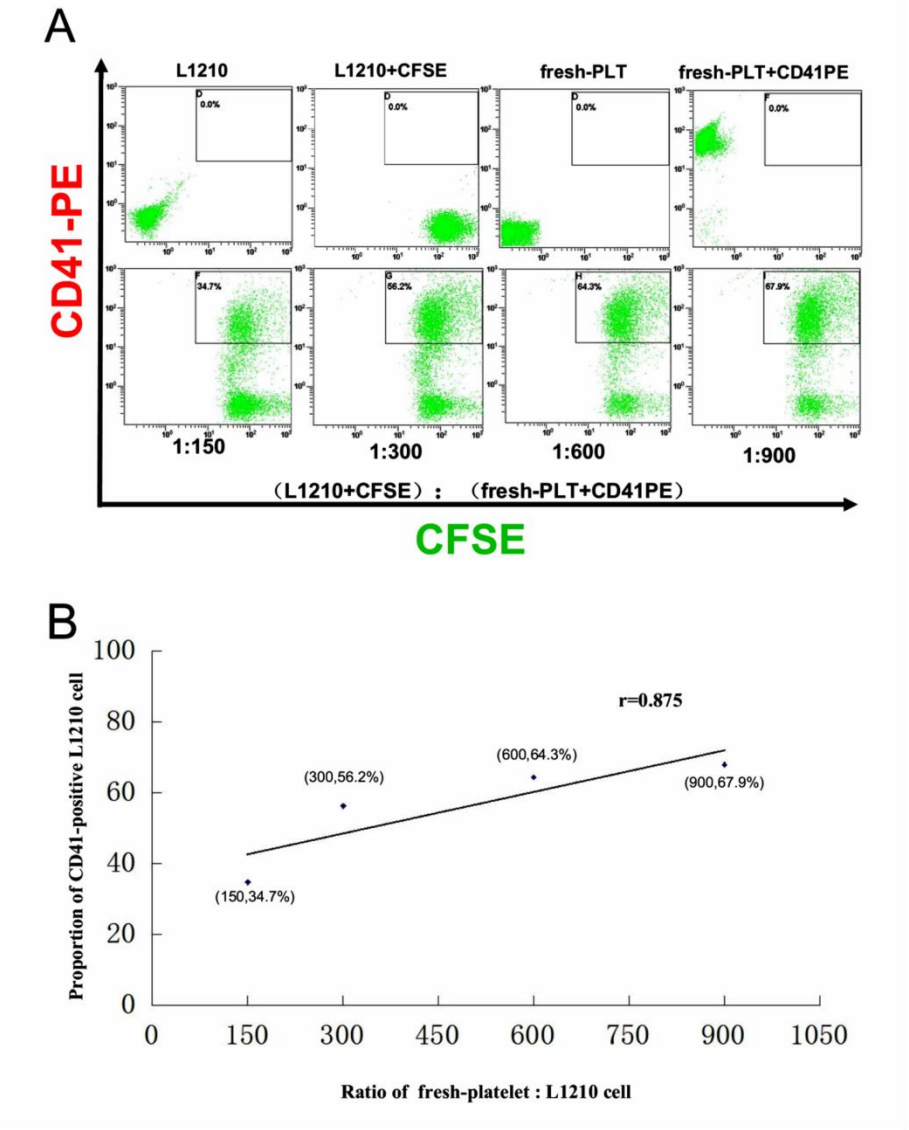

Figure 1. Formation of leukemia cells-platelets aggregates after coincubation. **A**: After coculture of CFSE labelled L1210 cells with platelets, the cells were harvested and stained with anti-mouse CD41-PE. Double positive events detected by FCM were for leukemia cells-platelets aggregates. **B**: The percentage of aggregation was positively correlated with the ratios of platelets to L1210 cells, with a linear regression rate $r = 0.875$.

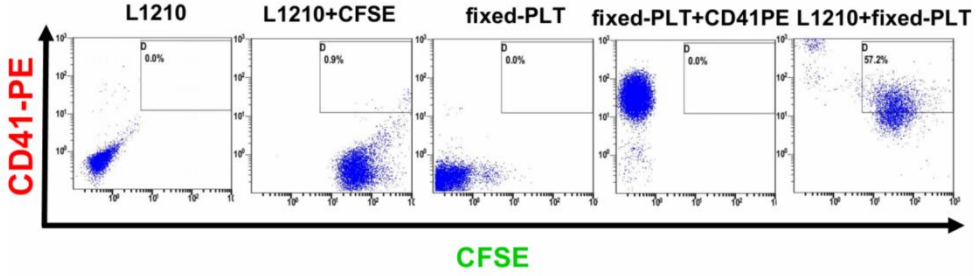

Figure 3. Formation of aggregates of leukemia cells with fixed platelets. CFSE-labeled L1210 cells were cocultured with fixed platelets at 1:300 at 37 ℃ for 30 minutes, and the harvested mixture was stained for anti-mouse CD41-PE. Please note that there was not a clear grouping for double stained L1210-platelet aggregates when fixed platelets were used instead of fresh ones.

Figure S1. Murine platelets, either fresh or fixed, formed aggregates with murine leukemia cells. Shown were screenshots of figures in our previous paper in Chinese language (but with English figure legends). Reuse of these figures was permitted by original journal. **Origin:** Hu Y, Shao L, Zhao L, Shen Y, Wu K, Wang Y. Platelets decreas the sensititity of leukemia cell L1210 to multiple druges via actibiting the AKT and ERK signaling pathway. [Zhong Guo Shi Yan Xue Ye Xue Za Zhi] (Journal of Experimental Hematology) 2016; 24(5): 1489-1494.

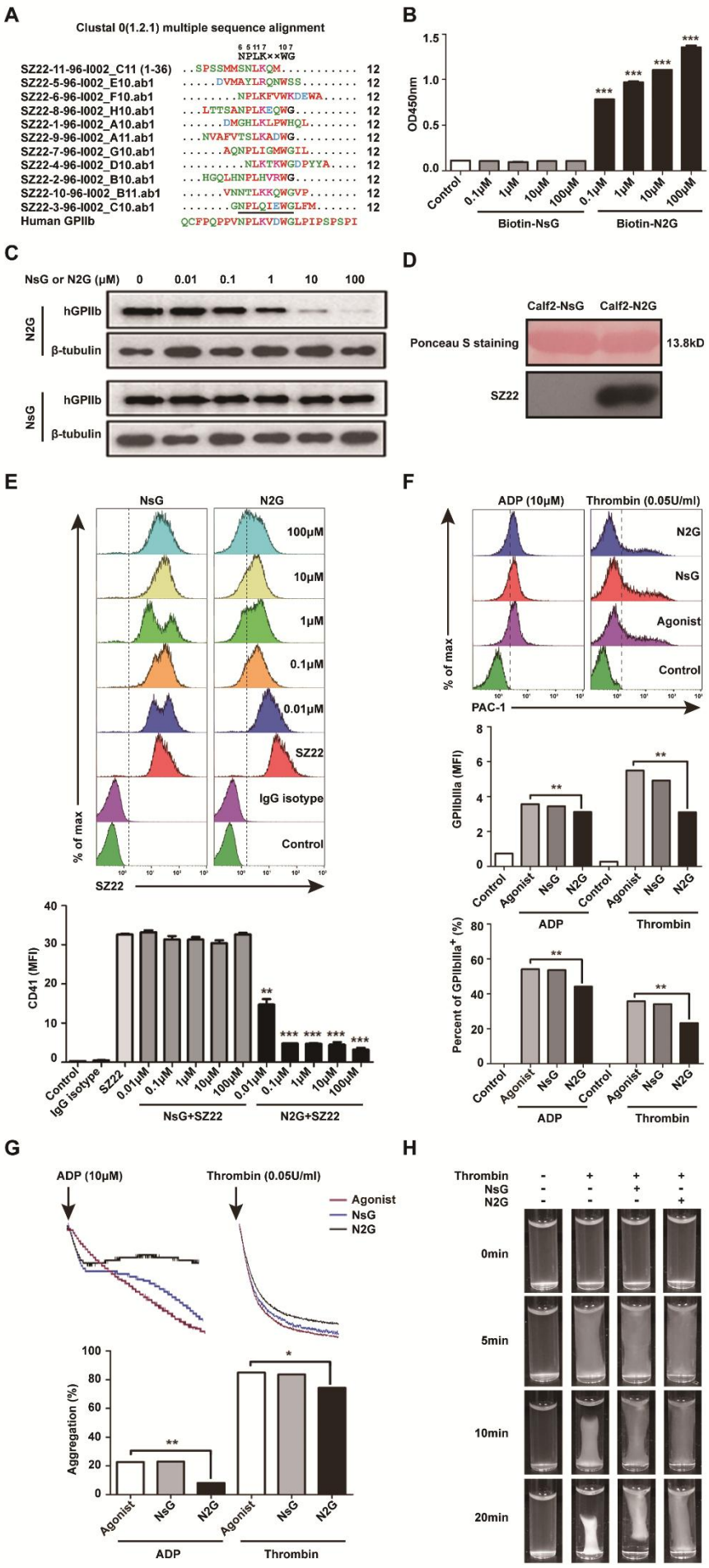

Figure S2. Identification of a novel epitope involved in platelets activation. (A) Clustal assay of sequences obtained by phage display screening against immobilized SZ22 antibodies revealed a conserved motif that aligned with human GPIIb. (B) Immobilized N2G-peptides were recognized by SZ22 in a dose-dependent manner. ELISA plates were coated with streptavidin (100 μg/ml, 100 μl/well) and different concentration of biotinylated synthetic N2G- or NsG-peptides were added. After washing and incubation with SZ22 (0.5 μg/ml), HRP conjugated goat-anti-mouse IgG was added. After developing with OD_{450} were read. (C) N2G-peptides blocked SZ22 binding onto hGPIIb in platelets proteins. N2G- or NsG-peptides of different concentrations were mixed with SZ22 (1 μg/ml) for 30 min and then the mixture was applied to blots of platelet proteins. (D) Binding of SZ22 with Calf2 domain relied on N2G epitope. Recombinant Calf2-N2G and Calf2-NsG were resolved side by side on SDS-PAGE and transferred to blot membrane for binding and development with SZ22. (E) N2G-peptides blocked SZ22 binding onto resting platelets. N2G- or NsG-peptides of different concentrations were mixed with SZ22 (1 μg/ml) for 30 min, and then the mixtures were utilized to stain platelets as measured by flow cytometry. Mean fluorescence intensity (MFI) were calculated for all platelets. (F) N2G-peptides inhibited ADP- or thrombin-induced GPIIbIIIa formation on platelets as measured with PAC-1 antibody. MFI of all platelets and percentage of PAC-1 positive platelets were calculated, with unstimulated platelets as negative control. (G) N2G-peptides (10 μM) inhibited ADP- or thrombin-induced platelet aggregation as measured using aggregometer. (H) N2G-peptides inhibited thrombin-induced clot retraction. Except for (A), all other panels were representatives of three experiments in each condition. For numerical indexes, data were shown as mean ± standard deviation (SD). For indicated comparisons, Student's *t*-test was utilized. *p<0.05, **p<0.01, ***p<0.001.

A. Calf2-N2G:

CATATGgctgaagctcaggttgaactgcgtggtaactctttcccggcttctctggttgtt
M A E A Q V E L R G N S F P A S L V V
gctgctgaagaaggtgaacgtgaacagaactctctggactcttggggtccgaaagttgaa
A A E E G E R E Q N S L D S W G P K V E
cacacctacgaactgcacaacaacggtccgggtaccgttaacggtctgcacctgtctatc
H T Y E L H N N G P G T V N G L H L S I
cacctgccgggtcagtctcagccgtctgacctgctgtacatcctggacatccagccgcag
H L P G Q S Q P S D L L Y I L D I Q P Q
ggtggtctgcagtgcttcccgcagccgccggttaacccgctgaaagttgactggggtctg
G G L Q C F P Q P P V N P L K V D W G L
ccgatcccgtctccgtctccgatccacccggctcaccacaaacgtAAGCTTgcggccgca
P I P S P S P I H P A H H K R *K L A A A*
ctcgagcaccaccaccaccaccactga
L E H H H H H H -

B. Calf2-NsG:

CATATGgctgaagctcaggttgaactgcgtggtaactctttcccggcttctctggttgtt
M A E A Q V E L R G N S F P A S L V V
gctgctgaagaaggtgaacgtgaacagaactctctggactcttggggtccgaaagttgaa
A A E E G E R E Q N S L D S W G P K V E
cacacctacgaactgcacaacaacggtccgggtaccgttaacggtctgcacctgtctatc
H T Y E L H N N G P G T V N G L H L S I
cacctgccgggtcagtctcagccgtctgacctgctgtacatcctggacatccagccgcag
H L P G Q S Q P S D L L Y I L D I Q P Q
ggtggtctgcagtgcttcccgcagccgccggttaacccgtggaaagttgacctgggtctg
G G L Q C F P Q P P V N P W K V D L G L
ccgatcccgtctccgtctccgatccacccggctcaccacaaacgtAAGCTTgcggccgca
P I P S P S P I H P A H H K R *K L A A A*
ctcgagcaccaccaccaccaccactga
L E H H H H H H -

Figure S3. Synthetic DNA fragments coding for recombinant wild type (Calf2-N2G) or mutant type (Calf2-NsG) Calf2 domains. (A) For Calf2 of wild-type amino acid sequences, namely containing a wild-type N2G epitope (NPLKVDWG). (B) For Calf2 mutant in which the L835 and W839 corresponding to N2G epitope was switched to form a control epitope (NsG, NPWKVDLG). Nucleotide sequences in CAPITAL are for two endonuclease restriction sites. *Amino acid initials in italic* were from vector pET24a. Amino acids in blue font highlighted the difference between Calf2-N2G and Calf2-NsG sequences.

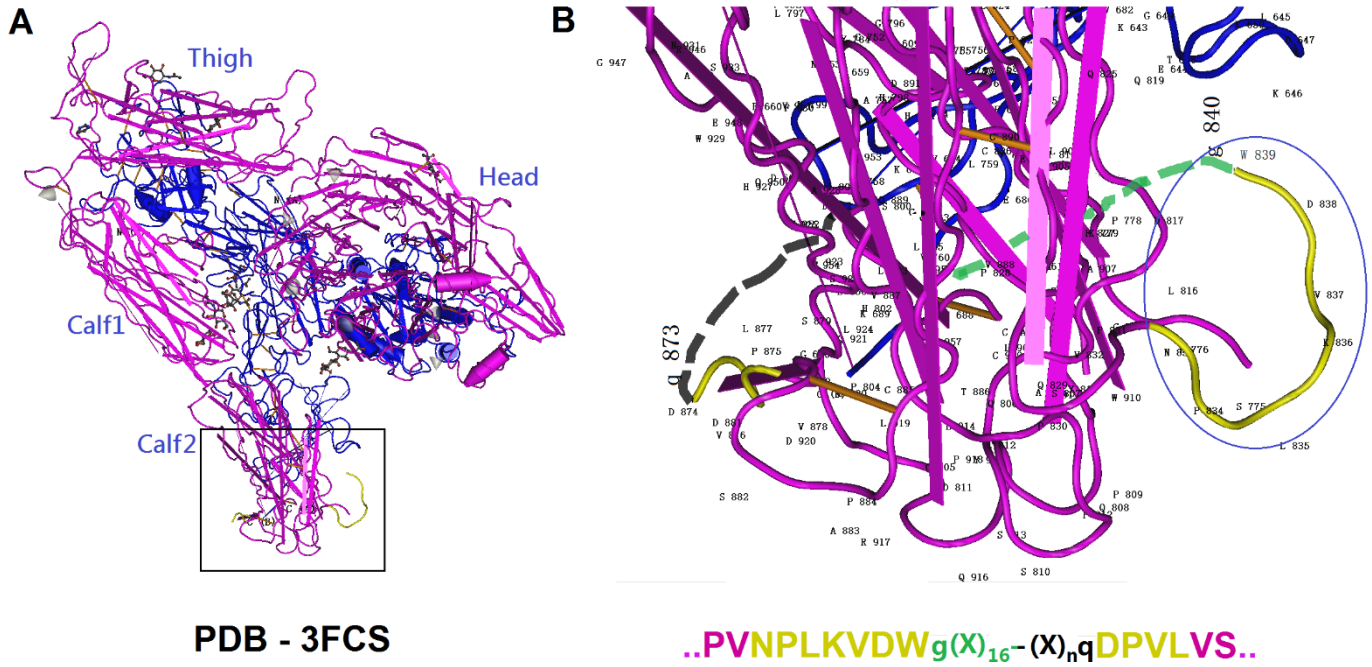

Figure S4. Illustration of N2G epitope on GPIIbIIIa complex (PDB 3FCS). This model was obtained with proteins in rest (closed) status, with four main domains labeled [12]. Panel B was a closer view of the part in square of panel A. Dashed lines were added manually to propose the position of the missing part (loop), where green part was for the C-terminal of the heavy chain of mature GPIIb, and black part for the N-terminal of the light chain of mature GPIIb. N2G epitope (**NPLKVDWg**) was highlighted in a blue oval.

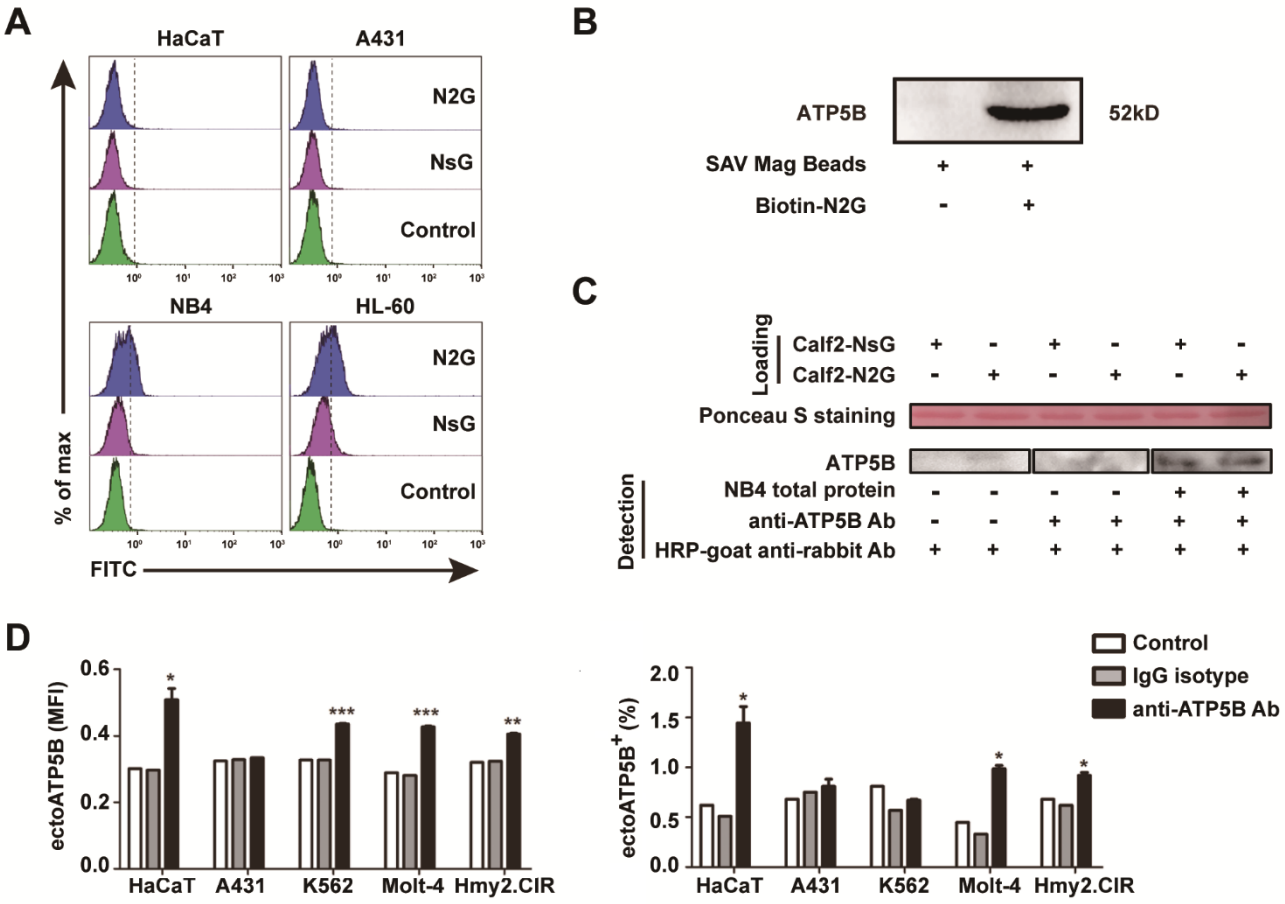

Figure S5. Binding of N2G-peptides onto leukemic cells was mediated by ectopic ATP5B. (A) FITC-conjugated N2G- or NsG-peptides (10 μM) were used to stain various cells for 30 min before flow cytometry. Only two representatives of leukemic or non-leukemic cancer cell lines were shown. (B) Biotinylated N2G-peptides (10 μM) were coated onto streptavidin (SAV) conjugated-MagBeads and mixed with membrane proteins prepared from NB4 cells under agitation for 2 h. After spinning, the bound proteins were dissociated with 1M NaCl and separated on SDS-PAGE. After transferring onto blotting membrane, anti-ATP5B was utilized for a routine western blotting. (C) Recombinant Calf2-N2G and Calf2-NsG fragments were run on SDS-PAGE and transferred to nitrocellulose membrane. Ponceau S staining confirmed the presence of the fragment proteins. Then NB4 membrane proteins (100 μg) were applied onto the blotting membrane overnight, followed by incubation with anti-ATP5B antibodies (5 μg/ml) and HRP-goat anti-rabbit IgG. To check the specificity of Calf2-N2G binding with ATP5B, controls without NB4 proteins or anti-ATP5B antibodies were run side by side. (D) Measurement of ectoATP5B expression on five other cell lines by using flow cytometry. Cells were incubated with anti-ATP5B (5 μg/ml) at 4°C for 30 min followed by PE donkey anti-rabbit IgG. Cells unstained cells or stained with IgG isotype control as primary antibody were used as controls. Please note that though staining with anti-ATP5B of these five cells might give statistically significant increases over isotype controls, the actual positive percentages for them (e.g. 1.4% for HaCaT) were much less than that for NB4 cells (over 80%, refer to Figure 2A), indicating an absence of ectopic ATP5B in these cells. In all panels, shown were representatives of three repeats in each. For (D), mean ± SD was obtained from two duplicates in each sample. *p<0.05；**p<0.01；***p<0.001 vs IgG isotype group, Student's *t*-test.

A. Features of wild type ATP5B protein

```
Cn3D-surface                    MLGFVGRVAAAPASGALRRLTPSASLPPAQLLLRAAPTAVHPVRDYAAQTSPSPKAGAAT  60
FBCPred (BCRPREDS Server 1.0)   MLGFVGRVAAAPASGALRRLTPSASLPPAQLLLRAAPTAVHPVRDYAAQTSPSPKAGAAT  60
Bepipred (Linear Epitope)       MLGFVGRVAAAPASGALRRLTPSASLPPAQLLLRAAPTAVHPVRDYAAQTSPSPKAGAAT  60

Cn3D-surface                    GRIVAVIGAVVDVQFDEGLPPILNALEVQGRETRLVLEVAQHLGESTVRTIAMDGTEGLV  120
FBCPred (BCRPREDS Server 1.0)   GRIVAVIGAVVDVQFDEGLPPILNALEVQGRETRLVLEVAQHLGESTVRTIAMDGTEGLV  120
Bepipred (Linear Epitope)       GRIVAVIGAVVDVQFDEGLPPILNALEVQGRETRLVLEVAQHLGESTVRTIAMDGTEGLV  120

Cn3D-surface                    RGQKVLDSGAPIKIPVGPETLGRIMNVIGEPIDERGPIKTKQFAPIHAEAPEFMEMSVEQ  180
FBCPred (BCRPREDS Server 1.0)   RGQKVLDSGAPIKIPVGPETLGRIMNVIGEPIDERGPIKTKQFAPIHAEAPEFMEMSVEQ  180
Bepipred (Linear Epitope)       RGQKVLDSGAPIKIPVGPETLGRIMNVIGEPIDERGPIKTKQFAPIHAEAPEFMEMSVEQ  180

Cn3D-surface                    EILVTGIKVVDLLAPYAKGGKIGLFGGAGVGKTVLIMELINNVAKAHGGYSVFAGVGERT  240
FBCPred (BCRPREDS Server 1.0)   EILVTGIKVVDLLAPYAKGGKIGLFGGAGVGKTVLIMELINNVAKAHGGYSVFAGVGERT  240
Bepipred (Linear Epitope)       EILVTGIKVVDLLAPYAKGGKIGLFGGAGVGKTVLIMELINNVAKAHGGYSVFAGVGERT  240

Cn3D-surface                    REGNDLYHEMIESGVINLKDATSKVALVYGQMNEPPGARARVALTGLTVAEYFRDQEGQD  300
FBCPred (BCRPREDS Server 1.0)   REGNDLYHEMIESGVINLKDATSKVALVYGQMNEPPGARARVALTGLTVAEYFRDQEGQD  300
Bepipred (Linear Epitope)       REGNDLYHEMIESGVINLKDATSKVALVYGQMNEPPGARARVALTGLTVAEYFRDQEGQD  300

Cn3D-surface                    VLLFIDNIFRFTQAGSEVSALLGRIPSAVGYQPTLATDMGTMQERITTTKKGSITSVQAI  360
FBCPred (BCRPREDS Server 1.0)   VLLFIDNIFRFTQAGSEVSALLGRIPSAVGYQPTLATDMGTMQERITTTKKGSITSVQAI  360
Bepipred (Linear Epitope)       VLLFIDNIFRFTQAGSEVSALLGRIPSAVGYQPTLATDMGTMQERITTTKKGSITSVQAI  360

Cn3D-surface                    YVPADDLTDPAPATTFAHLDATTVLSRAIAELGIYPAVDPLDSTSRIMDPNIVGSEHYDV  420
FBCPred (BCRPREDS Server 1.0)   YVPADDLTDPAPATTFAHLDATTVLSRAIAELGIYPAVDPLDSTSRIMDPNIVGSEHYDV  420
Bepipred (Linear Epitope)       YVPADDLTDPAPATTFAHLDATTVLSRAIAELGIYPAVDPLDSTSRIMDPNIVGSEHYDV  420

Cn3D-surface                    ARGVQKILQDYKSLQDIIAILGMDELSEEDKLTVSRARKIQRFLSQPFQVAEVFTGHMGK  480
FBCPred (BCRPREDS Server 1.0)   ARGVQKILQDYKSLQDIIAILGMDELSEEDKLTVSRARKIQRFLSQPFQVAEVFTGHMGK  480
Bepipred (Linear Epitope)       ARGVQKILQDYKSLQDIIAILGMDELSEEDKLTVSRARKIQRFLSQPFQVAEVFTGHMGK  480

Cn3D-surface                    LVPLKETIKGFQQILAGEYDHLPEQAFYMVGPIEEAVAKADKLAEEHSS             529
FBCPred (BCRPREDS Server 1.0)   LVPLKETIKGFQQILAGEYDHLPEQAFYMVGPIEEAVAKADKLAEEHSS             529
Bepipred (Linear Epitope)       LVPLKETIKGFQQILAGEYDHLPEQAFYMVGPIEEAVAKADKLAEEHSS             529
```

B. Recombinant ATP5B epitopes tandem (rATP5BesT, 247aa) and its coding DNA fragment

```
CATATGcatcttggtgaatcaaccgttcgtaccattgcaatggatggtactgaaggcctg
    M  H  L  G  E  S  T  V  R  T  I  A  M  D  G  T  E  G  L
gttcgtggtcagaaagttttagacagcggtgcacccattaaaattccggttggtcctgaa
 V  R  G  Q  K  V  L  D  S  G  A  P  I  K  I  P  V  G  P  E
accttaggtcgtataatgaatgttattggtgagccgattgatgaaagaggaccgattaaa
 T  L  G  R  I  M  N  V  I  G  E  P  I  D  E  R  G  P  I  K
accaaacagttcgcaccgatacacgcagaagcaccggagtttatggagatgagcgtggaa
 T  K  Q  F  A  P  I  H  A  E  A  P  E  F  M  E  M  S  V  E
caagaaattctggtgaccggtatagcaggagttggagaacggacaagagaaggcaacgac
 Q  E  I  L  V  T  G  I  A  G  V  G  E  R  T  R  E  G  N  D
```

ctgtatcatgaaatgattgaaagcggtgttattaatctgaaagatgccacaagcaaacag

L Y H E M I E S G V I N L K D A T S K Q

gcaggaagcgaagttagcgcgttactgggaagaataccaagcgcagttggataccagcct

A G S E V S A L L G R I P S A V G Y Q P

accttagcaacctatgtgccagcagatgacctgaccgatccggcaccagcaaccacattt

T L A T Y V P A D D L T D P A P A T T F

gcatatccggcagttgatcccttagatagcaccagccggattatggatccgaatattgtt

A Y P A V D P L D S T S R I M D P N I V

gggagtgaacactatgatgttgcaagaggtgccgaagttttaccggacacatgggtaaa

G S E H Y D V A R G A E V F T G H M G K

ctggttccgctgaaggaaaccattaaaggctttcagcagattctggcaggtgaatatgat

L V P L K E T I K G F Q Q I L A G E Y D

catctgccggagcaggcattttatatggttggtcctattgaagaagcagttgcaaaagca

H L P E Q A F Y M V G P I E E A V A K A

gataaactggcagaagaacacagcagccatcaccatcaccatcatcatcaccattaa

D K L A E E H S S *H H H H H H H H H H* -

tagcAAGCTT

\- *x x*

Figure S6. Design of a recombinant ATP5B protein analogue containing main surface B-epitopes of ATP5B. (A) Analysis of wild type ATP5B to obtain sequences to be included in proposed recombinant ATP5B analogue. Sequences in green were those located on surface of the whole ATP synthase complex as suggested by its 3D structure (PDB 1E79), while those sequences in blue were located on interface of ATP5B subunits with other subunits. Sequences in red were predicted immune B-epitopes. Underlined sequences were those included in final design. (B) Synthetic DNA fragment coding for the designed recombinant ATP5B epitopes tandem (rATP5BesT). Nucleotide sequences in CAPITAL are for two endonuclease restriction sites. *Amino acid initials in italic* are non-ATP5B original.

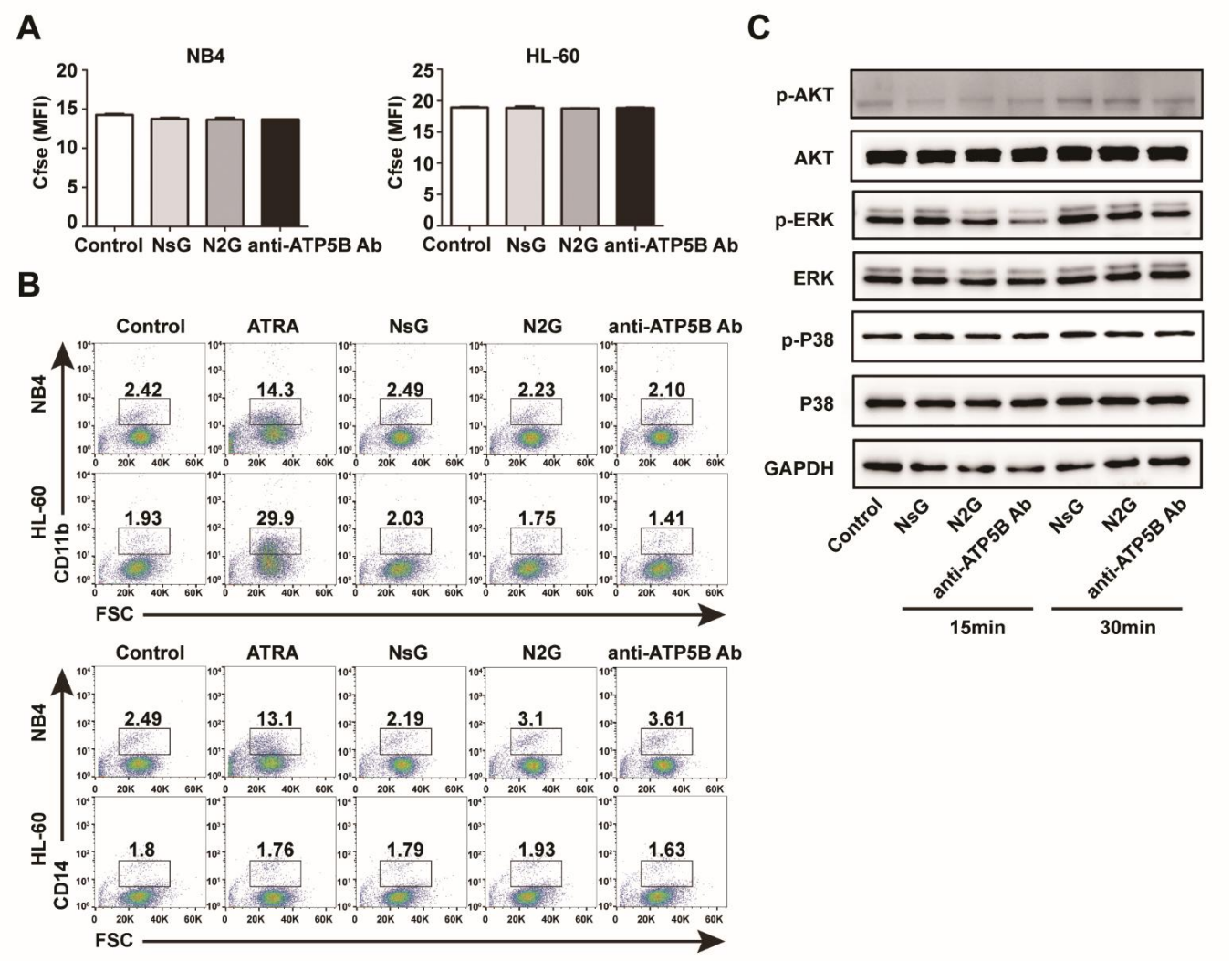

Figure S7. N2G-peptides showed no or minimal effects on proliferation, differentiation or signaling molecules of leukemic cells. (A) NB4 and HL-60 cells were labelled with CFSE (0.2 μM) and cultured with N2G- or NsG-peptides (10 μM) or anti-ATP5B antibodies (5 μg/ml) for 48 h at 37 ℃, and the fluorescence intensity of the culture was measured by flow cytometry. (B) NB4 and HL-60 cells were cultured with N2G- or NsG-peptides (10 μM) or anti-ATP5B antibodies (5 μg/ml) for 48 h at 37 ℃, and then subjected to staining for anti-CD11b or anti-CD14. Untreated cells were run as "control" and cells treated with all-trans retinoic acid (ATRA, 1 μM) as positive control. *$p<0.05$; ***$p<0.001$ vs control group, all by Student's *t*-test. (C) NB4 cells were cultured with N2G- or NsG-peptides (10 μM) or anti-ATP5B antibodies (5 μg/ml) at 37 ℃ for 15 or 30 min, and total proteins were extracted for western blotting of AKT, p-AKT, p-ERK1/2, ERK1/2, p-P38 or P38. All experiments were done three times and representative ones were shown.

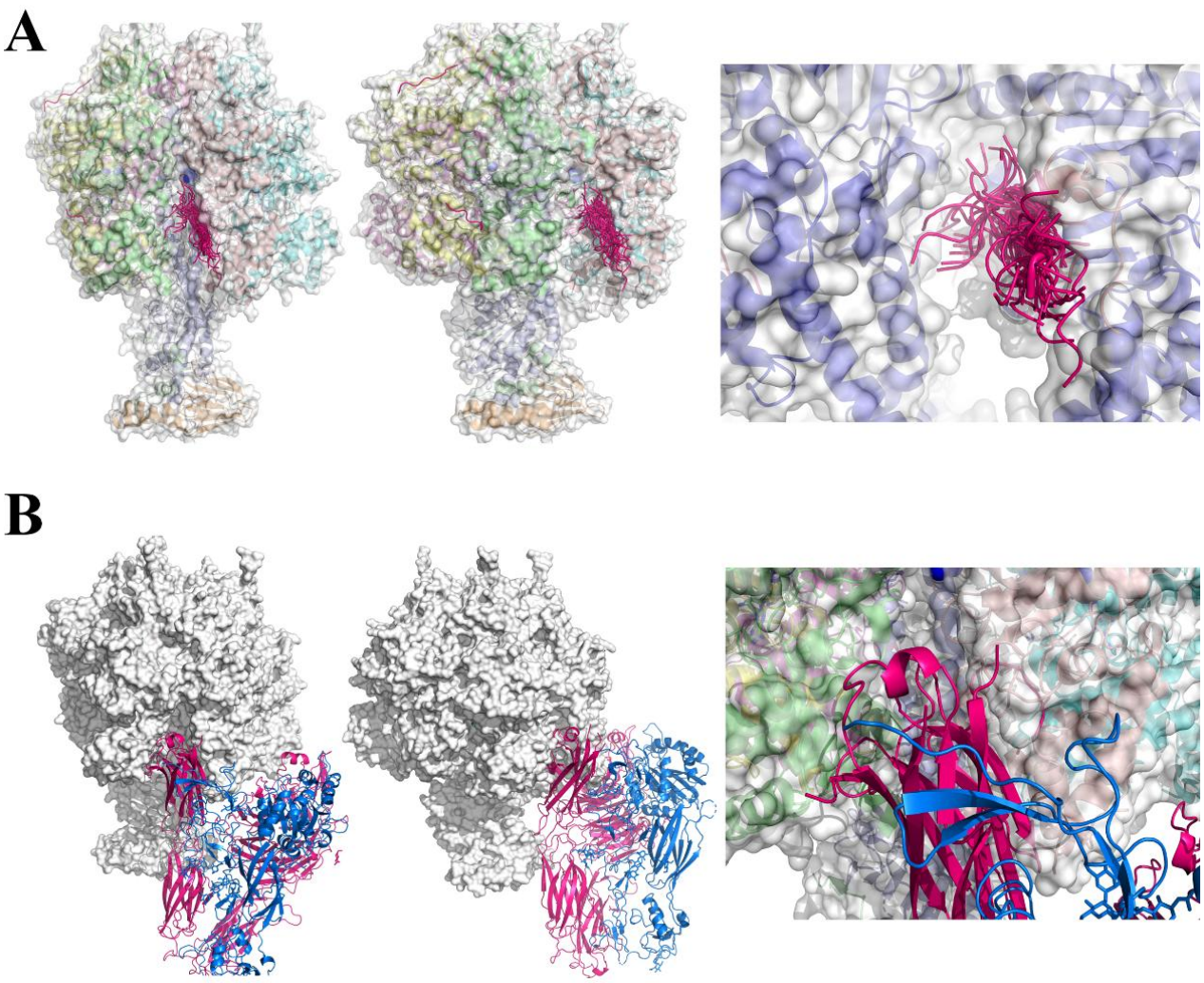

Figure S8. Structural modeling of ATP synthase–GPIIb interactions. (A) Identification of N2G-peptide binding sites on the surface of ATP synthase (PDB 1e79). Forty four of the top 50 best poses (threads in hot pink) are located onto a grove on one of the ATP synthase subunits beta (chain E, *1E79_E*) as shown in front (*left*), side (*middle*) and close views (*right*). The binding site on *1E79_E* involves residues 158-168, 335-347 and 416-425 of chain E. (B) The model of ATP synthase–GPIIb complex in front (*left*), side (*middle*) and close views (*right*).

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