Nuclear translocation of PKC-alpha is associated with cell cycle arrest and erythroid differentiation in myelodysplastic syndromes (MDSs)

Nuclear Translocation of PKCalpha is Associated with Cell Cycle Arrest and Erythroid Differentiation in Myelodysplastic Syndromes (MDS)

Alessandro Poli[1,2,a], Stefano Ratti[1,a], Carlo Finelli[3], Sara Mongiorgi[1], Cristina Clissa[3,4], Annalisa Lonetti[1,5], Alessandra Cappellini[6], Alessia Catozzi[1], Marilena Barraco[3], Pann-Ghill Suh[7], Lucia Manzoli[1], James A. McCubrey[8], Lucio Cocco[1,*], Matilde Y. Follo[1,*]

[1]Cellular Signalling Laboratory, Institute of Human Anatomy, DIBINEM, University of Bologna, Bologna, Italy; [2]Istituto Nazionale Genetica Molecolare "Romeo e Enrica Invernizzi", Milano, Italy; [3]Institute of Hematology "L. e A. Seràgnoli", S. Orsola-Malpighi Hospital, Bologna, Italy; [4]Hematology and Stem Cell Transplant Center, San Salvatore Hospital, Pesaro (PU), Italy; [5]Department of Pediatrics "Lalla Seràgnoli", Sant'Orsola-Malpighi Hospital, University of Bologna, Bologna, Italy; [6]Department of Human Social Sciences and Health,University of Cassino, Cassino (FR), Italy; [7]School of Life Sciences, UNIST, Ulsan, Republic of Korea; [8]Department of Microbiology and Immunology, Brody School of Medicine, East Carolina University, Greenville, NC, USA.

[a]These authors equally contributed to this work

*__Corresponding authors__: Drs. Lucio Cocco and Matilde Y. Follo, Cellular Signalling Laboratory, Institute of Human Anatomy, DIBINEM, University of Bologna, via Irnerio 48, 40126 Bologna, Italy. Phone: +39-0512091639; Fax: +39-051251735; email: lucio.cocco@unibo.it; matilde.follo@unibo.it

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ABBREVIATIONS

MDS: Myelodysplastic syndromes

del(5q): Deletion of the long arm of the chromosome 5

PI-PLC: Phosphoinositide-specific Phospholipase C

PKC: Protein Kinase C

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

PI: Phosphatidylinositol

IP3: inositol 1,4,5-trisphosphate

DAG: Diacylglycerol

AML: Acute myeloid leukemia

EPO: Erythropoietin

PB: Peripheral blood

MNCs: Mononuclear cells

WHO: World Health Organization

R-IPSS: Revised-International Prognostic Scoring System

IWG: International Working Group

CR: Complete Remission

PR: Partial Remission

HI: Hematologic Improvement

HI-E: Hematologic Improvement along the Erythroid Lineage

HRP: Horseradish Peroxidase

PP2CA: Protein Phosphatase 2A catalytic subunit alpha

GPA: Glycophorin A

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

CI: Confidence interval

ABSTRACT

PI-PLCbeta1 is involved in cell proliferation, differentiation and MDS pathogenesis. Moreover, the increased activity of PI-PLCbeta1 reduces the expression of PKCalpha that, in turn, delays the cell proliferation and is linked to erythropoiesis. Lenalidomide is currently used in del(5q) low-risk MDS patients, where it can suppress the del(5q) clone and restore a normal erythropoiesis.

Here we studied the effect of Lenalidomide on 16 low-risk del(5q) MDS patients, del(5q) and non-del(5q) cell lines, mainly focusing on erythropoiesis, cell cycle and PI-PLCbeta1/PKCalpha signalling.

Overall, 11 subjects were clinically evaluable: ten cases (90%) showed a favorable response (5 CR, 3 HI-E and 2 HI-E with cytogenetic response), and the remaining case had a stable disease. At a molecular level, both responder patients and del(5q) cells showed a specific induction of erythropoiesis and a nuclear translocation of PKCalpha. Moreover, Lenalidomide could induce a selective G0/G1 arrest of cell cycle in del(5q) cells, slowing the rate proliferation of this cell clone.

Altogether, our results could not only better explain the role of inositide-dependent signalling in erythropoiesis, but it could also lead to a better comprehension of the Lenalidomide effect on del(5q) MDS and pave the way to innovative targeted therapies.

Keywords: PKCalpha, Nucleus, Myelodysplastic Syndromes, Lenalidomide, Erythropoiesis

INTRODUCTION

Lenalidomide is an immunomodulating drug that is currently used in patients diagnosed with myelodysplastic syndromes (MDS) and bearing a deletion of the long arm of the chromosome 5 [del(5q)] (1). Indeed, in these low-risk MDS, Lenalidomide may suppress the del(5q) clone and restore a normal erythropoiesis (2, 3). Moreover, in Lenalidomide-sensitive del(5q) cell lines, Akt phosphorylation is inhibited and a cell cycle arrest is detected (4). Interestingly, Akt signalling is specifically activated in high-risk MDS, where it induces cell proliferation, and in low-risk MDS, where it is involved in erythropoiesis (5-7). In addition, during both erythropoiesis and erythropoietin-induced erythroid differentiation of CD34+ progenitor cells, phosphoinositides phosphorylated by Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) can act as second messengers and directly activate Akt and certain isoforms of Protein Kinase C (PKC), particularly PKCalpha (8).

PKCalpha is a downstream target of Phosphoinositide-specific Phospholipase C (PI-PLC) beta1 (9), a key enzyme of the Phosphatidylinositol (PI) metabolism (10-12). Indeed, the PI cycle usually includes the PLCs (13, 14), a class of enzymes able to hydrolyze phosphatidylinositol 4,5-biphosphate (PIP2) to produce inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), i.e. important second messengers involved in the activation of PKCs (15). Therefore, the PI cycle plays an essential role in the regulation of cell proliferation and differentiation (16-18), but is also implicated in immunomodulation processes (19).

Interestingly, PI-PLCbeta1 is involved in MDS progression to AML and response to epigenetic drugs (20-26). Moreover, the nuclear splicing variant of PI-PLCbeta1 is a negative regulator of erythroid differentiation and is reduced in both EPO responder low-risk MDS patients and hematopoietic progenitors induced to erythroid differentiation (7, 27). Furthermore, increased PI-PLCbeta1 activity reduces PKCalpha levels that, in turn, can induce a delay in cell proliferation (28). Notably, the above-mentioned decrease of PKCalpha has been found in total lysates of the human K562 erythroleukemia cell line, but the behaviour of PKCalpha could change in nuclear and cytoplasmic fractions. Indeed, the cellular localization of the PI metabolism is extremely important, as its enzymes may have a different regulation and function according to their localization (29-32).

Stemming from these data, here we investigated the molecular effect of Lenalidomide on erythroid differentiation of low-risk MDS cells and PI-PLCbeta1/PKCalpha signalling pathway. As we used mononuclear cells from del(5q) MDS patients, where del(5q) and non-del(5q) cells are both present, we also tested Namalwa CSN.70 and U937 hematopoietic cell lines, that have a del(5q) and a non-del(5q) karyotype, respectively. We particularly focused on the topographic localization of PI-PLCbeta1 and PKCalpha during Lenalidomide-induced erythroid differentiation, as the presence of

these enzymes in a particular cellular district could explain a different activation of signalling pathways during Lenalidomide therapy, thus leading to the comprehension of a new molecular mechanism for Lenalidomide therapy and the identification of possible molecular markers that could be used to develop innovative targeted therapies for del(5q) MDS patients.

MATERIALS AND METHODS

Patient Characteristics. Peripheral blood mononuclear cells (PBMNCs) from 16 patients with del(5q) MDS treated with Lenalidomide alone, who had given informed consent according to the Declaration of Helsinki, were examined (Table 1). All the samples came from the Institute of Hematology "L. e A. Seràgnoli" of the S. Orsola-Malpighi Hospital, Bologna, Italy. Patients, whose median age was 77 years (range 69-90 years), were observed between 2010 and 2016 and the median follow-up was 24 months (range 1 to 86 months). MDS diagnosis was defined according to the WHO classification (33) while, according to the R-IPSS (34), patients were divided into two risk subgroups: intermediate risk (n=6) and low-risk (n=10). However, throughout the text, all of the MDS patients are defined as low-risk MDS. Patient demographics and disease characteristics are summarized in Table 1.

Patient Treatment and Evaluation of Response. At first, 16 patients with del(5q) underwent Lenalidomide treatment (10 mg/die, days 1-21), but 2 patients died before the 4th cycle (one for a stroke and one for a brain tumor) and other 3 patients did not reach 4 cycles of therapy for other reasons. Therefore, only 11 patients were considered clinically evaluable for hematologic response, and for them both clinical and molecular analyses were carried out. We recorded the response to treatment (according to the revised IWG response criteria (35)), survival (calculated from the start of Lenalidomide) and causes of death (Table 1). Data were censored when patients died or were lost during follow-up. Patients who achieved Complete Remission (CR), Partial Remission (PR) or any Hematologic Improvement (HI) according to IWG criteria (35) were considered responders, whereas all the other outcomes were defined as non responders. The duration of response was assessed in patients who showed a clinical response to treatment.

Isolation of Mononuclear Cells From Peripheral Blood Samples. For in vitro experiments, peripheral blood (PB) mononuclear cells (MNCs) were isolated by Ficoll-Paque (GE Healthcare, Waukesha, WI, USA) density-gradient centrifugation, according to the manufacturer's instructions.

All analyses were performed on samples from patients at baseline, and subsequently once a month during Lenalidomide therapy. Also MNCs from healthy subjects were extracted.

Antibodies and reagents. The following antibodies and reagents were purchased from commercial sources. Mouse monoclonal to Cyclin D3 (#2936), rabbit polyclonals to Cyclin E (#2925), Histone 3A (H3A, #9715), HRP-conjugated anti-rabbit IgG (#707), HRP-conjugated anti-mouse IgG (#7076) and the Phototope®-HRP Western Blot Detection System (#7071) were from Cell Signaling Technology (Beverly, MA, USA). Rabbit PP2CA (sc-130237), rabbit Cyclin D3 (sc-182), mouse PKCalpha (sc-8393) and mouse PI-PLCbeta1 (sc-5291) antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA). Mouse Beta-Tubulin was from Sigma-Aldrich (St Louis, MO, USA). Phycoerythrin (PE)-conjugated mouse monoclonal to CD71 or Glycophorin A were from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). FITC-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (#F2883), Cy3-conjugated $F(ab')_2$ fragment of goat anti-rabbit IgG (#C2306) were from Sigma-Aldrich (St. Louis, MO, USA).

Cell cultures. Human Namalwa CSN.70 Burkitt lymphoma cells (i.e. del(5q) cells) and U937 histiocytic lymphoma cells (i.e. non-del(5q) cells) were cultured at 37°C with 5% CO_2 in RPMI 1640 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum and streptomycin/penicillin at an optimal cell density of 0.3-0.8 x 10^6 cells/mL. Cells were treated with 1µM Lenalidomide (CDS022536, Sigma-Aldrich) for 10 days, in order to give cells a concentration comparable to the plasma concentration reached in clinical uses. Samples were taken at days 6, 8 and 10 to monitor the molecular effect of Lenalidomide.

Flow cytometric analysis of cell cycle. Cells were cultivated in RPMI medium (Cambrex Bio Science) with Lenalidomide. The subdiploid DNA content was evaluated using an FC500 Dual Laser Flow Cytometer with the appropriate software (System II, Beckman Coulter), as reported elsewhere (36). At least 10,000 events/sample were acquired.

Flow cytometric detection of Glycophorin A and CD71 levels. For the detection of Glycophorin A (GPA) and CD71 surface antigens, cells were cultivated in RPMI medium (Cambrex Bio Science) with Lenalidomide. The percentage of positive cells was quantified using an FC500 Dual Laser Flow Cytometer with the appropriate software (System II, Beckman Coulter), as reported elsewhere (37). At least 10,000 events/sample were acquired.

Nucleic Acids Extraction. Total RNA was isolated from cell lines and total MNCs from MDS patients and healthy subjects by using the RNeasy Mini Kit (Qiagen Ltd, Valencia, CA, USA) according to the manufacturer's protocol, then RNA was retro-transcribed as described elsewhere (38).

Analysis of Gene and Protein Expression. The expression of PI-PLCbeta1a, PI-PLCbeta1b, Beta-Globin and Gamma-Globin was quantified by using a specific TaqMan Real-Time PCR method (Applied Biosystems, Foster City, CA, USA), as described elsewhere (39). A pool of healthy subjects was used as an internal reference, whilst GAPDH was the housekeeping gene. Due to the low amount of MDS cells, protein expression was examined only by immunocytochemical analyses on MNCs, as previously illustrated (40). For cell lines, we carried out a Western blot analysis, and the nuclear/cytoplasmic fractionation was performed as already reported (41). Beta-tubulin was used as a control of equal protein loading in total lysates, whereas purity of nuclear and cytoplasmic fractions was tested using either Beta-tubulin (cytoplasmic marker) or H3A (nuclear marker) antibodies.

Statistical Analyses. All the analyses were performed by using the GraphPad Prism Software (v.4.0, La Jolla, CA, USA). Dunnett's test post-ANOVA was used to compare continuous values. Tests were considered statistically significant when the p-value was <0.05.

RESULTS

Patients Outcome. Between July 2009 and December 2016, 16 low-risk del(5q) MDS patients were treated with Lenalidomide. Eleven patients were evaluable for response. According to the revised IWG criteria (34), ten patients (90%) showed a favorable response to the treatment (5 CR, 3 HI-E and 2 HI-E with cytogenetic response). The remaining patient had a stable disease (Table 1).

Lenalidomide Effect on Globin Genes and PI-PLCbeta1 in Low-Risk MDS Patients. The ratio between Gamma and Beta-Globin mRNAs was quantified in low-risk MDS at baseline and during the therapy (Fig 1). A reduction of Gamma/Beta-Globin ratio was detected in responder patients, with a statistically significant difference between pre- and post-treatment (Student's t-test, $p<0.05$ vs. baseline, 95% CI +0,85 to +1,85). Also the amount of both PI-PLCbeta1 splicing variants was assessed (Fig 1): PI-PLCbeta1a significantly increased only in the late cycles of therapy (Student's

t-test, $p<0.05$ vs. baseline, 95% CI -0,63 to +0,05), while PI-PLCbeta1b mRNA was not significantly induced by the therapy (Student's t-test, $p>0.05$ vs. baseline, 95% CI -0,38 to +0,19).

Lenalidomide Effect on PI-PLCbeta1 and PKCalpha Protein Expression in Low-Risk MDS Patients. After Lenalidomide therapy, PKCalpha localized within the nucleus, especially in cells not expressing high levels of PP2CA, whereas in the same cells PI-PLCbeta1 seemed to be mainly cytoplasmic (Fig 2).

Flow Cytometric Analysis of Cell Cycle in del(5q) and non-del(5q) Cell Lines. Lenalidomide induced a significant accumulation in the G0/G1 phase of Namalwa CSN.70 cells (i.e. del(5q) cells) after 6 and 8 days of treatment with Lenalidomide (+16,6% and +19,3%, respectively), before being reduced at day 10 of the treatment. On the contrary, U937 cells (i.e. non-del(5q) cells) showed a slight increase of G0/G1 phase of cell cycle only after 8 days of treatment (+4,1%), before being reduced at day 10 of the treatment (Fig 3a, Fig 3b).

Lenalidomide Effect on Cell Cycle Protein Expression in del(5q) and non-del(5q) Cell Lines. Namalwa CSN.70 cells (i.e. del(5q) cells) displayed a significant increase of p21 and p27, a slight decrease of Cyclin D3 and an almost constant low amount of Cyclin E. On the contrary, the expression of p21, p27 and Cyclin D3 was maintained in U937 cells (i.e. non-del(5q) cells), whereas Cyclin E was induced in the last days of treatment with Lenalidomide (Fig 3).

Flow Cytometric Analysis of Erythropoiesis Activation in del(5q) and non-del(5q) Cell Lines. Namalwa CSN.70 cells (i.e. del(5q) cells) displayed a significant increase of GPA (+29,72%), but not of CD71 (-10,06%) after 8 days of treatment. Conversely, the treatment with Lenalidomide induced a significant increase of both GPA and CD71 in U937 cells (i.e. non-del(5q) cells) after 8 days of treatment (+12,88% and +17,34%, respectively), which was maintained at day 10 of treatment. (Fig 4).

Lenalidomide Effect on PI-PLCbeta1 and Globin Genes in del(5q) and non-del(5q) Cell Lines. As Fig 5 shows, Namalwa CSN.70 cells (i.e. del(5q) cells) treated with Lenalidomide showed a significant decrease of Gamma/Beta-Globin ratio (Student's t-test, $p<0.05$ vs. baseline, 95% CI -0,04 to -1,63). In contrast, PI-PLCbeta1a showed an increase only in the last days of treatment of Namalwa CSN.70 cells (Student's t-test, $p>0.05$ vs. baseline, 95% CI -0,87 to +0,74), whereas the amount of PI-PLCbeta1b mRNA was almost constant (Student's t-test, $p>0.05$ vs. baseline, 95% CI

-0,02 to +0,02). As for U937 cells (i.e. non-del(5q) cells), they displayed an increase of Gamma/Beta Globin ratio, although without a statistically significant difference between pre- and post-treatment (Student's t-test, $p>0.05$ vs. baseline, 95% CI +0,01 to +0,07). U937 cells also showed a late increase of PI-PLCbeta1a (Student's t-test, $p>0.05$ vs. baseline, 95% CI +0,01 to +0,05, while PI-PLCbeta1b was almost constant (Student's t-test, $p>0.05$ vs. baseline, 95% CI -0,01 to +0,02).

Lenalidomide Effect on the Expression of PI-PLCbeta1 and PKCalpha in del(5q) and non-del(5q) Cell Lines. Namalwa CSN.70 cells (i.e. del(5q) cells) showed a very slight increase of PI-PLCbeta1 in the last days of treatment with Lenalidomide, whilst PKCalpha was significantly reduced. On the contrary, U937 cells (i.e. non-del(5q) cells) displayed an almost constant amount of PI-PLCbeta1 and PKCalpha during Lenalidomide treatment (Fig 6).

Lenalidomide Effect on the Expression of PI-PLCbeta1 and PKCalpha in Nuclear and Cytoplasmic Fractions of del(5q) and non-del(5q) Cell Lines. After 8 days of treatment, PKCalpha appeared to be highly localized inside the nucleus of Namalwa CSN.70 cells (i.e. del(5q) cells) and it was barely expressed in the cytoplasmic fraction of this cell line, where PI-PLCbeta1 was detectable, especially at day 10 of treatment. On the contrary, U937 cells did not show a nuclear translocation of PKCalpha or a cytoplasmic localization of PI-PLCbeta1 after Lenalidomide treatment (Fig 7).

DISCUSSION

Nuclear inositide signalling pathways are deregulated in MDS, and nuclear PI-PLCbeta1 is a negative regulator of erythroid differentiation. Moreover, nuclear PI-PLCbeta1 specifically targets PKCalpha, which regulates proliferation and differentiation of human erythroleukemia cells (42).

Lenalidomide is currently used in MDS patients bearing a deletion of chromosome 5q, who show a favourable outcome in the vast majority of cases. Its molecular mechanisms in del(5q) patients are still unclear, although Lenalidomide improves their erythropoiesis, which is characteristically deregulated, and possibly arrest their clonal cell proliferation, as some cases also show a cytogenetical remission and the restoration of a normal karyotype.

In this study we focused on the molecular effect of Lenalidomide on inositide-dependent signalling, and especially on the PI-PLCbeta1/PKCalpha axis. At first, we investigated the effect of

Lenalidomide in mononuclear cells obtained from del(5q) low-risk MDS patients. In our case series, 5/16 patients early discontinued Lenalidomide, and for these patients neither a clinical assessment of Lenalidomide effect, nor a molecular analysis, were possible. Among the remaining patients, 10 subjects responded to Lenalidomide, whereas the non responder patient showed a stable disease after treatment. At a molecular level, responder patients showed an activation of erythropoiesis, in that the Gamma/Beta-Globin ratio decreased, as compared with baseline. Moreover, these subjects displayed a specific increase of PI-PLCbeta1a splicing variant, but not PI-PLCbeta1b, in the last cycles of therapy. Interestingly, PI-PLCbeta1a is mainly localized in the cytoplasm, and is not directly associated with inhibition of erythroid differentiation, while nuclear PI-PLCbeta1 is a negative regulator of erythropoiesis. The protein expression of PI-PLCbeta1, as well as its downstream target PKCalpha, have also been investigated. In particular, we performed a double immunostaining with PP2CA and PI-PLCbeta1 or PKCalpha on MDS mononuclear cells: as PP2CA gene is localized in the 5q chromosome, cells showing both proteins were considered as non-del(5q)cells, whereas cells with a low amount of PP2CA were del(5q) cells. Surprisingly, we detected a distinct behaviour between del(5q) and non-del(5q) MDS cells, in that PI-PLCbeta1 seemed to be localized mainly in the cytoplasm of del(5q) cells, while PKCalpha translocated to the nucleus after Lenalidomide therapy.

To better discriminate between del(5q) and non-del(5q) cells, we studied the effect of Lenalidomide on hematopoietic cell lines, using Namalwa CSN.70 cells, showing a del(5q) karyotype, and U937 cells, showing a normal 5q chromosome. Only Namalwa CSN.70 cells showed an arrest of cell cycle in the G0/G1 phase, which corresponded to an increase of p21, p27 and a slight decreased expression of cyclin D3 and cyclin E. On the contrary, in non-del(5q) cells (i.e. U937 cells), Lenalidomide did not significantly affect cell cycle. As for the erythroid effect, Namalwa CSN.70 cells showed a decrease of Gamma/Beta-Globin ratio, an increase of GPA and a reduction of CD71 during Lenalidomide treatment, confirming the induction of erythropoiesis. On the contrary, U937 cells treated with Lenalidomide showed a higher amount not only of Globin genes, but also of both GPA and CD71 markers. Our data on cell lines also show that Lenalidomide induces a nuclear translocation of PKCalpha, which has also been associated with erythroid differentiation.

Altogether, our data show that Lenalidomide can induce a selective arrest of cell cycle in G0/G1 phase of del(5q) cells, thus slowing the rate proliferation of this cell clone. On the contrary, p21, p27, cyclins, Globin genes, CD71 and GPA are specifically activated in non-del(5q) cells, possibly leading to a normal cell proliferation and erythroid differentiation. Therefore, Lenalidomide seems to specifically slow the proliferation of the del(5q) clone, promoting instead its erythroid differentiation. In addition, our data show that Lenalidomide effect on del(5q) cells is specifically

associated with the nuclear translocation of PKCalpha. Interestingly, PKCalpha is a downstream target of PI-PLCbeta1, and in our cases it could be induced by a specific increase of PI-PLCbeta1a. Therefore, this finding may be important not only to understand the inositide-dependent signalling in erythropoiesis, but it could also lead to a better comprehension of the Lenalidomide effect on del(5q) MDS and pave the way to innovative targeted therapies.

AUTHORS CONTRIBUTIONS: A. Poli, S. Ratti, S. Mongiorgi, A. Lonetti, A. Cappellini, A. Catozzi, M.Y. Follo performed the research; C. Finelli, L. Manzoli, L. Cocco and M.Y. Follo designed the research study; C. Clissa, M. Barraco, P.G. Suh and J.A. McCubrey contributed essential reagents or tools and provided clinical samples and data; A. Poli, S. Ratti, C. Finelli, S. Mongiorgi, L. Cocco and M.Y. Follo analysed the data; A. Poli, C. Finelli, L. Cocco and M.Y. Follo wrote the paper. All authors revised it critically and approved the final version.

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FIGURE LEGENDS

Fig 1. Lenalidomide Reduces Gamma/Beta Globin Ratio and Induces PI-PLCbeta1a mRNA in Responder MDS. The Gamma/Beta-Globin ratio significantly decreases during the therapy. As for PI-PLCbeta1, the PLCbeta1a splicing variant is induced by Lenalidomide in the last cycles of treatment, while the PI-PLCbeta1b splicing variant is not affected (*$p<0.05$ vs T0). Data are representative of at least three independent experiments.

Fig 2. Lenalidomide induces Cytoplasmic PI-PLCbeta1 and Nuclear PKCalpha in MDS Responder Patients. Representative immunocytochemical analysis of mononuclear cells from MDS responder patients treated with Lenalidomide. Original magnification×600. Nuclei are visualized by DAPI staining (blue signal). (A) Levels of PI-PLCbeta1 in MDS at baseline and after Lenalidomide (Lena) therapy (green signal). The identification of del(5q) cells was done with a Cy3-conjugated anti-PP2CA antibody (red signal). The merged image for PI-PLCbeta1 and PP2CA staining indicates colocalization of the two antigens (yellow signal). Arrows indicate the del(5q) cells. (B) Levels of PKCalpha in MDS at baseline and after Lenalidomide (Lena) therapy (green signal). The identification of del(5q) cells was done with a Cy3-conjugated anti-PP2CA antibody (red signal). The merged image for PKCalpha and PP2CA staining indicates colocalization of the two antigens (yellow signal). Data are representative of at least two independent experiments. Arrows indicate the del(5q) cells.

Fig 3. Lenalidomide Induces a Cell Cycle Arrest in the G0/G1 Phase in Namalwa CSN.70 del(5q) Cells. (A) Flow cytometric analysis of PI-stained cells treated with 1μM Lenalidomide (LENA) for up to 10 days (as indicated). Histograms report the cell percentage for each phase (*$p<0.05$ vs T0). Lenalidomide significantly increased the G0/G1 cell fraction only in Namalwa CSN.70 cells (NAM, i.e. del(5q) cells), with the consequent decrease of the other cell cycle phases. The treatment not perturb the cell cycle of the U937 cell line (i.e. non-del(5q) cells). Data are representative of at least three independent experiments. (B) Western blotting documented that, in Namalwa CSN.70 cells (i.e. del(5q) cells), Lenalidomide induced the expression of p27 and p21, along with a slight reduction of Cyclin D3 and Cyclin E. On the contrary, U937 cells (i.e. non-del(5q) cells) were not significantly affected. Antibody to Beta-Tubulin served as a loading control. Molecular weights are indicated on the right. Data are representative of at least three independent experiments.

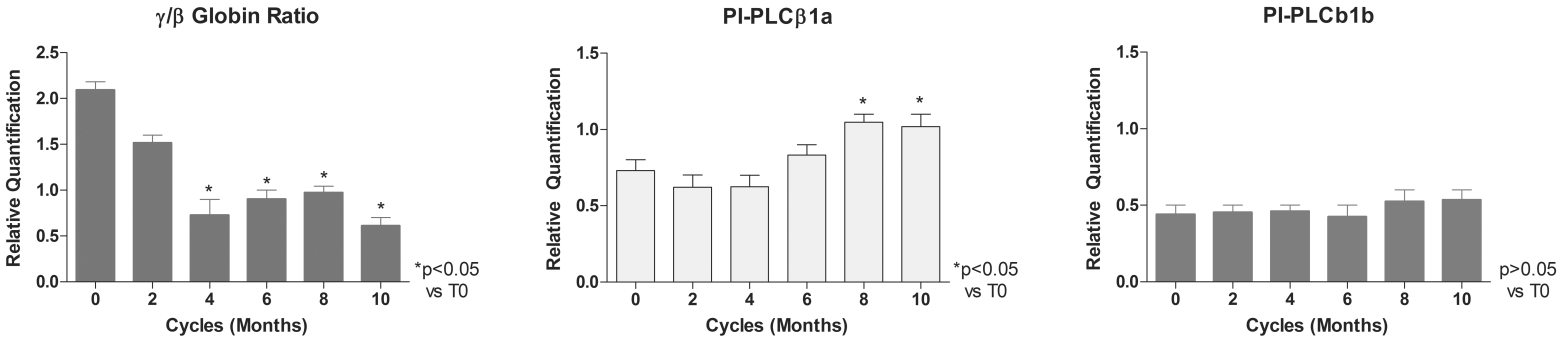
Fig 4. Lenalidomide Increases the Expression of Erythoid-Specific Surface Markers in del(5q) Cells. (A) Flow cytometric analysis of surface Glycophorin A (GPA) and CD71 expression in Namalwa CSN.70 (NAM, i.e. del(5q) cells) treated with Lenalidomide (LENA). At day 8 of treatment GPA is significantly induced, while CD71 positive cells decrease. Data are representative of at least three independent experiments. (B) Flow cytometric analysis of surface GPA and CD71 expression in U937 cells (i.e. non-del(5q) cells) treated with Lenalidomide (LENA). Both GPA and CD71 surface markers increased. Data are representative of at least three independent experiments.

Fig 5. Lenalidomide Reduces Gamma/Beta Globin Ratio and Induces PI-PLCbeta1a mRNA in del(5q) Cells. In Namalwa CSN.70 cells (i.e. del(5q) cells), the Gamma/Beta-Globin ratio significantly decreases during the therapy, while U937 cells (i.e. non-del(5q) cells) are not significantly affected. On the other hand, the expression of the PI-PLCbeta1a splicing variant increases in both cell lines by Lenalidomide (Lena) in the last days of treatment, although this is not statistically significant, while PI-PLCbeta1b splicing variant is not affected (*$p<0.05$ vs T0).

Fig 6. Lenalidomide Induces PI-PLCbeta1 and Decreases PKCalpha Protein Expression in del(5q) Cells. Western Blotting analyses of Namalwa CSN.70 (i.e. del(5q) cells) and U937 cell lines (i.e. non-del(5q) cells) treated with Lenalidomide. The treatment slightly increased the levels of PI-PLCbeta1 in Namalwa CSN.70 cells, which also showed a decrease of PKCalpha. On the contrary, the amount of PI-PLCbeta1 and PKCalpha was almost constant, or at least slightly induced in the last days of treatment, in U937 cells. Antibody to Beta-Tubulin served as a loading control. Molecular weights are indicated on the right. Data are representative of at least three independent experiments.

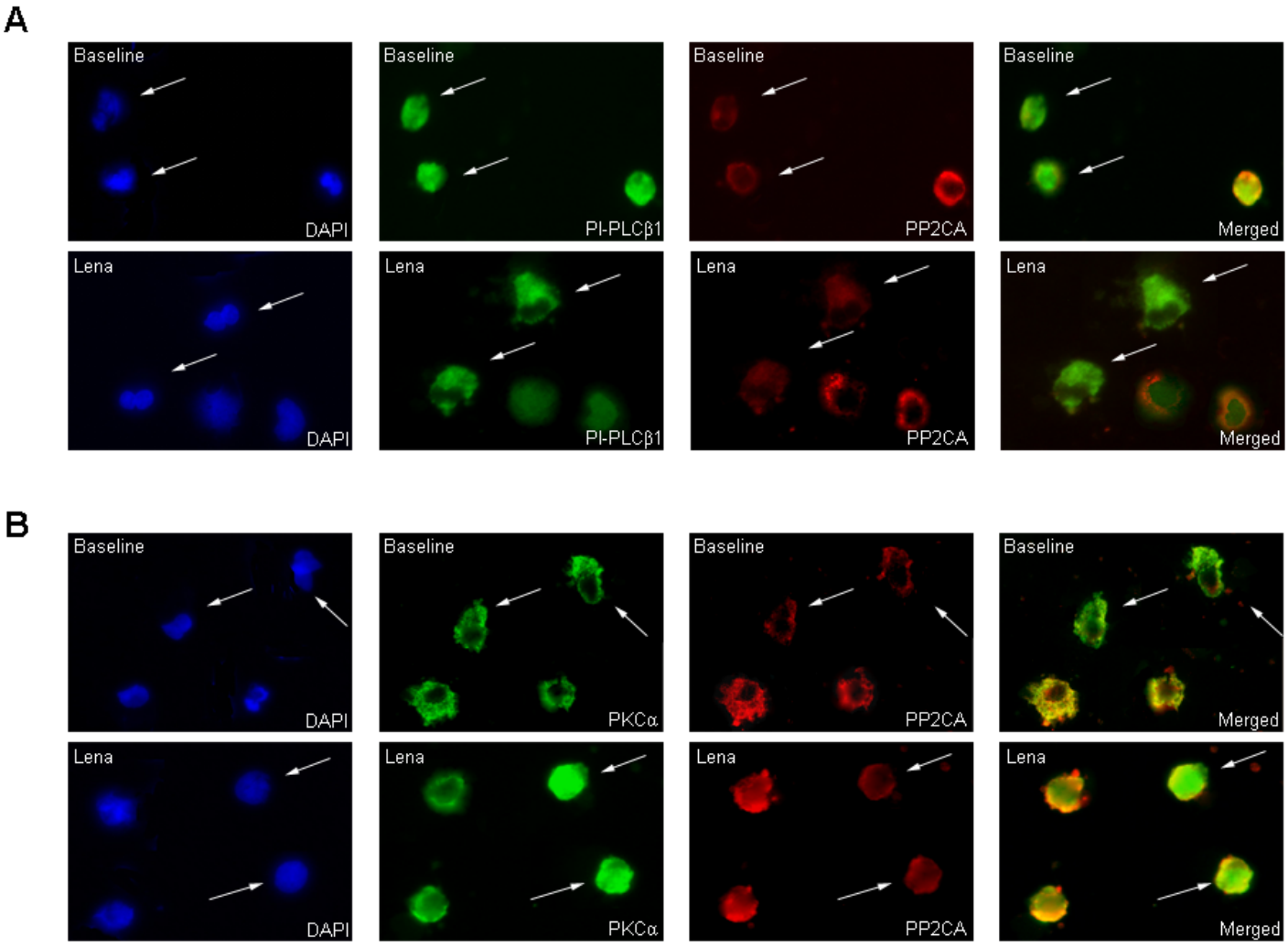
Fig 7. PKCalpha is Highly Localized into the Nucleus of del(5q) Cells after Lenalidomide Treatment. (A) Nuclei and cytoplasms of Namalwa CSN.70 cells (i.e. del(5q) cells) before and after treatment with Lenalidomide were separated. Immunoblot assay indicated PKCalpha as highly present in the nuclear compartment, while PI-PLCbeta1 seemed to be mainly localized in the cytoplasm. (B) Nuclei and cytoplasms of U937 cells (i.e. non-del(5q) cells) before and after treatment with Lenalidomide were separated. Immunoblot assay indicated that neither PKCalpha nor PI-PLCbeta1 were affected. For all experiments, antibody to Beta-Tubulin served as a loading control and a control for cytoplasm purity, whereas H3A antibody served as a loading control and a control for nuclear purity. Molecular weights are indicated on the right. Data are representative of at least three independent experiments.

Figure 1



Poli et al. Figure 1

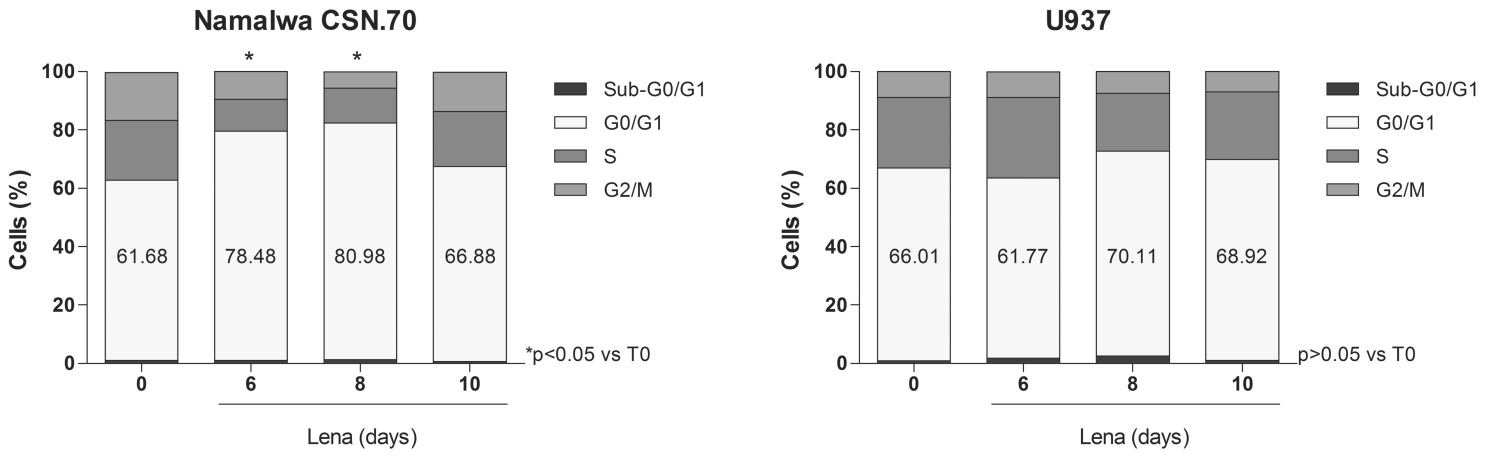
Figure 2



Poli A. et al. Figure 2

Figure 3

A



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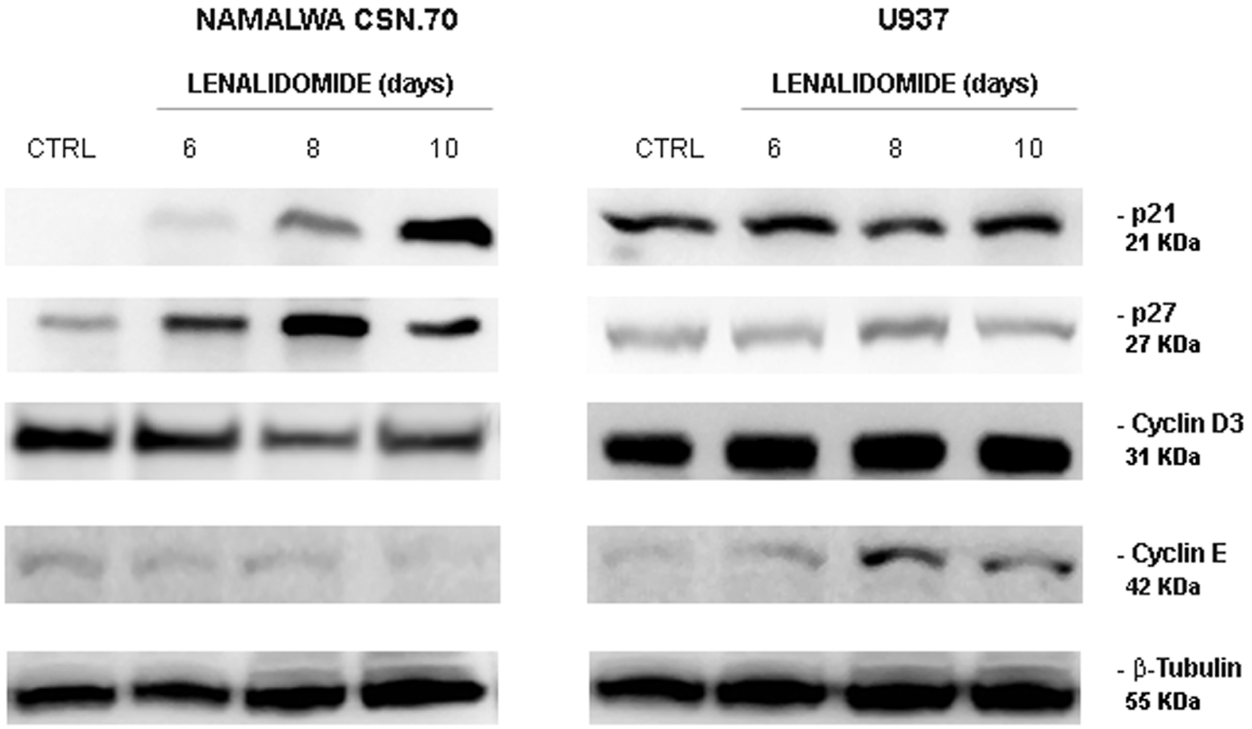
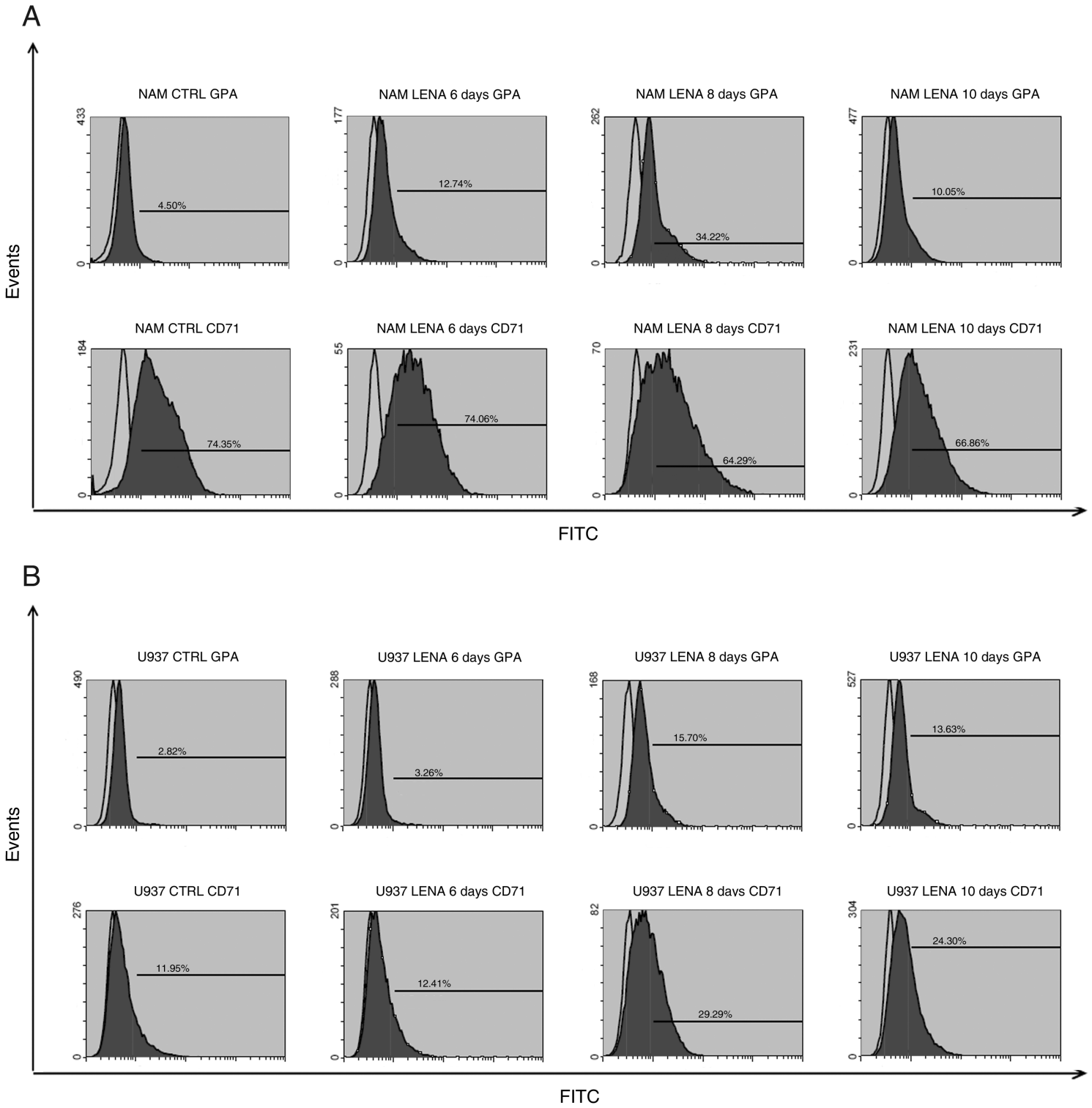


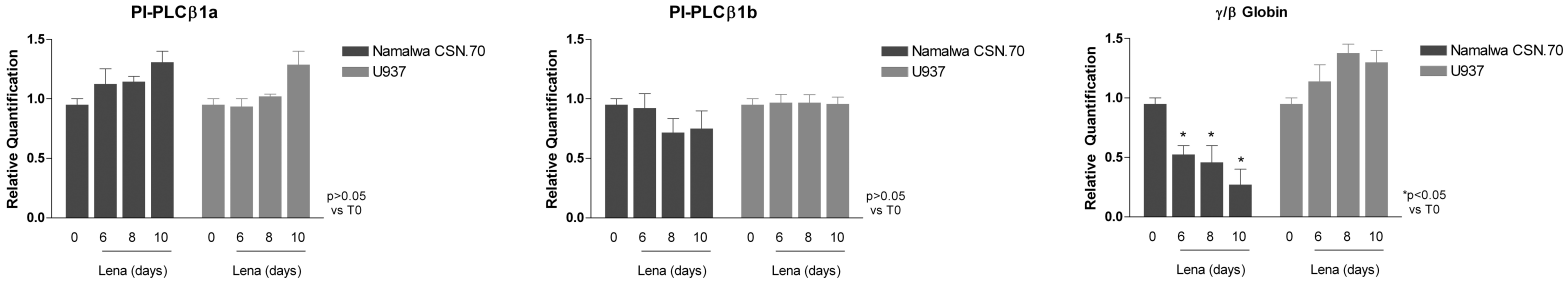
Poli A. et al. Figure 3

Figure 4



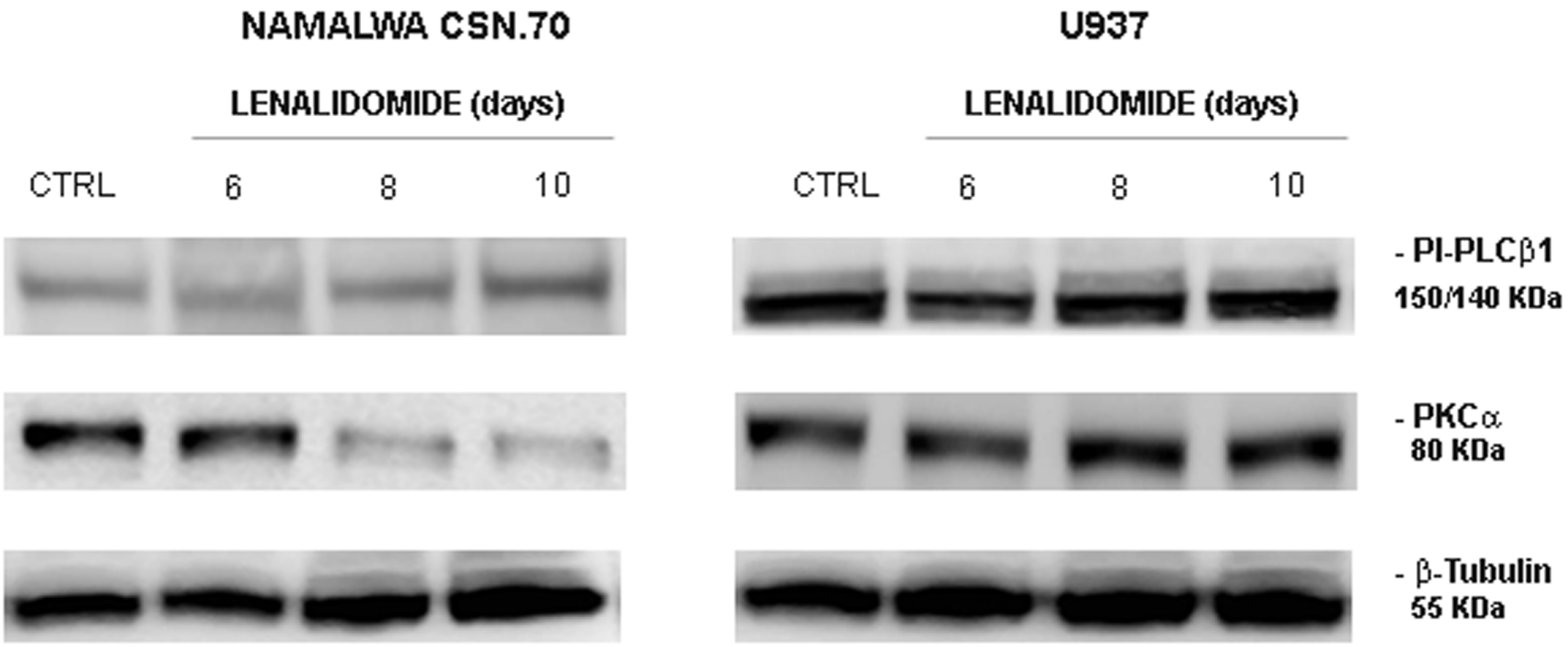
Poli A. et al. Figure 4

Figure 5



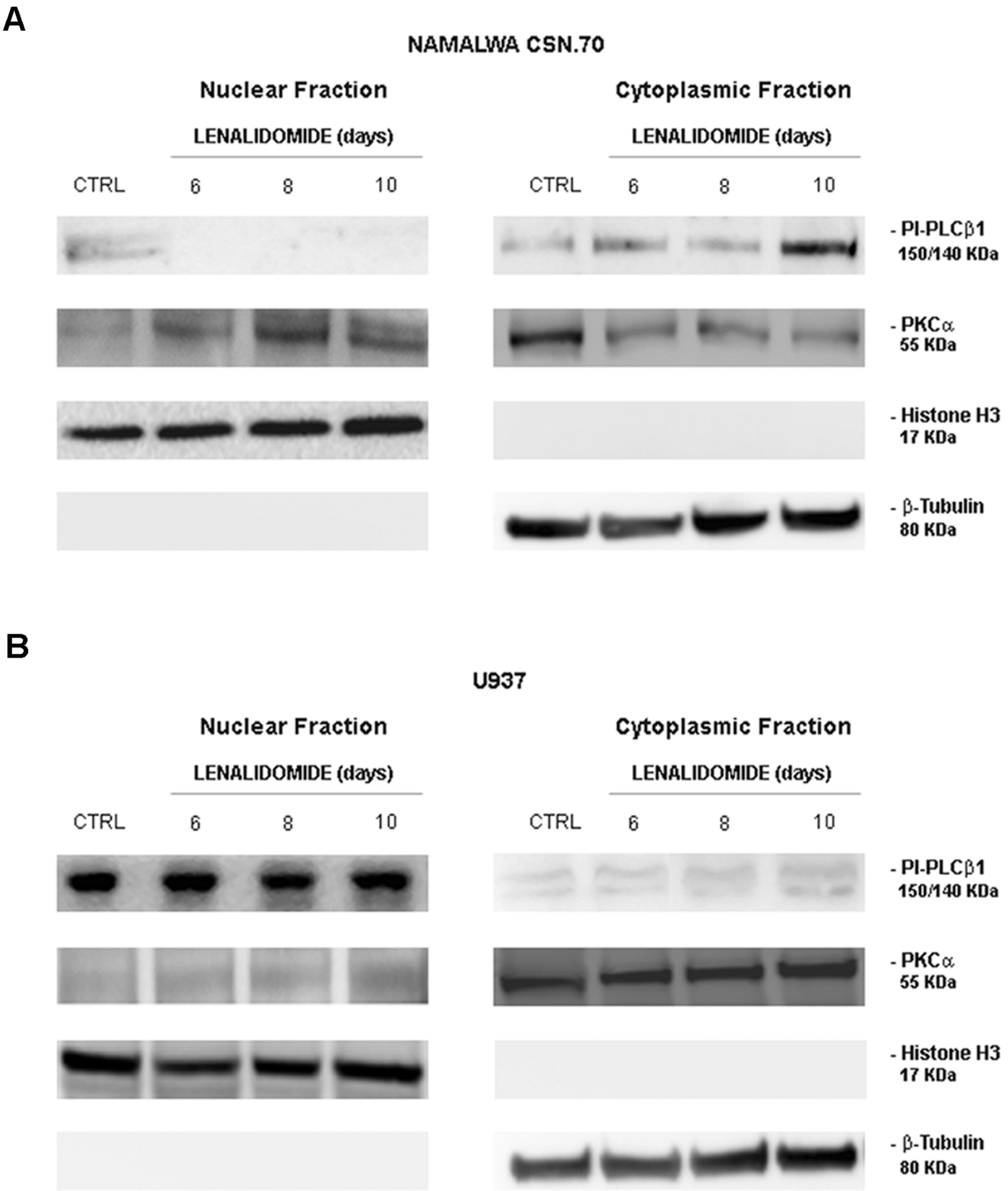
Poli et al. Figure 5

Figure 6



Poli A. et al. Figure 6

Figure 7



Poli A. et al. Figure 7