The anti-tumoral effect of lenalidomide is increased in vivo by hypoxia-inducible factor (HIF)-1α inhibition in myeloma cells.

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We investigated the effect of stable suppression of hypoxia inducible factor (HIF)-1α in myeloma cells on sensitivity to lenalidomide (LEN) *in vivo*. We found that the *in vivo* anti-tumoral effect of LEN is enhanced by HIF-1α suppression in myeloma cells.

It has been reported that HIF-1α is over-expressed by myeloma cells[1-4] and that HIF-1α suppression significantly blocks myeloma-induced angiogenesis, and reduces both tumoral burden and bone destruction *in vivo* in multiple myeloma (MM) mouse models.[3] The potential effects of HIF-1α modulation on drug sensitivity in MM cells are not known and are currently under investigation. The immunomodulatory drugs (IMiDs®), including LEN, are a class of drugs derived from thalidomide[4] able to exert anti-myeloma effects by the selective Cereblon-dependent destruction of IKZF proteins,[5,6] either through a direct action on MM cell proliferation and survival,[7] or through indirect immunomodulatory and anti-angiogenic effects.[7]

Previous data indicated that HIF-1α inhibition did not increase the anti-proliferative *in vitro* effect of bortezomib on MM cells;[2,3] this drug induces a strong downregulation of HIF-1α in MM cells.[2] We recently reported that HIF-1α knockdown in the human myeloma cell line (HMCL) JJN3 potentiated the *in vitro* effect of LEN treatment (48-72 h) on cell proliferation through a significant upregulation of p27 without changing cell viability.[3] It has been consistently reported that LEN only slightly down-regulated HIF-1α expression in MM cells.[2] Such evidence has provided the rationale to investigate the effect of stable suppression of HIF-1α in myeloma cells on LEN sensitivity *in vivo*.

Therefore, in the present study, we first inhibited HIF-1α in three HMCLs (JJN3, OPM2 and H929) using an anti-HIF1α lentiviral shRNA pool, as previously described.[3] H929 showed a very high mortality rate after anti-HIF1α lentiviral infection (*data not shown*) and were not used for the subsequent *in vivo* experiments.

We assessed the effect of LEN in combination with HIF-1α inhibition in a non-obese diabetic/severe combined immunodeficiency (NOD/SCID) subcutaneous *in vivo* mouse model.[3] Different groups of animals (5 animals in each group) of two sets of independent experiments were injected with JJN3 pLKO.1 (empty vector) or JJN3 anti-HIF1α. When tumors became palpable (approx. 7-10 days after injection) mice were treated with LEN 5 mg/kg (Selleckchem, Houston, TX, USA) or vehicle (DMSO) using the intraperitoneal route. The same mouse model, grouping strategy and treatment conditions were used for OPM2 pLKO.1 or OPM2 anti-HIF1α.

After three weeks, we evaluated tumor volume and weight, as previously described,[3] and immunohistochemistry was used to evaluate the microvascular density (MVD) and checked by CD34 immunostaining (Santa Cruz, Dallas, TX, USA).[3] In addition, in the first set of mice experiments, the expression of p27 (Abcam, Cambridge, UK) was evaluated by immunohistochemistry. The expression of S-phase kinase-associated protein 2 (SKP2), a p27 inhibitor, expression of the HIF-1α target key mediator of glycolysis and tumoral growth, Hexokinase II (HK2), and levels of pERK 1/2, and total Caspase-3 (Casp-3) were evaluated in the *ex vivo* plasmacytoma lysates by western blot using the following antibodies: SKP2, Casp-3 (Santa Cruz, Dallas, TX, USA), HK2, pERK 1/2, (Cell Signaling, Danvers, MA, USA). β-actin was used as internal control (Millipore, Darmstadt, Germany). Immunoblots were performed as previously described.[8]

As previously published,[3] we found that HIF-1α inhibition decreased the tumoral burden compared to JJN3 pLKO.1 mice. Moreover, HIF-1α suppression potentiated LEN treatment with an additive effect, inducing a reduction of tumor volume in mice injected with JJN3 anti-HIF1α as compared to JJN3 pLKO.1 after LEN treatment (Figure 1A). Average variation of tumor volume ± standard deviation was: JJN3 pLKO.1 plus LEN *versus* JJN3 pLKO.1 plus vehicle -62±8%; JJN3 anti-HIF1α *versus* JJN3 pLKO.1 plus vehicle -60±12%; JJN3 anti-HIF1α plus LEN *versus* JJN3 pLKO.1 plus vehicle -91±11%. These data were confirmed with OPM2 (Figure 1B) suggesting that the effect of HIF-1α inhibition on LEN treatment was not specific for JJN3.

The p27 nuclear expression was significantly increased by LEN treatment in JJN3 anti-HIF1α as compared to JJN3 pLKO.1 mice (mean±SD: 13.3±2% *vs.* 8±2%; P=0.05) and compared to mice not treated with JJN3 anti-HIF1α (mean±SD: 13.3±2% *vs.* 6.8±1.6%; P=0.006). Figure 1C shows p27 expression in one representative tumor grown in mouse for each group of treatment.

Western blot analysis on plasmacytomas showed that LEN in combination with HIF-1α inhibition significantly reduced the expression of p-ERK1/2, total Casp-3, HK2 and the p27 inhibitor, SKP2 (Figure 1D). These data suggest that HIF-1α inhibition may increase the *in vivo* anti-tumoral effect of LEN through modulation of p27 signaling, and consequently cell proliferation and survival. Based on these *in vivo* data, we further checked the *in vitro* effect of long-term LEN treatment on JJN3 pLKO.1 and JJN3 anti-HIF1α viability by MTT assay (Alexis Biochemical, San Diego, CA, USA). We showed that, after six days of LEN treatment, HIF-1α inhibition led to an increased sensitivity of JJN3 to LEN (Figure 1E); JJN3 is known to be resistant to this drug.[9]

Since it is well known that LEN exerts its anti-myeloma effect targeting the IKZF proteins,[5,6] we checked whether the *in vivo* effect of the combination of HIF-1α suppression and LEN treatment could be mediated by modulation of these proteins. Expression of IKZF1 (Santa Cruz, Dallas, TX, USA), IKZF3 (Novus Biological, Cambridge, UK), and IRF4 (DAKO, Milan, Italy) were evaluated by western blot in JJN3 anti-HIF1α and JJN3 pLKO.1 treated *in vitro* with LEN (2-10 μM) or vehicle (DMSO) for 72 h. Interestingly, after LEN treatment at 2 and 10 μM, we found that neither IKZF1 nor IKZF3 was differentially expressed, whereas IRF4 was down-regulated in JJN3 anti-HIF1α as compared to JJN3 pLKO.1 (Figure 1G and H). This suggests that, besides being a IKZF3 target, IRF4 could be a downstream target of HIF-1α through a HIF-1a knock-down-dependent downregulation of NF-κB.[10,11] Moreover, our data indicate that modulation of IRF4 is involved in the increased sensitivity to LEN by anti-HIF-1α suppression in LEN-resistant MM cells.

We evaluated a possible combinatory effect *in vivo* and, as expected, we found that both the number of CD34 positive vessels and MVD were reduced in mice colonized by JJN3 HIF-1α compared to JJN3 pLKO.1, as previously reported.[3] On the other hand, LEN treatment did not lead to a further significant reduction in the number of CD34 positive vessels and the MVD (Figure 2A), as shown in one representative *ex vivo* plasmacytoma for each group of treatment (Figure 2B).

Indeed, to understand the lack of LEN effect on angio-

genesis in our *in vivo* model, we investigated the expression of the main pro-angiogenic molecules *in vitro* by an angiogenesis PCR array (Roche, Milan, Italy). Accordingly to our *in vivo* results on the plasmacytoma vascularization, we did not demonstrate any significant inhibitory effect of LEN treatment on these molecules in JJN3 anti-HIF1α compared to JJN3 pLKO.1, even after 72 h (Figure 2C). Interestingly, upregulation of CCL2, CCL3, PECAM1 and MMP9 expression was observed in JJN3 pLKO.1 after LEN treatment (Figure 2C). This upregulation was reduced by stable HIF-1α suppression in JJN3. In line with these observations, a paradoxical upregulation

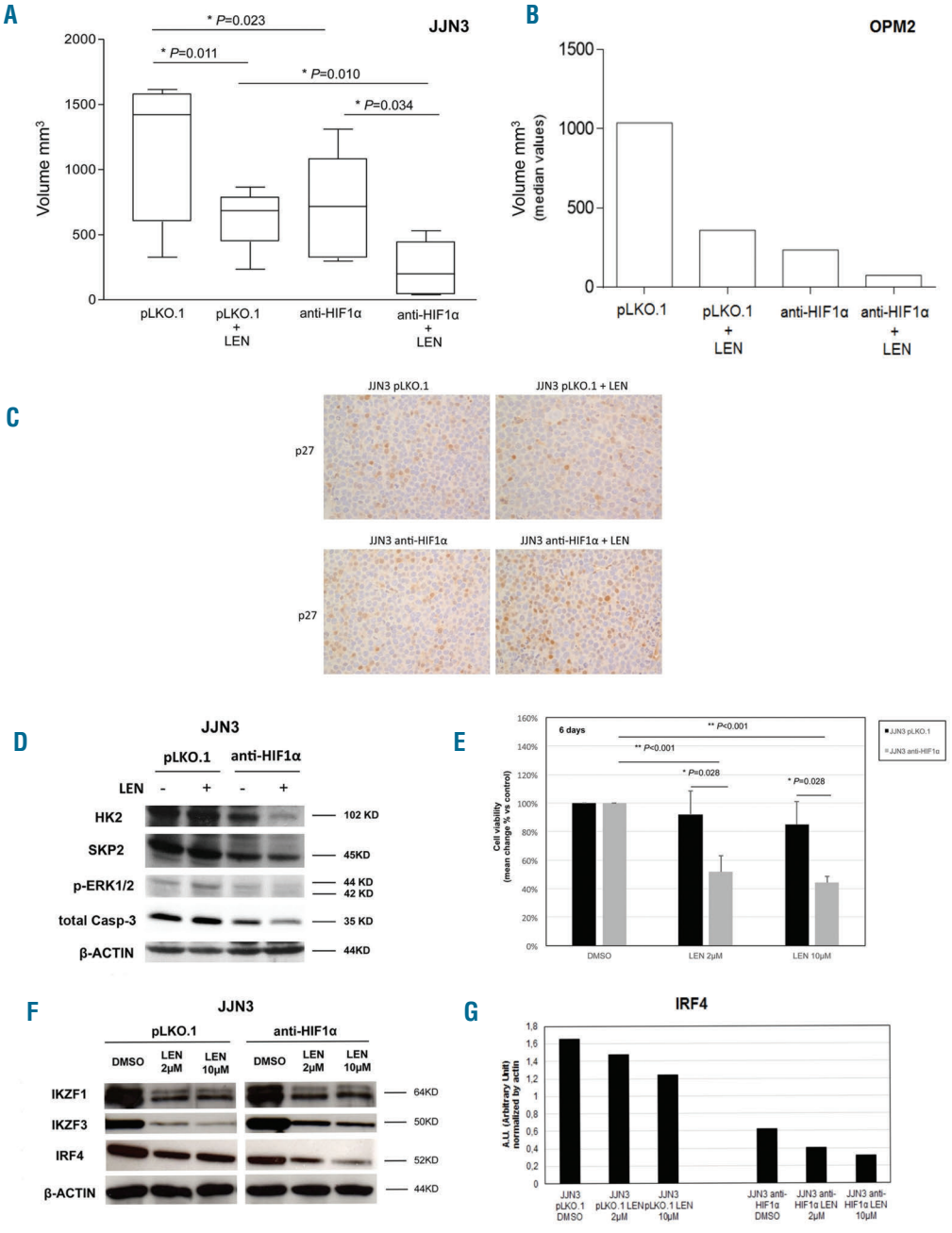

Figure 1. Stable inhibition of HIF-1α in myeloma cells significantly increased the anti-tumoral effect of lenalidomide (LEN) *in vivo*. Four groups of 5 NOD/SCID mice each were injected with JJN3 pLKO.1 or OPM2 pLKO.1 (empty vector) or JJN3 anti-HIF1α or OPM2 anti-HIF1α, obtained by anti-HIF1α lentiviral shRNA pool. From 7-10 days after injection, mice were treated with LEN (5 mg/kg) or vehicle (DMSO) 5 days per week *via* the intraperitoneal route. Tumor volume was evaluated after three weeks. (A) Box plot represents median volume of masses removed from all the mice inoculated with JJN3 pLKO.1 or JJN3 anti-HIF1α. Data were analyzed with Mann-Whitney test. (B) Graph bars represent the median volume of the masses removed from all the mice of each experimental group inoculated with OPM2 pLKO.1 or OPM2 anti-HIF1α. (B) Data were analyzed with Kruskal-Wallis test (*P*=0.022). (C) Plasmacytomas were processed and were analyzed by immunohistochemistry for expression of p27. Image is representative of each group (JJN3 pLKO.1, JJN3pLKO.1 + LEN, JJN3 anti-HIF1α and JJN3 anti-HIF1α + LEN) at 21 days. (D) Protein levels of HK2, SKP2, p-ERK1/2, total Casp-3 and β-actin were evaluated by western blot on *ex vivo* plasmacytoma total lysates from mice injected with JJN3 pLKO.1 or JJN3 anti-HIF1α treated with LEN or vehicle. (E) *In vitro* viability of JJN3 pLKO.1 or JJN3 anti-HIF1α treated with LEN (2 and 10μM) or vehicle (DMSO) for six days was evaluated by MTT assay. Graphs and bars represent mean±SD. Data were analyzed by Student's *t*-test. (F) Expression of IKZF1, IKZF3, IRF4, and β-actin (internal control) was evaluated in JJN3 pLKO.1 or JJN3 anti-HIF1α treated *in vitro* with LEN (2 and 10 μM) or vehicle (DMSO) for 72 h. (G) IRF4 protein bands were quantified by ImageJ open software and normalized by β-actin.

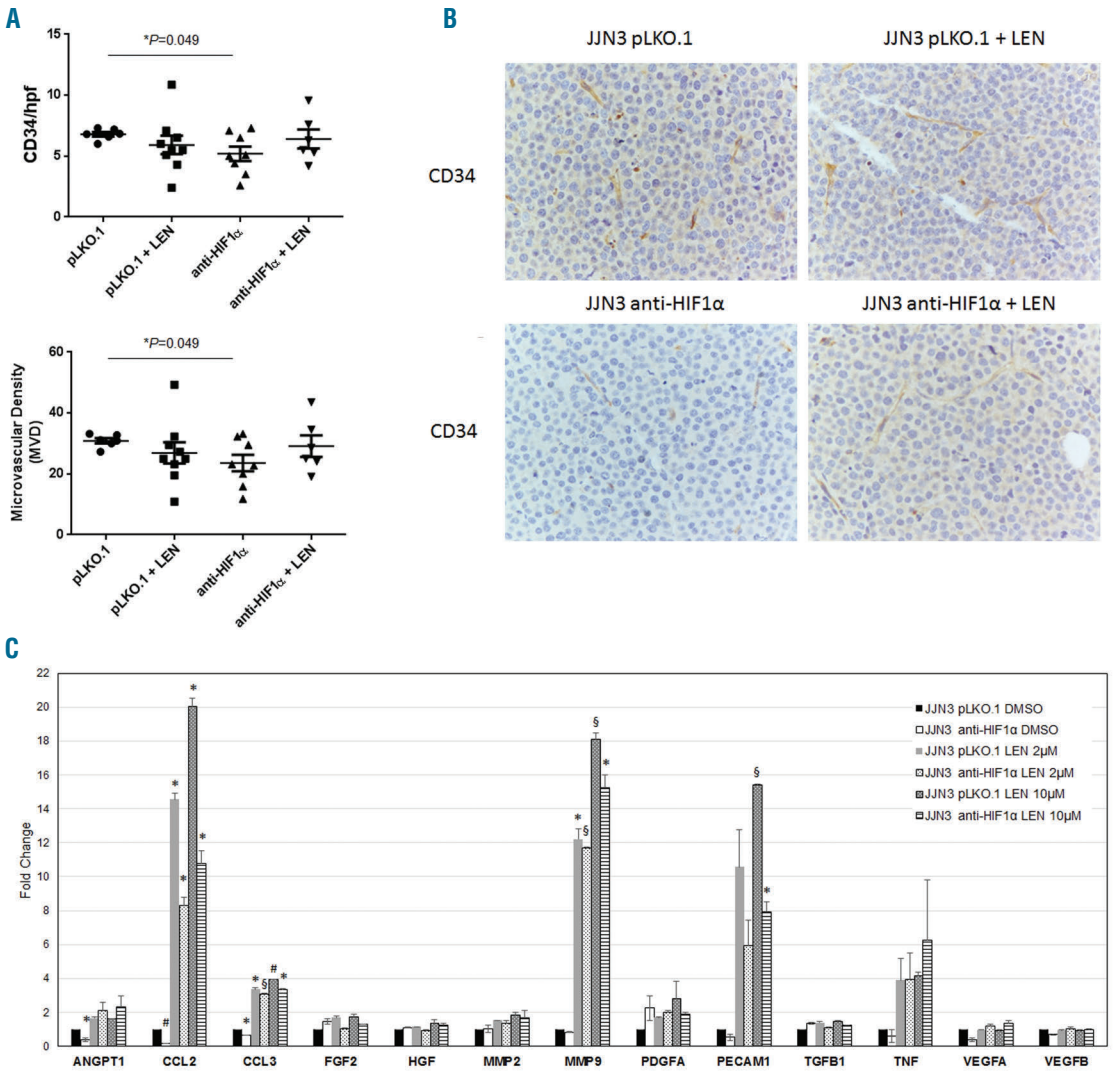

Figure 2. Effect of the combination of stable inhibition of HIF-1α and lenalidomide (LEN) treatment in myeloma cells on *in vivo* angiogenesis and on expression of the main pro-angiogenic molecules. The number of vessels positive to CD34 and the microvascular density (MVD) were evaluated by immunohistochemistry in the *ex vivo* JJN3 plasmacytomas removed from all the mice treated with LEN or vehicle (DMSO), as described above. (A) Plots and bars represent the single values and mean±SE of CD34 positive vessels and MVD, respectively, of the two independent sets of the *in vivo* experiments. Data were analyzed by two-tailed *t*-test. (B) Representative image of CD34 staining of each group of mice at 21 days (JJN3 pLKO.1, JJN3 pLKO.1 + LEN, JJN3 anti-HIF1α and JJN3 anti-HIF1α + LEN). (C) Expression of the main pro-angiogenic molecules were analyzed by PCR-array in JJN3 pLKO.1 or JJN3 anti-HIF1α treated *in vitro* with LEN (2 and 10 μM) or vehicle (DMSO) for 72 h. Graph bars represent the mean fold changes ± SD of two independent experiments calculated assuming JJN3 pLKO.1 DMSO as control condition. Data were analyzed by two-tailed *t*-test (**P*<0.05; §*P*<0.01; #*P*<0.001).

of pro-inflammatory and pro-angiogenic cytokines, such as TNF-α, has been previously reported.[12] Although a direct anti-angiogenic effect of LEN on endothelial cells has been demonstrated,[13] and also *in vivo* in a lymphoma mouse model,[14] other authors have failed to report a significant reduction in bone marrow MVD in MM patients treated with new drugs, including LEN.[15]

Overall, our data indicate that HIF-1α suppression in MM cells significantly increases the anti-MM *in vivo* effect of LEN, mainly through inhibition of proliferation signaling pathways rather than through an anti-angiogenic effect. These data suggest that there may be a rationale for a combined LEN and HIF-1α inhibition in MM therapy.

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