

Studying the role of the immune system on the antitumor activity of a Hedgehog inhibitor against murine osteosarcoma

Christophe Paget,¹ Helene Duret,¹ Shin Foong Ngiow,^{1,2,3} Maya Kansara,⁴ David M. Thomas⁴ and Mark J. Smyth^{1,2,*}

¹Cancer Immunology Program; Peter MacCallum Cancer Centre; East Melbourne, Australia; ²Sir Peter MacCallum Department of Oncology; University of Melbourne; Parkville, Australia; ³Department of Pathology; University of Melbourne; Parkville, Australia; ⁴Sarcoma Genomics and Genetics Laboratory; Peter MacCallum Cancer Centre; East Melbourne, Australia

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Recent evidence demonstrates that the efficacy of conventional anticancer therapies including chemotherapy requires a functional immune system. Here, we addressed the possibility that the antitumor effect of a selective Smoothed antagonist and Hedgehog (Hh) pathway inhibitor (LDE225), a promising anticancer drug, might at least partially depend on the immune system. To this aim, we used tumor cell lines derived from a murine model of radiation-induced osteosarcoma. In vitro treatment of osteosarcoma cells with LDE225 resulted in a decreased ability of tumor cells to proliferate, but had no effect on their viability. Flow cytometry analysis demonstrated that LDE225-treatment did not detectably modulate the immunogenicity of tumor cells. Moreover, LDE225 did not display any pro-apoptotic properties on osteosarcoma cells, highlighting that its antitumor profile mainly derives from a cytostatic effect. Furthermore, calreticulin exposure, a key feature of immunogenic cell death, was not provoked by LDE225, neither alone nor combined with recognized immunogenic drugs. Finally, the oral administration of LDE225 to osteosarcoma-bearing mice did significantly delay the tumor growth even in an immunocompromised setting. These data suggest that inhibiting Hh signaling can control osteosarcoma cell proliferation but does not modulate the immunogenic profile of these cells.

Introduction

Hedgehog (Hh) signaling regulates multiple genes that are important for both cellular growth and differentiation during embryonic development across multiple species. In healthy adult organisms, signaling via the Hh pathway is virtually shut down, or at least strongly reduced, due to the lack of Hh ligands. In mammals, three different Hh proteins have been identified so far: Sonic hedgehog (SHH), Indian hedgehog (IHH) and Desert hedgehog (DHH). In the absence of these proteins, the transmembrane receptor patched 1 (PTCH1) controls Smoothed (SMO) activation and the whole signaling cascade.¹ However, uncontrolled activation of the Hh pathway has been reported in several type of cancers including rhabdomyosarcoma, medulloblastoma, breast carcinoma, basal cell carcinoma, leukemia as well as lung, pancreatic and prostate cancer.^{2,3} Such an aberrant activation appears to depend on the ability of some transformed cells to produce Hh ligands or on particular mutations (including loss-of-function mutations of *PTCH1* or gain-of-function alterations of *SMO*). Accordingly, several Hh inhibitors have been engineered as potential anticancer drugs. Most of them are SMO antagonists and have provided promising results in different mouse models.^{4–6} Results from various clinical trials reinforce the possibility that

these compounds might be useful in cancer patients,^{7–9} although secondary mutations may lead to drug resistance.¹⁰ The ability of Hh inhibitors to prevent tumor development mainly relies on their anti-proliferative activity against transformed cells.

For a long time, immunity has been considered to play no or little role in the control of oncogenesis and tumor progression. Now, accumulating evidence demonstrates that the immune system is a fundamental player in oncology and an important determinant of patient prognosis and response to treatment.^{11,12} Recently, several studies have demonstrated that the efficacy of some conventional anticancer therapies, originally described to directly kill transformed cells, also relies on the activation of anticancer immune responses.^{13–15} Since some Hh inhibitors have been shown to exert pro-apoptotic effects on tumor cells in association with increased expression of FAS and natural killer (NK)-cell ligands,¹⁶ we have investigated the potential contribution of the immune system to the antitumor effects of the SMO antagonist LDE225 on osteosarcoma cells.

Results

LDE225 treatment reduced osteosarcoma cell proliferation but not induced cytotoxicity in vitro. Recent studies have

*Correspondence to: Mark J. Smyth; Email: mark.smyth@petermac.org
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demonstrated that human osteosarcomas display an overactivated Hh signaling,¹⁷ rendering the inhibition of this pathway an interesting approach to control disease progression.^{18,19} We first addressed the possibility that the SMO antagonist would be able to control cell lines generated from a murine model of the radiocarcinogen-induced osteosarcoma. Using two different *in vitro* assays (radioactivity and flow cytometry), we observed that LDE225 reduced the proliferation of two radiocarcinogen-induced osteosarcoma cell lines, namely OS5 and OS18 cells, in a dose-dependent manner (Fig. 1A). Of note, the cytostatic effect of the drug was more pronounced for OS18 than for OS5 cells. Previous studies had already demonstrated that Hh inhibitors can sensitize tumor cells to cytotoxicity or even kill them in a direct fashion.^{20,21} In contrast, we only observed a slight, but not significant, cytotoxic effect of LDE225 alone against osteosarcoma cells (Fig. 1B). Then, we hypothesized that the combination of LDE225 and conventional chemotherapeutics such as etoposide (Eto) or doxorubicin (Dox) might enhance the cytotoxic properties of these drugs. However, the addition of LDE225 did not increase the cytotoxic effect of Eto or Dox (Fig. 1C). Taken together, these results demonstrate that LDE225 exerts anticancer effects on osteosarcoma cells predominantly by preventing cell proliferation.

LDE225 treatment did not modulate the immunogenicity of osteosarcoma cells. Then, we addressed the possibility that a SMO antagonist might affect the immunogenicity of our cell lines. To this aim, we have screened (by cytofluorometry) the expression of numerous immunologic markers on vehicle- or LDE225-treated mouse osteosarcoma cells, including antigen-presenting molecules, NK cell ligands, co-stimulatory and inhibitory molecules. Since 5 μ M of LDE225 was the dose exerting the most potent cytostatic effect (Fig. 1A), we used this dose throughout the rest of our study. Interestingly, both OS5 and OS18 cells expressed a large panel of NK cell ligands (i.e., pan-Rae-1, CD155, DR5) rendering them potential targets for NK cell-mediated cytotoxicity (Fig. 2). However, LDE225 treatment did not enhance or decrease the level of expression of these molecules (Fig. 2). Of note, Fas was not expressed by OS5 and OS18 cells, and LDE225 exposure did not induce its expression (not shown). Moreover, both osteosarcoma cell lines also expressed the MHC Class I molecule H2-K^b and the MHC class Ib molecule CD1d, but not MHC Class II molecules. LDE225 had no effect on the expression of these molecules. We failed to detect CD40, CD70 and CD86 molecules on the surface of OS5 and OS18 cells and treatment with the SMO antagonist did not induce expression of these markers (Fig. 2). Finally, we observed that both cell lines expressed the immunosuppressive molecule PD-L1, but not PD-1 nor PD-L2. Once again, the spectrum of expression of these molecules was not modulated by LDE225. Together, these results indicate that osteosarcoma cell lines display a fairly immunogenic profile but LDE225 does not modulate this phenotype.

LDE225 did not modulate apoptosis or calreticulin exposure as induced by immunogenic drugs. Hh inhibitors can directly induce apoptosis.¹⁶ To gauge if LDE225 could induce the apoptotic demise of our osteosarcoma cells, we labeled LDE225-treated cells with Annexin-V and propidium iodide and subjected

them to flow cytometry to identify apoptotic cells. As depicted in Figure 3A, LDE225 did not induce apoptosis in OS18 cells, at any dose applied. As expected, we found that both Dox and (to a lesser extent) Eto induced a potent apoptotic response on osteosarcoma cells, in a dose-dependent manner (Fig. 3A). The combination of LDE225 with these conventional chemotherapeutics did not modulate their capacity to trigger apoptosis (Fig. 3A). These results suggest that LDE225 is neither able to induce apoptosis *per se* nor to modulate the pro-apoptotic potential of conventional drugs against osteosarcoma cells.

It is now well established that some conventional anticancer drugs such as anthracyclines can enhance the immunogenicity of tumors by inducing calreticulin (CRT) exposure as they trigger cell death. This is a process that can facilitate the uptake of dying tumor cells by neighboring antigen-presenting cells, a mandatory step to achieve an optimal antitumor response.²² To test the possibility that a SMO antagonist might have a similar effect on osteosarcoma cells, we assessed the amount of CRT exposed on the membrane of OS5 and OS18 cells upon LDE225 treatment. As depicted in Figure 3B (and not shown), LDE225 does not induce CRT exposure on these cell lines. Since anthracyclines (e.g., Dox) have been demonstrated to induce CRT exposure on a broad range of cancer cells, we have investigated this possibility on our murine osteosarcoma models. Cytofluorometric studies demonstrated indeed that Dox can induce the exposure of CRT on the surface of OS5 and OS18 cells. In line with a previous report,²² this effect was restricted and Eto, a topoisomerase inhibitor, failed in this respect. Next, we assessed if the simultaneous administration of LDE225 and Dox or Eto might increase/induce CRT exposure, finding that LDE225 does not alter CRT expression as triggered or not triggered by Dox and Eto, respectively (Fig. 3B).

LDE225 does not significantly modulate cytokine production as triggered by LPS or Con A in splenic cells. Since the Hh pathway is slightly active in resting immune cells,²³ we investigated the potential effect of LDE225 on the initiation/development of different immune responses. To this aim, spleen cells were cultured for two days with low doses of lipopolysaccharide (LPS, an agonist of Toll-like receptor 4) or concavalin A (Con A, a lymphocyte mitogen) in the presence or in the absence of LDE225. As expected, LPS (Fig. 4A) and Con A (Fig. 4B) treatment resulted in the production of a substantial amount of various cytokines including interferon γ (IFN γ), tumor necrosis factor α (TNF α), interleukin (IL)-2, IL-4 and IL-10 (Fig. 4 and data not shown). Interestingly, the addition of LDE225 inhibited the production of specific cytokines (IFN γ and IL-10) in response to LPS (Fig. 4A), but not Con A (Fig. 4B), in a dose-dependent manner. Of note, LDE225 treatment did not modulate proliferation induced by Con A (not shown). Taken together, these data suggest that LDE225 might modulate the development of immune responses, though to a minor extent.

The oral administration of LDE225 in osteosarcoma-bearing mice controls tumor progression independently from the immune system. LDE225 has been demonstrated to be promising in the control of tumor progression in various models.^{24,25} Thus, we investigated the *in vivo* effect of LDE225 on subcutaneously growing osteosarcomas. As depicted in Figure 5A, the

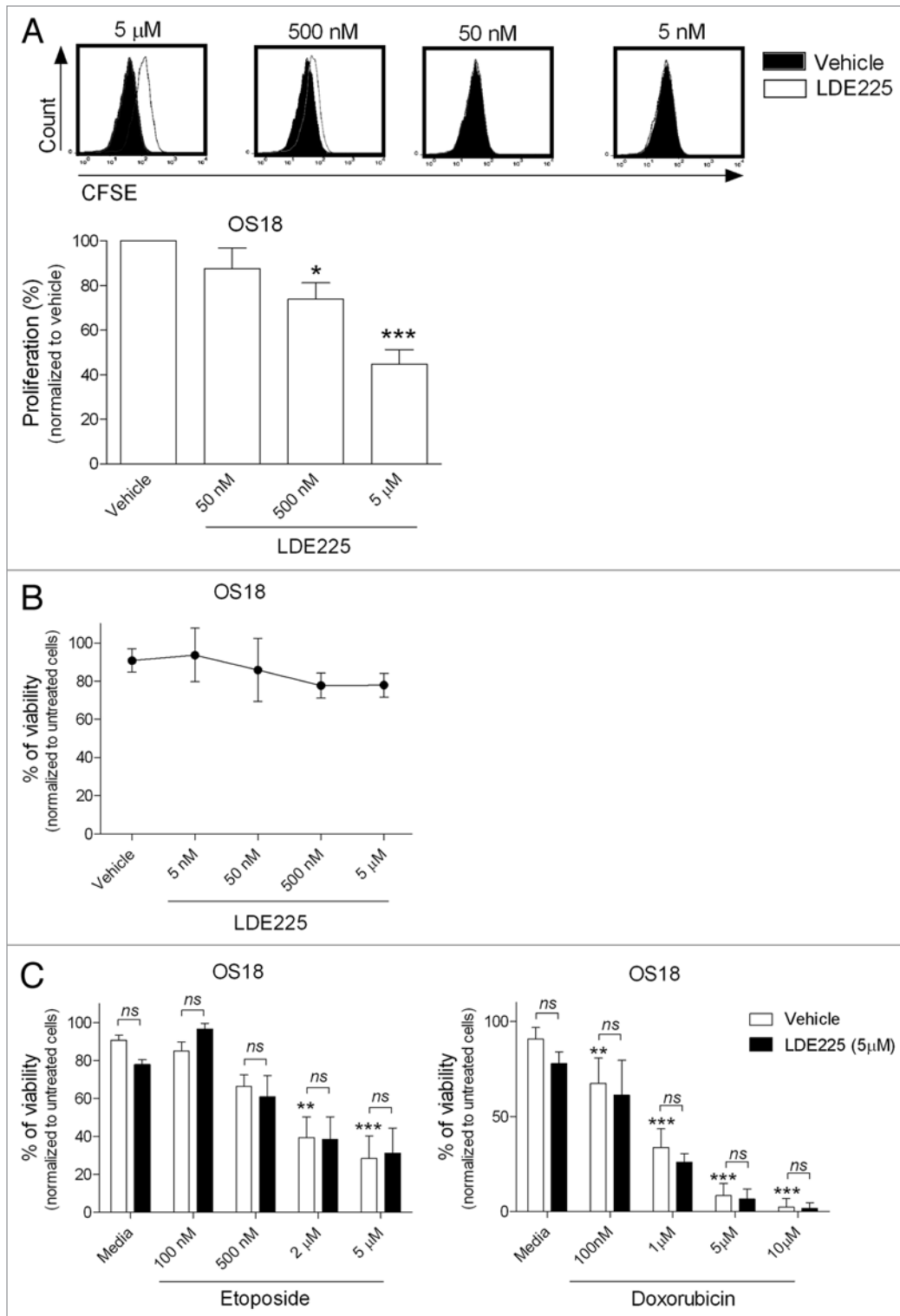


Figure 1. LDE225 reduced the proliferation ability of osteosarcoma cell lines. **(A)** Upper panel, CFSE-labeled ($2.5 \mu\text{M}$) 2×10^4 OS18 cells have been cultured for three days in complete media (α -MEM) in the absence (filled histograms) or in the presence (open histograms) of the indicated concentrations of LDE225. After three days, cells were monitored for CFSE dilution. Lower panel, OS18 cells were cultured in the absence or in the presence of LDE225 at the indicated doses. After 8 h, $0.5 \mu\text{Ci}$ of ^3H /well were added and radioactivity was measured after 48 h of culture using a liquid scintillation counter. Data represent means of 6 replicates per group \pm SEM, from $n = 2$ independent experiments. Differences in means were analyzed using a one-way ANOVA test. * $p < 0.05$, *** $p < 0.001$. **(B and C)** LDE225-treated or untreated OS18 cells were cultured for 24 h in the presence of various concentrations of either doxorubicin (Dox) or etoposide (Eto) and then viability was assessed using the CellTiter-Blue[®] reagent. Data represent means of $n = 3$ independent experiments performed in triplicates. Differences in means were analyzed using a one-way ANOVA test. ** $p < 0.01$, *** $p < 0.001$. Of note, in each experiment the amount of vehicle corresponds to the highest dose of LDE225 used.

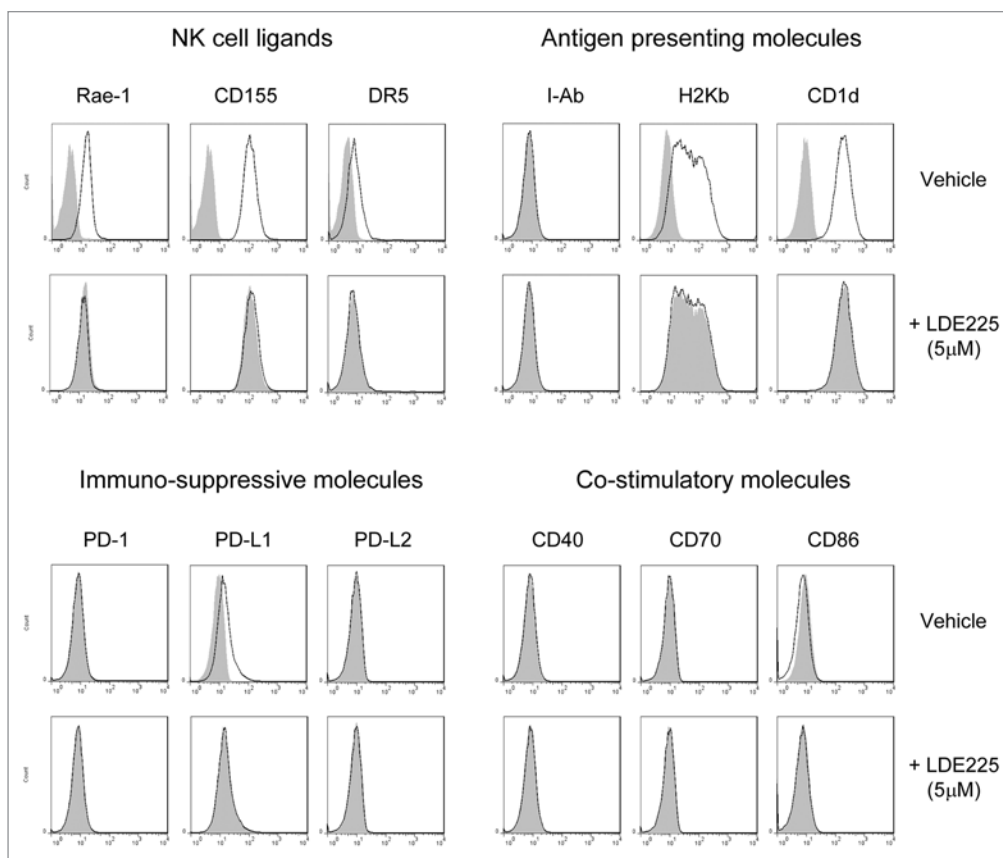


Figure 2. Phenotype of OS18 cells in the presence or absence of LDE225. Upper row, OS18 cells were monitored for the expression of markers including NK cell ligands (pan-Rae-1, CD155 and DR5), antigen presenting molecules (I-Ab, H2Kb and CD1d), immunosuppressive molecules (PD-1, PD-L1 and PD-L2) and co-stimulatory molecules (CD40, CD70 and CD86). All markers (open histograms) have been compared with their respective isotype controls (gray histograms). Lower row, marker expression has been assessed in the presence (open histograms) and in the absence (gray histograms) of LDE225 (5 μ M).

therapeutic regimen (which was started when tumor size reached ~ 15 mm²) with LDE225 (80 mg/kg/day) significantly delayed tumor growth. To assess *in vivo* the potential requirement of the immune system for the anticancer effects of LDE225, we used *Rag-1*^{-/-} mice, which lack mature B and T cells. Despite a trend for osteosarcomas to grow faster in these mice than in wild type (WT) mice, suggesting a role of the immune system in the natural control of these neoplasms, tumor growth was equivalently controlled in WT and *Rag-1*^{-/-} mice by LDE225 (Fig. 5B). Similar results were obtained using *Rag-2*^{-/-} \times γ c^{-/-} mice, which lack NK cells in addition of T and B cells (Fig. 5C). Taken together, our data demonstrate that immunocompromised mice fully respond to LDE225 treatment, hence suggesting that LDE225 efficacy does not rely on the host immune system.

Alleviation of immunosuppression does not modulate efficacy of LDE225. One of the limitations for effective anticancer immunotherapy is the rapid establishment of immunosuppression.²⁶ To investigate if the immune-independent mechanism of action of LDE225 might be explained by a pre-established immunosuppressive tumor microenvironment, we assessed the expression of different inhibitory molecules including TIM-3, PD-1 and CTLA-4 on tumor-infiltrating lymphocytes (TILs). We observed that tumor-associated CD4⁺ and CD8⁺ T cells

expressed these molecules at different levels (Fig. 6A), suggesting that they might constitute potential target for immunotherapy against osteosarcoma. The frequency of regulatory T cells ($\sim 40\%$ of all CD4⁺ T cells) also suggested an established immunosuppressive environment in the tumor (Fig. 6A). Of note, LDE225 treatment did not modulate the expression of TIM-3, PD-1 and CTLA-4 (data not shown). Interestingly, we observed that a monotherapy with anti-PD-1 monoclonal antibodies was more efficient against osteosarcoma *in vivo* than either anti-Tim-3 or anti-CTLA-4 treatments (Fig. 6B). The combination of anti-PD-1 with an anti-CD137 antibody (to re-stimulate exhausted T cells) resulted in even a greater antitumor effect (Fig. 6B). The addition of LDE225 to the anti-CD137 + anti-PD-1 immunotherapy did not modulate the efficacy of the treatment (Fig. 6B). These data indicate that immunosuppression does not affect the antitumor effects of LDE225.

Discussion

Harnessing the overactivation of Hh signaling in cancer is a promising targeted strategy. The requirement of the host immune system in the beneficial effect of Hh inhibitors has never been tested earlier. Our work demonstrates that the antitumor effects

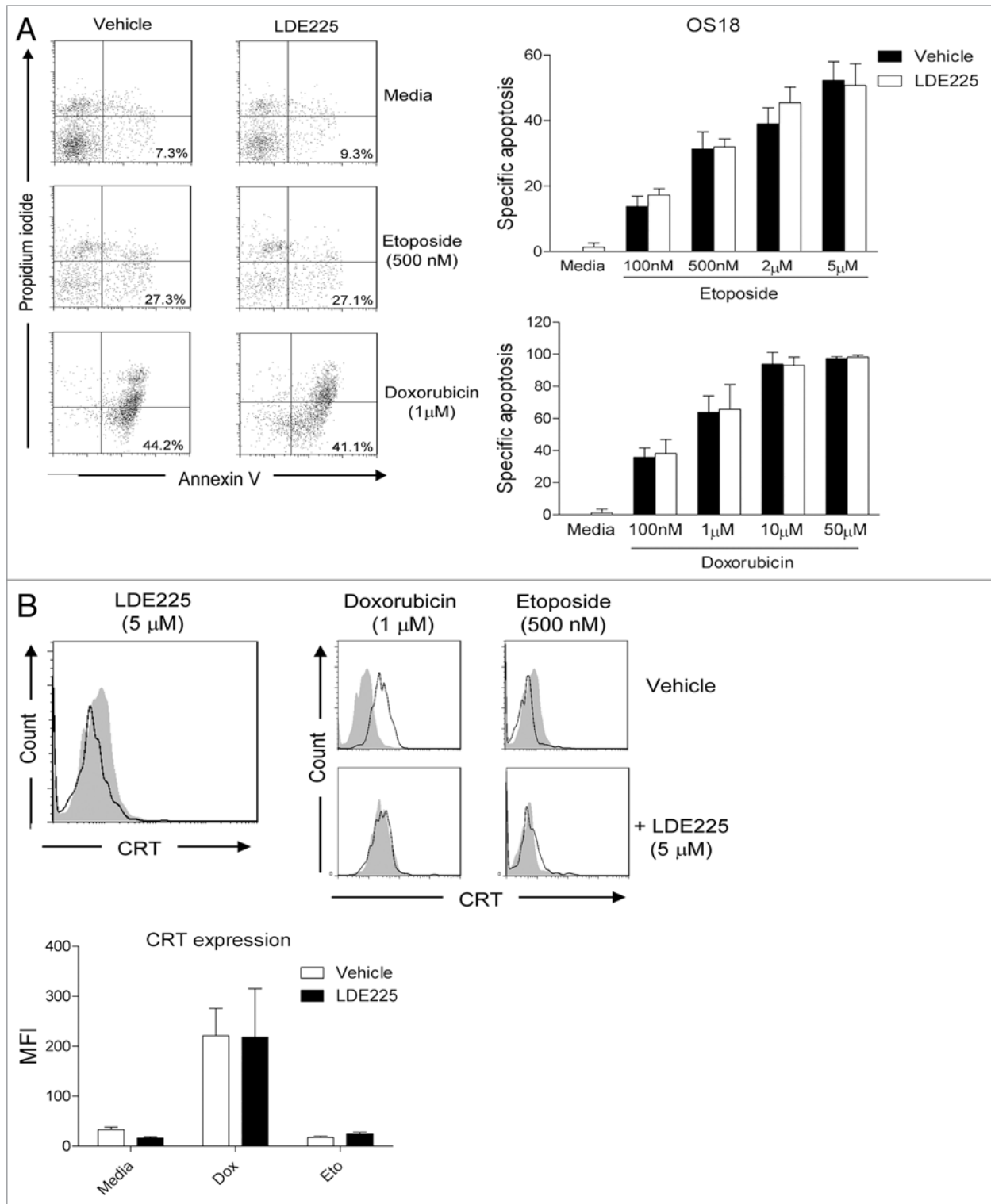


Figure 3. Effect of LDE225 on immunogenic cell death-induced apoptosis and calreticulin expression. **(A)** OS18 cells were cultured for 24 h with LDE225 (5 μ M) alone or in combination with either doxorubicin (Dox, 1 μ M) or etoposide (Eto, 500 nM) and apoptosis was assessed according to Annexin V/propidium iodide (PI) labeling. One representative experiment out of three is shown (left panel). Percentages \pm SD of apoptotic cells (AnnexinV⁺ PI⁺) are represented in the right panel. **(B)** OS18 cells were treated for 24 h with LDE225 (5 μ M), Dox (1 μ M), Eto (500 nM) or the indicated combinations and monitored for calreticulin expression. The mean of fluorescence intensity is indicated. One representative experiment out of three is shown (upper panel). The average \pm SEM of calreticulin expression on OS18 cells is shown in the lower panel (n = 9).

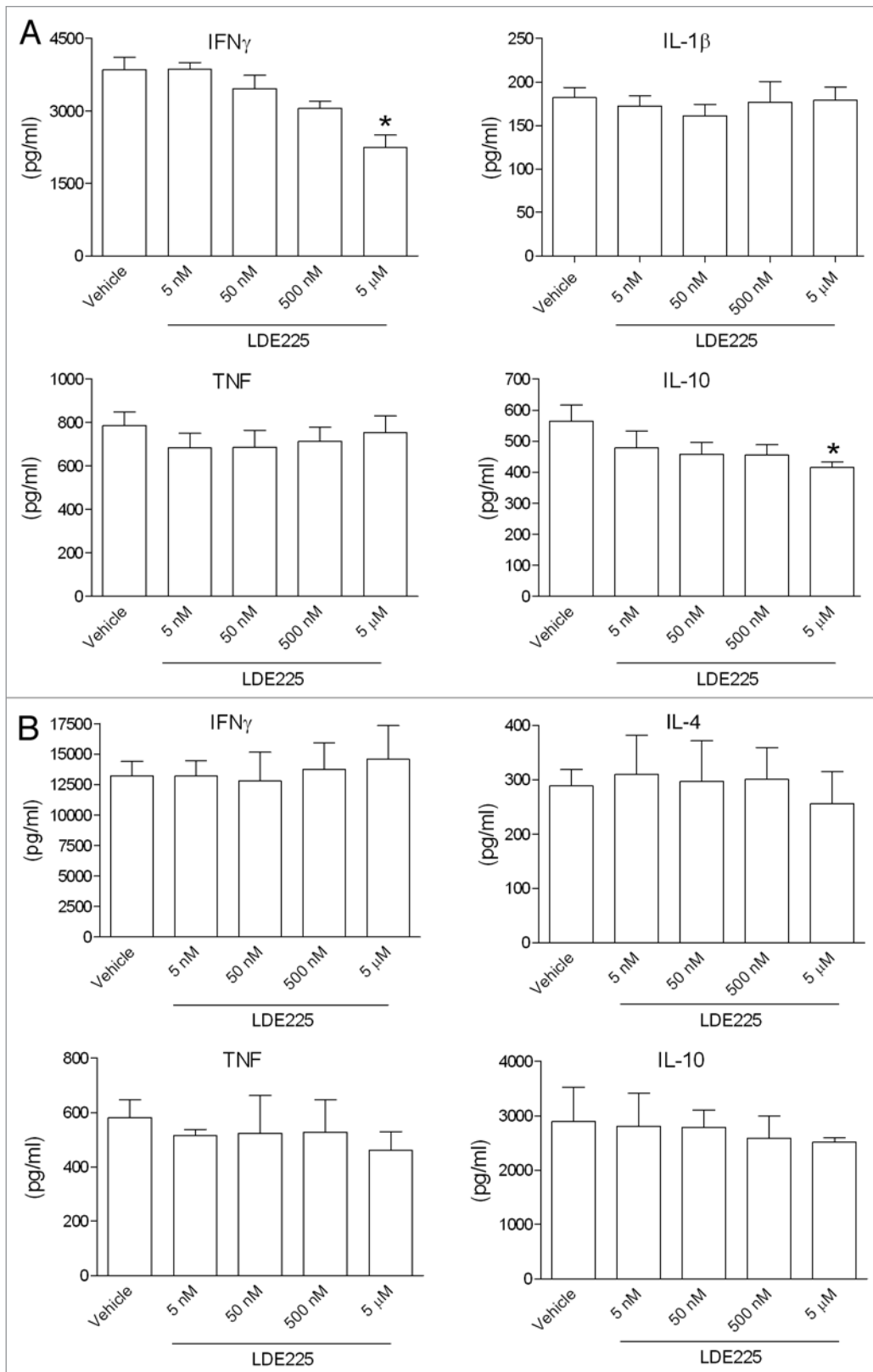


Figure 4. In vitro activity of LDE225 on the development of immune responses. **(A and B)** One million vehicle- or LDE225-treated spleen cells were cultured in presence or not of lipopolysaccharide (LPS, 50 ng/mL) **(A)** or concanavalin A (Con A, 2.5 μ g/mL) **(B)** and supernatants were collected after 48 h **(A)** or 72 h **(B)**. Cytokines in supernatants were assessed using the CBA system. The average \pm SD of one representative experiment out of two performed in triplicates is shown. Statistical analyses were performed using a one way ANOVA test followed by a Dunn's Multiple Comparison post-test. * $p < 0.05$.

of LDE225 against murine osteosarcomas neither rely on an increased immunogenicity of tumor cells nor on a fully competent immune system.

As previously shown with different type of cancer cells, we observed that LDE225 can control the proliferation of murine radiocarcinogen-induced osteosarcoma cell lines in vitro in a dose-dependent manner. This effect was not accompanied by a decrease in cell viability, indicating the cytostatic, rather than cytotoxic, nature of this Hh inhibitor. The anti-proliferative effects of different Hh inhibitors mainly rely on the induction of a cell cycle arrest in the G_0/G_1 phase.²⁷ Because the importance of the immune system is now widely accepted as a critical determinant for antitumor responses, we have investigated the potential modulation of tumor immunogenicity by LDE225. Interestingly, phenotyping studies suggested that our osteosarcoma cell lines are quite immunogenic, in particular considering the expression of different NK cell ligands and antigen-presenting molecules. However, LDE225 failed to modulate these markers. Despite recent reports highlighting an apoptotic effect of different Hh inhibitors,¹⁶ we were unable to demonstrate any pro-apoptotic or chemosensitizing effect of LDE225, either on its own or combined with other pro-apoptotic compounds. The reasons of this discrepancy remain unknown, but may relate to the intrinsic biology of the cell lines used in our study. Consistent with this, a seminal study has demonstrated that the anti-proliferative activity of some Hh inhibitors (e.g., cyclopamine) was not necessarily accompanied by the apoptotic demise of target cells,²⁸ suggesting that the effects of Hh inhibitors may vary in different cell lines. One of the key processes of immunogenic cell death is the exposure of CRT on the surface of pre-apoptotic tumor cells.²² Consistent with the lack of a pro-apoptotic activity, we failed to detect CRT on the surface of LDE225-treated osteosarcoma cells. As expected, the treatment of the tumor cells with an anthracycline (i.e., Dox) resulted in the appearance of CRT at the plasma membrane, while Eto failed to do so.²²

The role of the Hh signaling in T-cell development is now well documented but its role in the activation/regulation of peripheral immune responses remains poorly understood.²⁹ Here, we have investigated the influence of LDE225 on immune responses as induced by either LPS or ConA. Interestingly, while LDE225 did not modulate the cytokine profile produced by splenic cells in response to ConA, Hh inhibition reduced the LPS-stimulated production of IL-10 and IFN γ (but not that of IL-4, IL-2, IL-1 β or TNF). This suggests that the blockade of Hh signaling might selectively influence innate immunity. Of note, LDE225 did not induce the death of splenic cells in the conditions used in our study. It will be interesting to investigate how and at which stage of the development of immune responses LDE225 plays a role.

LDE225 has already shown potent in vivo effects against a range of murine cancers.^{24,25} Here, we extended the spectrum of activity of this drug to a mouse model of established osteosarcoma. LDE225 treatment by oral gavage significantly delayed the progression of osteosarcomas established by two distinct radiocarcinogen-induced cell lines. In line with our in vitro data, the anticancer efficacy of the Hh inhibitor persisted in strongly immunocompromised mice. Our data suggest that LDE225 might be efficient irrespective of the immunological status of the patient,

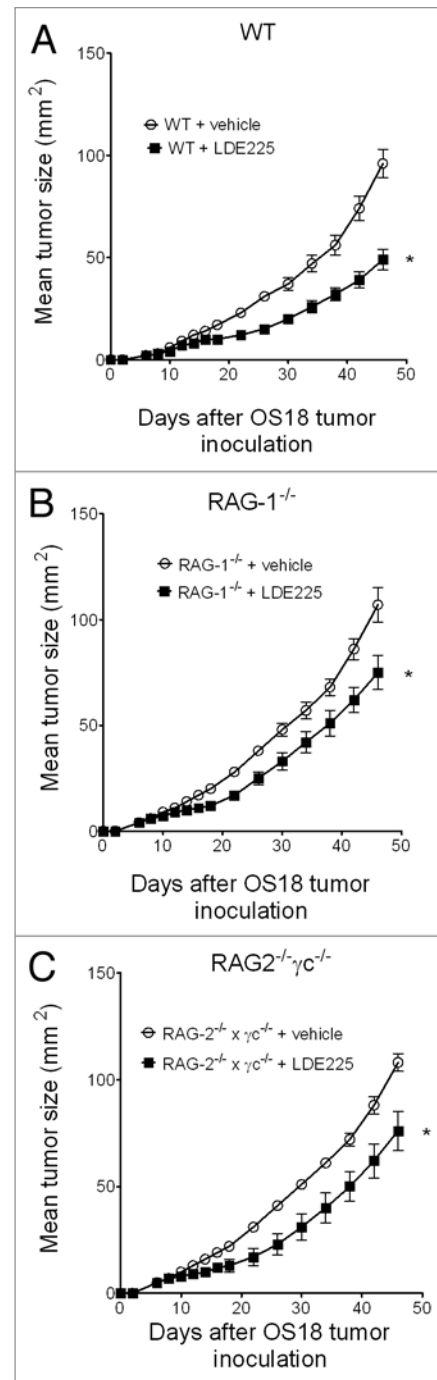


Figure 5. In vivo activity of LDE225 in the context of host immunity. (A–C) Groups of 5 wild type (WT) (A), *Rag-1^{-/-}* (B), *Rag-2^{-/-} x γ C^{-/-}* (C) mice were inoculated s.c. with 1×10^6 OS18 cells. Mice then received either vehicle or LDE225 (80 mg/kg) daily on days 6–10 and 13–17 after tumor cell inoculation. Tumor size was measured as indicated. Data represent means of 5 mice per group \pm standard errors. Statistical analyses were performed at the indicated time point using a Mann-Whitney test (* $p < 0.05$).

an observation that is relevant because cancer development has been correlated with primary or acquired immunodeficiency, and because most of the treatments used to cure cancer lead to immune deregulation. This hypothesis is reinforced by the fact

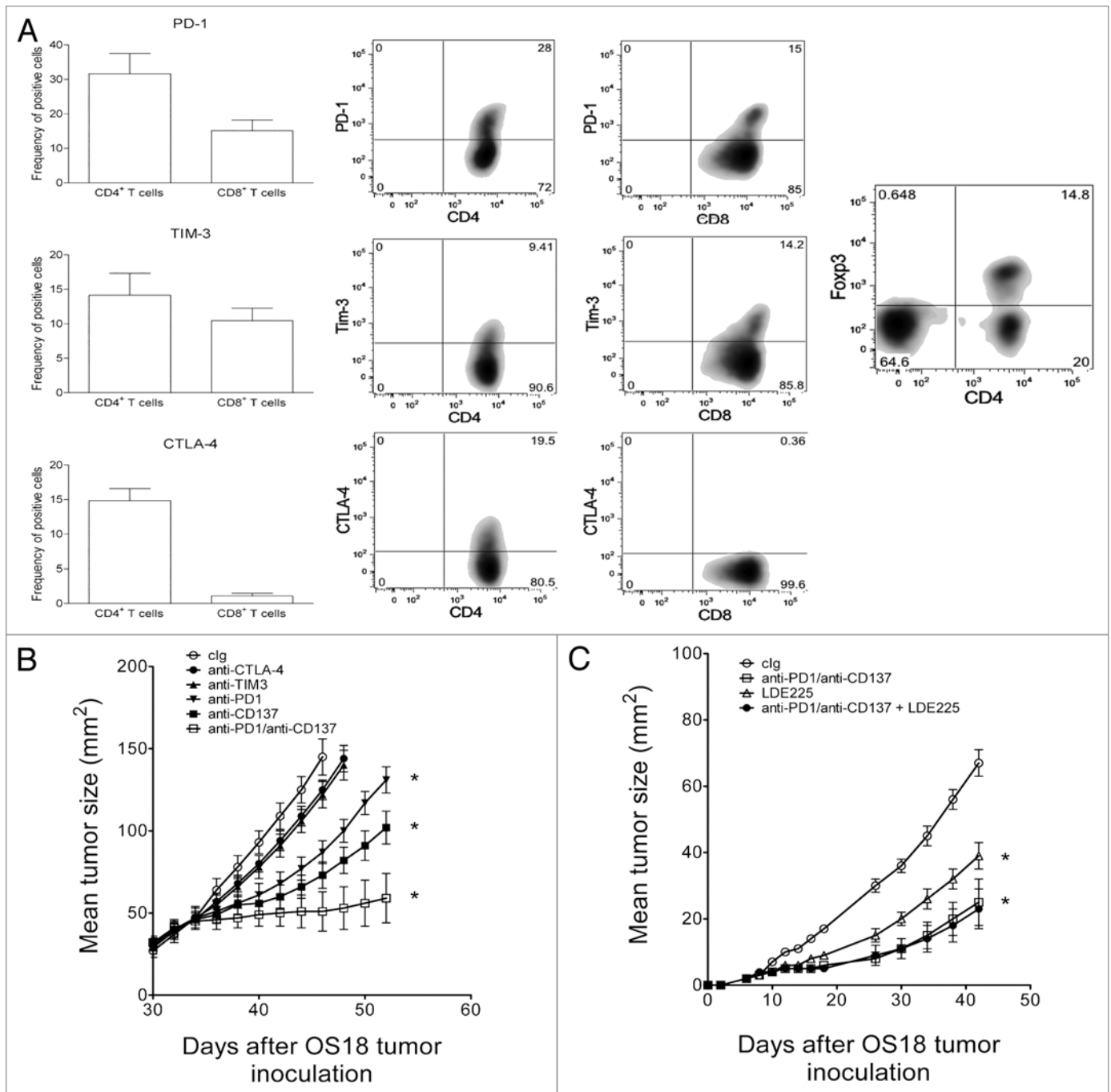


Figure 6. Effect of immunotherapy alone and in combination with LDE225. (A–C) Groups of 5 wild type (WT) mice were inoculated s.c. with 1×10^6 OS18 cells. Tumours were harvested and tumor-infiltrating lymphocytes (TILs) were analyzed for Tim-3, PD-1 and CTLA-4 expression on CD4⁺ and CD8⁺ T cells and regulatory T cell (CD4⁺ FOXP3⁺) frequency (A). Mice received control immunoglobulins (clg), anti-CTLA-4, anti-Tim-3, anti-PD-1, anti-CD137 or anti-PD-1/anti-CD137 combination (100 μ g i.p each) on days 34, 38, 42 after tumor cell inoculation (B). Mice received as indicated either vehicle or LDE225 (80 mg/kg) daily on days 6–10 and 13–17 and/or clg or anti-PD1/anti-CD137 on days 6, 10 and 14 after tumor cell inoculation (C). Tumor size was measured as indicated. Data represent means of 5 mice per group \pm SEM. Statistical analyses were performed at the indicated time point using a Mann-Whitney test. * $p < 0.05$, as compared with clg.

that relieving immunosuppression failed to augment the anticancer effects of LDE225. Moreover, these results may suggest that, in the context of an effective immunotherapy protocol, a drug targeting the oncogenic properties of tumor cells (e.g., cell proliferation)

does not provide any additional antitumor effect. The possibility to generalize this interpretation to other targeted therapies needs to be tested in other immunotherapeutic approaches or other types of cancer (e.g., hematological malignancies).

Overall, this study demonstrates that LDE225 controls the proliferation of osteosarcoma cells and could represent a therapeutic alternative for patients affected by profound immunodeficiency.

Materials and Methods

Mice. Wild type (WT) C57BL/6 mice were purchased from the Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia) and maintained at the Peter MacCallum Cancer Centre (Peter Mac). All C57BL/6 gene-targeted mice were bred and maintained at the Peter Mac as previously described.³⁰ Mice aged 8–12 weeks were used in all experiments, according to Peter Mac Animal Experimental Ethics Committee guidelines.

Cell lines and reagents. OS5 and OS18 osteosarcoma cells were isolated from the hind limbs of mice which received four intra-peritoneal injections of the radiocarcinogen ⁴⁵Ca as previously described.³¹ OS5 and OS18 cells were maintained and cultured in α -MEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM Glutamax[®], 100 U/mL penicillin, 100 μ g/mL streptomycin and 100 μ M ascorbate-2-phosphate. Cell lines were routinely tested for mycoplasma contamination. LDE225 has been kindly provided by Novartis.

CFSE proliferation assays. Tumor cells were labeled with 2.5 μ M of CFSE (Invitrogen, C34554) for 15 min at 37°C. Thereafter, labeled cells were washed and cultured in the presence or not of different concentrations of LDE225. After 3 days, cells were monitored for CFSE dilution using FACS LSR II (BD Biosciences).

³H thymidine proliferation assays. Tumor cells were plated in the presence or in the absence of LDE225 at different doses. After 8 h, 0.5 μ Ci of ³H/well were added and radioactivity was measured after 48 h of culture using a Tri-Carb 2910TR liquid scintillation counter (PerkinElmer).

Assessment of cell viability. The viability of tumor cells after treatment with LDE225 was monitored using the CellTiter-Blue[®] reagent from Promega (G8080). Briefly, OS5 and OS18 cells were cultured in the presence or in the absence of LDE225 and, after 24 h, 20 μ L of CellTiter-Blue[®] reagent were added. Two hrs later, the viability of cells was assessed by their ability to convert a redox dye, resazurin, into a fluorescent product, resorufin. Fluorescence was monitored on a VersaMax plate reader using a 560/590 nm filter set (Molecular Devices).

Flow cytometry (phenotype and apoptosis assay). Single cell suspensions were analyzed using FACS LSR II (BD Biosciences). Cells were first stained with anti-CD16/32 (2.4G2) for 10 min at 4°C, then with specifically conjugated antibodies for 30 min at 4°C. PD-1 (J43), PD-L1 (MIH5), PD-L2 (TY25), H2-K^b (AF6–88.5), CD1d (1B1), I-Ab (AF6–120.1), CD155 (4.24.1), pan-Rae-1 (186107), DR5 (MD5–1), CD40 (3/23), CD70 (FR70), CD86 (GL1) and streptavidin-allophycocyanin as well as their respective isotype controls were purchased from BD Biosciences, BioLegend, R&D System or eBiosciences. Assessment of calreticulin (Rabbit polyclonal, Abcam, ab4) expression was monitored on cells pre-fixed with 0.25% paraformaldehyde. Non-viable

cells were excluded on the basis of staining with 7-aminoactinomycin D (7-AAD) or Fluorogold (BD PharMingen). Data sets were analyzed using the Flowjo software (Tree Star). Cells were assessed for apoptosis using Annexin V (added in Annexin V binding buffer for 15 min at room temperature). Propidium iodide (1 μ g/mL) was added immediately prior to analysis.

Spleen cell activation and detection of cytokines. Splenic mononuclear cells from naïve mice were prepared by classical procedures. Vehicle- or LDE225-treated spleen cells were cultured in presence or not of lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (50 ng/mL) (Sigma, L2630) or concanavalin A (Con A) from *Canavalia ensiformis* (2.5 μ g/mL) (Sigma, C5275). Supernatants were collected after 48 (for LPS) or 72 (for Con A) hrs of culture. Cytokines in supernatants were detected using the BD Cytometric Bead Array (CBA) system (BD Bioscience). Acquisition was performed on an LSR-II. A total of 300 bead events for each cytokine were collected. Analysis was performed using FCAP array.

Tumor models. Mice were injected with 1×10^6 OS18 cells. In some cases, mice were treated with control immunoglobulins (cIg, Mac-4), anti-Tim-3 (RMT3–23),³⁰ antagonistic anti-PD-1 (CD279; RMP1–14),³² antagonistic anti-CTLA-4 (CD152; UC10–4F10 kindly provided by Jeffrey Bluestone) and anti-CD137 (3H3)³³ antibodies.

Tumor-infiltrating lymphocytes. Tumors harvested from mice were digested and cell suspensions were then used for flow cytometry analysis as previously described.³⁰ For surface staining, tumor-infiltrating lymphocytes (TILs) were stained with A750-anti-CD45.2, PECy7-anti-CD4, FITC-anti-CD3, eF450-anti-CD8, FITC-anti-PD-1, PE-anti-Tim-3 (clone 8B.2C12 and RMT3–23 respectively) and APC-anti-CTLA-4 (after permeabilization) in the presence of 2.4G2 (anti-CD16/32, to block Fc-receptors) on ice. 7-AAD was added immediately before flow cytometry analysis. Tim-3 and PD-1 expression on CD8⁺ or CD4⁺ T cells was limited to CD3⁺CD45.2⁺7AAD⁻ cells. For intracellular staining, TILs were fixed using the BD Cytofix/Cytoperm reagent and stained with APC-CTLA-4 (eBioscience), suspended in BD Perm/Wash Buffer.

Statistical analysis. Results are expressed as means \pm SD. The statistical significance of differences between experimental groups was calculated by one-way ANOVA or unpaired Student's t-tests (GraphPad Prism 5 Software). Results with a p value < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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