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ORIGINAL ARTICLE

Immunological hallmarks of *cis*-DDP-resistant Lewis lung carcinoma cells

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Abstract

Purpose Tumor cell resistance to platinum-based chemotherapeutic agents is one of the major hurdles to successful cancer treatment with these drugs, and is associated with alterations in tumor cell immune evasion and immunomodulatory properties. Immunocyte targeting is considered as a relevant approach to fight drug-resistant cancer. In this study, immunological hallmarks of *cis*-DDP-resistant Lewis lung carcinoma cells (LLC/R9) were investigated.

Methods Immunological features of LLC/R9 cells cultured in vitro in normoxic and hypoxic conditions as well as of those that were grown in vivo were examined. The expression of immunologically relevant genes was evaluated by RT-PCR. Tumor cell susceptibility to the macrophage contact tumoricidal activity and NK-mediated cytolysis was investigated in MTT test. TNF-α-mediated tumor cell apoptosis as well as macrophage phagocytosis, oxidative metabolism, and CD206 expression after the treatment with conditioned media from normoxic and hypoxic tumor cells were studied by flow cytometry. Flow cytometry was also used to characterize dendritic cell maturity.

Results When growing in vitro, LLC/R9 were characterized by slightly increased immunosuppressive cytokine gene expression. Transition to in vivo growth was associated with the enhancement of transcription of these genes in tumor cells. LLC/R9 cells had lowered sensitivity to contact-dependent macrophage-mediated cytotoxicity and to the TNFα-mediated apoptosis in vitro. Conditioned media from hypoxic LLC/R9 cells stimulated reactive oxygen species generation and CD206 expression in non-sensitized macrophages. Acquisition of drug resistance by LLC/R9 cells was associated with their increased sensitivity to NK-cell-mediated cytolysis. Meanwhile, the treatment of LLCR/9-bearing animals with generated ex vivo and loaded with LLC/R9 cell-lysate dendritic cells (DCs) resulted in profoundly enhanced tumor metastasizing.

Conclusion Decreased sensitivity to macrophage cytolysis, polarizing effect on DCs maturation along with increased susceptibility to NK-cell cytotoxic action promote extensive local growth of chemoresistant LLC/R9 tumors in vivo, but hamper their metastasizing.

Keywords Lewis lung carcinoma · *cis*-DDP · Chemoresistance · Macrophage-mediated cytotoxicity · NK-cell-mediated cytolysis · Dendritic cells

Introduction

Tumor cell resistance to chemotherapeutic agents is one of the major hurdles to successful cancer treatment. The development of chemoresistance is a result of metabolic plasticity and heterogeneity inherent to tumor cell population [1–3]. Chemoresistant tumor cells are characterized by the dramatic changes in key hallmarks including immune evasion and immunomodulatory mechanisms. Dynamic bidirectional interplay between tumor cells and immunocytes has become considered as a crucial factor influencing tumor growth and outcome of the therapy [4–6]. Chemotherapeutic

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agents can induce changes in transcriptome and metabolome of survived tumor cells which enable them to escape from immune surveillance and antitumor immunity as a whole [7–9]. *Cis*-diamminedichloro-platinum(II) (*cis*-DDP) is one of the most commonly used broad-spectrum chemotherapeutic agents. However, treatment with *cis*-DDP initiates cellular self-defense system with drastic epigenetic and genetic alterations in tumor cells and with the development of the resistance. Resistance is a major limitation of *cis*-DDP-based cancer chemotherapy [10–12]. *Cis*-DDP-resistant tumor cells acquire a range of phenotypic and functional alterations including those responsible for the immune evasion and immunomodulation. For instance, PDL1 expression by cisplatin-resistant tumor cells is up-regulated and therefore protect them from the effectors of adaptive immunity [13]. Tumor cells, resistant to cisplatin, have stem-like features, as platinum-based compounds are more effective at eliminating of differentiated malignant cells. Cancer stem cells, as is known, are characterized by potent immuno-evading machinery [14–16]. Cisplatin activates a plethora of transcription factors and modifies cytokine profile expressed by tumor cells. This cytokine milieu provides autocrine and paracrine proliferative and antiapoptotic signals for tumor cells. In addition, cytokines produced by cisplatin-resistant tumor cells polarize immunocytes in tumor microenvironment and in distant locations, and create favorable immunological niches for tumor local growth and metastasizing [17, 18]. Thus, the crosstalk between immune cells and tumor cells is one of the most important factors in sustained growth of chemoresistant tumor [19, 20]. However, there are still multiple gaps in our understanding of immunological hallmarks of cisplatin-resistant cells. Unlike tumor cells, immune cells are genetically stable and thus represent an attractive therapeutic target with reduced risk of resistance and tumor recurrence. Understanding immunological hallmarks of tumor cells that survived after the genotoxic cisplatin stress will aid in the rational design of combined treatment with the use of cytotoxic drug and immunotherapeutic agents for preventing tumor escape from the immunosurveillance and for improving therapeutic response.

Previously, we have described some biological properties of *cis*-DDP-resistant Lewis lung carcinoma cells (LLC/R9) that were generated from the wild-type LLC strain after nine sequential *cis*-DDP courses in vivo [21, 22]. LLC/R9 tumors are characterized by high (as compared with LLC) growth rate. In addition, LLC/R9 is distinguished by a threefold lower metastatic potential in comparison with wild-type LLC cells. Higher angiogenic potential of LLC/R9 cells as compared to LLC was also revealed and confirmed by (1) 1.5-fold increased rate of VEGF secretion; (2) more pronounced ability to induce vessel growth on chick embryo chorioallantoic membrane; and (3) higher sensitivity to the action of antiangiogenic agents [23, 24]. Manifestation of the paraneoplastic hematological syndrome was observed in animals with drug-resistant LLC/R9 variant, and was attributed to the ability of cancer cells to secrete VEGF in a high rate [25]. Paraneoplastic syndrome in LLC/R9-bearing animals was associated with alterations in central and peripheral lymphoid organs. This fact along with current literature data [26, 27] allowed us to suppose systemic immunomodulatory effect of drug-resistant tumor.

The aim of this work was to investigate immunological hallmarks of *cis*-DDP-resistant variant of Lewis lung carcinoma LLC/R9.

Materials and methods

Collection of conditioned media from normoxic and hypoxic tumor cells

The wild-type and chemoresistant tumor cell lines (LLC and LLC/R9 correspondingly) were kindly provided by National Bank of Cell Lines and Transplanted Tumors of IEPOR NASU. The cells were cultured in vitro in RPMI medium (Sigma, USA) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 40 mg/ml gentamycin at 37 °C in humidified atmosphere with 5% CO_2.

Hypoxia induction experiments were performed as described earlier with slight modifications [28]. Briefly, LLC and LLC/R9 cells were subjected to either normoxia (21% O_2) or hypoxia (3% O_2) for 16 h. Normoxia was controlled by using a humidified 5% CO_2/air incubator, and hypoxia—by pregassing RPMI for 30 min in a sealed hypoxic work station with 5% CO_2/balance N_2 gas mix and subsequent culture in a humidified hypoxic (CO_2/N_2) incubator. After the incubation for indicated time period, conditioned media (CM) were harvested, centrifuged to remove cell debris, and stored in aliquots at − 20 °C.

To investigate the effect of CM from normoxic and hypoxic LLC and LLC/R9 cells on non-sensitized peritoneal macrophage functional profile, phagocytes were cultured with CM for 24 h at 37 °C in humidified atmosphere with 5% CO_2. Phagocytic activity, intracellular reactive oxygen species (ROS) generation and the expression of nonopsonic scavenger receptor CD206 were analyzed by flow cytometry (FACS). Phagocytic activity was assayed with the use of FITC-labelled *Staphylococcus aureus* Cowan I cells as described earlier [28]. CD206 expression was examined with the use of CD206—Alexa Fluor 647 (MR5D3; BioLegend).

RT-PCR

For RT-PCR studies, LLC and LLC/R9 cells were cultured in vitro as described above as well as were isolated from primary tumor nodules in animal at days 10, 16, and 20 after

the tumor cell inoculation. For this purpose, tumor tissue from LLC- and LLC/R9-bearing mice ($n=3$ for each tumor) was excised, minced into 1–2 mm pieces using a scalpel and placed in dissociation buffer (100 U/ml Collagenase IV and 100 µg/ml DNase in RPMI + 10% FBS) for 30 min at 37 °C. Cell suspension was then filtered through the 70 µm cell strainer to get rid of big cell aggregates. Erythrocytes were lysed by treatment with ammonium chloride.

Total RNA was isolated from tumor cells using the 'Ribo-Sorb' kit (AmpliSens, Moscow, Russia). Extracted RNA was transcribed into cDNA using M-MuLV reverse transcriptase (Fermentas; Vilnius, Lithuania) following the recommendations of the manufacturer. The cDNA concentration was analyzed with absorbance at 260 nm. Equal aliquots of cDNA samples were taken for semi-quantitative analysis of the expression level and primers for the GAPDH gene were used in the same reaction vessel to circumvent false negative results. For each reaction, the PCR mixture contained Taq Buffer with $(NH_4)_2SO_4$, 2.5 µM Mg_2+, 0.2 µM each dNTP, 1U Taq polymerase (Fermentas), 50 µM each primer from a pair, and 2 µl cDNA. The following primers were used (fw, forward, rv, reverse):

- fw TAP1: 5′GGACTGTCAGCAGCGGCAACC-3′
- rv TAP1: 5′CAAGGCCTTT CATGTTTGAGGG-3′
- fw TAP2: 5′CAGGATGCAGTGGCCAGGGCG-3′
- rv TAP2: 5′TAGATACACGTCTTTTTCCAGG-3′
- fw TGFβ: 5′ACACGGAATACAGGGCTTTCGATT CA3′
- rv TGFβ: 5′CTTGGGCTTGCGACCCAGTAGTA3′
- fw VEGF: 5′CTGTGCAGGCTGCTGTAACG3′
- rv VEGF: 5′GTTCCCGAAACCCTGAGGGAG3′
- fw IL10: 5′ATGCAGGACTTTAAGGGTTACTTG GGTT3′
- rv IL10: 5′ATTTCGGAGAGAGGTACAAACGAG GTTT3′
- fw MMP2: 5′AGATCTTCTTCTTCAAGGACCGGTT3′
- rv MMP2: 5′GGCTGGTCAGTGGCTTGGGGTA3′
- fw MMP9: 5′GTTTTTGATGCTATTGCTGAGATC CA3′
- rv MMP9: 5′CCCACATTTGACGTCCAGAGAAGAA3′
- fw MT-MMP: 5′GGATACCCAATGCCCATTGGCCA3′
- rv MT-MMP: 5′CCATTGGGCATCCAGAAGAGAGC3′
- fw GAPDH: 5′GCCAAGGTCATCCATGACAACTTT GG-3′
- rv GAPDH: 5′GCCTGCTTCACCACCTTCTTGATG TC-3′

PCR included an initial denaturation step (94 °C, 4 min) and 30 amplification cycles (93 °C, 35 s; 60 °C, 35 s; 72 °C, 35 s). PCR products were visualized by electrophoresis in 2% agarose on RAF buffer, using a UV transilluminator and ethidium bromide staining. Semi-quantitative analysis of the expression level was performed using TotalLab TL120 (Nonlinear Dynamics Inc., Newcastle, United Kingdom). The GAPDH mRNA/cell served as an internal control. For Table 1, expression was normalized to GAPDH. Each PCR data point for genes, mentioned above, was divided by the respective individual GAPDH results. Mean and SD were calculated with normalized values, and statistical significance was evaluated by the Student two-tailed *t* test.

Isolation of murine peritoneal macrophages (PMs)

Murine PMs were isolated without preliminary stimulation as described previously [28]. Intact mice (male, 8 weeks, bred at animal facility of IEPOR NASU) were sacrificed and PMs were harvested using phosphate buffered saline containing 100 U/mL of heparin. Cells were centrifuged at 300*g* for 5 min at 4 °C, washed twice with serum-free DMEM, and resuspended in DMEM containing 10% FCS and 40 µg/mL gentamycin.

TNFα-mediated apoptosis evaluation

LLC/R9 an LLC cells were exposed to TNFα at the growing concentrations for 24 and 48 h. TNFα-mediated apoptosis was examined by FACS after propidium iodide staining as described previously [29].

Cytotoxicity assay

To analyse cytotoxic activity of PMs and mononuclear splenic leukocytes, modified MTT assay was performed as described earlier [30]. Tumor cells were cultured in vitro as described above. Mononuclear splenic leukocytes were obtained from splenocyte suspension by centrifugation (200*g*, 40 min) in Ficoll–Hypaque density gradient ($\rho=1.077$). PMs were isolated without preliminary stimulation as described above. To perform cytotoxic assay cancer cells were placed in 96-well plates (3×10^5 cells/well), and mononuclear splenic leukocytes or PMs were added at 40:1 and 20:1 *E:T* ratio. Cells were incubated in RPMI-1640 medium supplemented with gentamycin sulfate (100 µg/ml) and maintained at 37 °C for 4 h (with mononuclear splenic leukocytes) or for 18 h (with PMs) in 5% CO_2 atmosphere. After incubation, MTT (Sigma) was added to a final concentration of 0.5 mg/ml and cells were cultured for additional 3 h. Cells were centrifuged (5810R, Eppendorf) at 4000 rpm for 10 min. Medium was collected, precipitated blue formasan crystals were dissolved in 100 µl DMSO. Optical density was determined on a Multiscan RC at 570 nm, and the background signal was subtracted at 620 nm. Cytotoxic activity of

Table 1 RT-PCR analysis of immunologically relevant genes in drug-resistant LLC/R9 cells

Tumor cell line	Growth conditions	Normalized value of mRNA							
		TGF-β	VEGF	IL-10	MMP2	MMP9	MT-MMP	TAP1	TAP2
LLC	In vitro	41.0 ± 2.1	39.0 ± 1.6	41.0 ± 5.0	37.0 ± 1.0	43.0 ± 1.0	47.0 ± 1.8	35.0 ± 4.4	26.6 ± 6.4
	In vivo (days)								
	10	18.8 ± 7.1*	22.5 ± 3.2*	23.0 ± 6.1*	19.5 ± 4.3*	21.5 ± 1.9*	32.5 ± 5.7*	37.3 ± 8.8	16.5 ± 3.7
	16	34.1 ± 7.1	35.0 ± 4.9	19.0 ± 1.1*	18.8 ± 6.3*	27.5 ± 7.5*	49.6 ± 6.7	49.3 ± 8.0	56.5 ± 7.5*
	20	49.0 ± 6.0	30.7 ± 2.4*	38.0 ± 4.0	21.5 ± 8.9*	25.6 ± 7.2*	49.0 ± 2.0	56.8 ± 5.4*	46.0 ± 4.0*
	25	46.3 ± 8.1	25.7 ± 3.3*	35.9 ± 2.9	10.0 ± 5.0*	29.0 ± 8.7*	45.0 ± 8.5	76.0 ± 8.9*	57.3 ± 8.1*
LLC/R9	In vitro	50.0 ± 3.0$^{\#}$	44.5 ± 1.5	Un-detectable$^{\#}$	Un-detectable$^{\#}$	27.5 ± 1.5	25.0 ± 1.0$^{\#}$	44.5 ± 6.5	30.2 ± 6.1
	In vivo (days)								
	10	34.7 ± 7.1$^{*,\#}$	51.0 ± 2.0$^{*,\#}$	28.1 ± 5.2*	1.0 ± 0.5$^{\#}$	21.5 ± 1.5*	18.7 ± 2.3$^{\#}$	74.7 ± 5.3$^{*,\#}$	43.5 ± 0.9$^{*,\#}$
	16	22.0 ± 2.5$^{*,\#}$	47.0 ± 3.0$^{\#}$	17.3 ± 4.2*	Un-detectable$^{\#}$	19.5 ± 6.1	10.4 ± 6.9$^{*,\#}$	44.6 ± 7.0	35.3 ± 5.4$^{\#}$
	20	10.5 ± 5.1$^{*,\#}$	42.8 ± 8.1	18.2 ± 8.4$^{*,\#}$	Un-detectable$^{\#}$	22.4 ± 9,2	29.5 ± 2.5$^{\#}$	27.5 ± 2.5$^{*,\#}$	19.0 ± 2.6$^{*,\#}$
	25	43.0 ± 5.3	38.5 ± 7.6	23.9 ± 9.9*	Un-detectable$^{\#}$	15.4 ± 9.9	19.5 ± 6.7$^{\#}$	18.2 ± 4.1$^{*,\#}$	18.3 ± 4.7$^{*,\#}$

Semi-quantitative RT-PCR was performed using specific primers listed in "Materials and methods". Total cellular RNA from LLC and LLC/R9 cells was extracted and subjected to RT-PCR analysis. Amplification of the GAPDH cDNA served as an internal control. Data are presented as average of mean of triplicate in four independent experiments after normalization to GAPDH ± SD. *$P < 0.05$ compared with in vitro growth of corresponding cell line; $^{\#}P < 0.05$ compared with corresponding value of wild-type cells

the studied cells was characterized by cytotoxicity index that was calculated using the formula: Cytotoxicity index $= (1 - \varepsilon/\varepsilon_c) \times 100\%$, where ε_c and ε are the extinctions of control and test sample, respectively. All experiments were performed in triplicates.

Intracellular ROS-generation assay

ROS levels were measured as described earlier using 2′7′-dichlorodihydro-fluorescein diacetate (carboxy-H_2DCFDA, Invitrogen), which is converted into a non-fluorescent derivative (carboxy-H_2DCF) by intracellular esterases [28]. After the treatment of PMs with CM from LLC and LLC/R9 for 60 min, cells were washed with PBS and incubated with PBS containing 10 μM carboxy-H_2DCFDA for 20 min at 37 °C. The loading buffer was then removed, and a short recovery time was allowed for the cellular esterases to hydrolyze the acetoxymethyl ester or acetate groups and render the dye responsive to oxidation. Then, the cells were returned to prewarmed growth medium and incubated for 20 min at 37 °C. Cells were washed twice with PBS, trypsinized, and collected in 1 ml PBS. The cells were then analyzed by FACS (excitation: 488 nm, emission: 525 nm). Only living cells, gated according to scatter parameters, were used for the analysis. All experiments were performed in triplicates.

Dendritic cells protocol

Dendritic cell isolation was performed as described earlier [31] with slight modifications. Spleens were carefully excised from C57Bl/6 mice, sliced into small pieces, and single-cell suspension was prepared by passing through cell sieve. Erythrocytes were lysed by treatment with ammonium chloride. Remaining unfractionated cell populations were plated at a density of 5×10^5 cells/ml on plastic dishes in RPMI 1640 containing 10% FCS and supplemented with 200 mmol/L L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 mM 5-mercaptoethanol for 24 h at 37 °C in humidified atmosphere with 5% CO_2. Suspended and weakly adherent cells were then removed and fractioned on 14.5% metrizamide gradient. The interface cell fraction contained predominantly (more than 80%) dendritic cells (DC).

To determine DC phenotype, the expression of accessory molecules was analyzed by FACS. For this purpose, cells were washed and stained with PE-labelled anti-mouse CD54 and APC-labelled anti-mouse CD86 antibodies (PharMingen, USA).

DC tumor vaccine

For antigen pulsing DC were incubated for 4 h at 37 °C in the presence of 0.05 mg/ml of tumor cell lysate. Tumor cell lysate (LLC or LLC/R9) for DC pulsing was prepared in several steps. Single-cell suspensions were obtained by passing of tumor tissue pieces through cell sieve. These suspensions were frozen at − 20 °C in plastic dishes (layer thickness 5–8 mm). Frozen cell suspensions were then subjected to vacuum freeze drying (0.66 Pa) for 24 h in lyophilizer "MA-950" (Hungary). After this, freeze-dried suspensions were subjected to mechano-chemical activation with the use of vibro mill MMVE-0.005 ("Hephaestus", Russia) for 5 min and prepared for DC pulsing in Ringer's solution. The immunization procedure was as follows: antigen-pulsed DC were administered at a dose of 2×10^5 cells into the retro-orbital sine of tumor-bearing mice, in a volume of 50 µL, according to a protocol described by Maldonado-López et al. [32].

Tumor models

The studies were conducted on male C57Bl/6 mice (bred at animal facility of IEPOR NASU) weighting 18–22 g and aged 2–2.5 months, which were fed diets with normal protein contents. The investigation of the effect of dendritic cell (DC) loaded with tumor cell lysates (DC tumor vaccine) on growth and metastasizing of LLC and LLC/R9 was carried out using four groups of tumor-bearing mice ($n = 10$ animals per group) randomized by weight before cancer cell inoculation. Group 1—control animals bearing LLC; group 2—animals bearing LLC and treated with DC tumor vaccine; group 3—control animals bearing LLC/R9; group 4—animals bearing LLC/R9 and treated with DC tumor vaccine. Tumor cells were inoculated subcutaneously into the right hind paw (1×10^6 cells per animal in 0.1 ml of Hanks' solution) as described previously [25]. DC tumor vaccine was injected tree times at 3 day interval starting from 12th day after the tumor cell transplantation. The tumor growth was characterized by linear dimensions of primary tumor focus measured each third day with the use of callipers starting from the 5th day after tumor cell inoculation. At each time point, the characteristics of tumor growth were averaged for each group. Mean values and standard errors were calculated and were used for figure presentation.

Mice were sacrificed on the 25th day after cancer cell inoculation by exposure to a lethal dose of CO_2 followed by cervical dislocation. The number of metastatic nodules on the surface of five lung lobules of each mouse was determined using a binocular with dimension glass. The volume of primary tumor (V_i mm^3) at time point t_i and total volume of lung metastases (V_m mm^3) for each mouse were calculated using the following formulas:

$$V_i = \pi \times d_i^3 / 6$$

$$V_m = \frac{\pi}{6} \times \left(\sum_j n_j \times {d_m}^3(j) \right);$$

$$d_m(j) = 0.5 \times j$$

$$j = 1, \ldots 10$$

where d_i is the linear size (mm) of the tumor at timepoint t_i and n_j is the number of metastases with diameter $d_m(j)$ (mm).

Animal study protocols and operation procedures were approved by the Animal Ethics Committee of IEPOR NASU.

Splenectomy and sham laparotomy

Male C57Bl/6 mice were randomized by weight and divided into four groups ($n = 10$ animals per group): group 1—laparotomized animals bearing LLC; group 2—splenectomized animals bearing LLC; group 3—laparotomized animals bearing LLC/R9; group 4—splenectomized animals bearing LLC/R9. Mice were anesthetized by ketamine (25 mg/kg intraperitoneally) before the surgery. Splenectomy was performed via a lateral laparotomy incision. Sham laparotomy was performed in control animals (group 1 and 3). The spleen in control mice was carefully removed outside of the incision, remained extracorporeal for 3 min, and returned, and the laparotomy was closed. Non-opioid analgesics were injected intraperitoneally at the end of splenectomy and sham laparotomy to all mice. 14 days later LLC and LLC/R9 cells were inoculated subcutaneously into the right hind paw of animals from experimental and control groups. Tumor growth and metastasizing were evaluated as described above.

Statistics

All data are expressed as mean ± SD. The Student t test was used to determine the statistical significance of the difference. A $P < 0.05$ was considered significant.

Results

Evaluation of immunologically relevant genes in LLC/R9 cells

Using real-time RT-PCR, the expression of VEGF and seven other immunologically relevant genes was analyzed for cells of chemoresistant variant of LLC (LLC/R9) in comparison with wild-type LLC cells (TGFβ, IL10, MMP2, MMP9,

MT-MMP as well as transporters associated with antigen processing TAP1 and TAP2). To estimate the effect of in vivo growth of tumor cells on the expression of these genes, we used two different cancer cell populations in the experiments: cells that were culturing in vitro and cells that were isolated from primary tumor nodules in animal at days 10, 16, and 20 after the tumor cells transplantation. Distinct expression patterns of VEGF genes as well as other immunologically relevant genes, mentioned above, were revealed in chemoresistant variant LLC/R9 (Table 1). In spite of the fact that drug-resistant LLC/R9 cells are distinguished by significantly higher rate of VEGF secretion in comparison with wild-type cells [22, 25], the level of VEGF mRNA in these cells was only slightly increased as compared with LLC cells. Post-transcriptional regulation of VEGF expression (alternative splicing of primary transcript; mRNA instability in normoxic condition and its stabilization in hypoxic condition; miRNA-mediated regulation etc.) can be the reason of this phenomenon [33, 34]. Quite a different picture was observed in cells isolated from tumor tissue. Transition to in vivo growth was associated with sharp down-regulation of VEGF gene in wild-type cells; whereas the expression of the gene in chemoresistant cancer cells isolated from primary tumor nodules was increased in comparison with that in in vitro cultured cells and was higher than that in wild-type cells at all time points. In LLC/R9 cells that were grown in vitro, genes of MMPs were significantly down-regulated as compared to wild type of LLC cells: MT-MMP—by 1.9 times, MMP9—by 1.6 times, and MMP2 mRNA was totally inhibited. Throughout all stages of in vivo growth, the level of MMPs mRNA did not differ significantly in LLC/R9 cells. In wild-type LLC cells from tumor nodules, the level of MMPs mRNA was substantially lower than that in their culturing counterparts, but anyway was higher than that in chemoresistant cells from tumor nodules. Gene of immunoinhibitory IL10 in cultured in vitro LLC/R9 cells was totally inhibited in comparison with wild cells. Transition to in vivo growth was associated with substantial increase of IL10 mRNA level in drug-resistant cells. The level of mRNA of another immunoinhibitory cytokine TGFβ in in vitro cultured LLC/R9 cells was slightly higher than that in wild-type cells. Transition to in vivo growth caused moderate progressive decrease of TGFβ gene expression in drug-resistant cells. In contrast, in wild-type cells down-regulation of TGFβ expression on the initial stage of in vivo growth was followed by substantial up-regulation on late stage.

Expression of TAP genes in chemoresistant cells growing in vitro did not differ from that in wild-type cells. Transition to in vivo growth was accompanied by the increase of the level of TAPs mRNA in LLC/R9 cells at the initial stage of tumor focus forming (day 10 after tumor cell transplantation). Follow-up stages of in vivo growth were characterized by progressive decrease of TAP mRNA expression in drug-resistant cells.

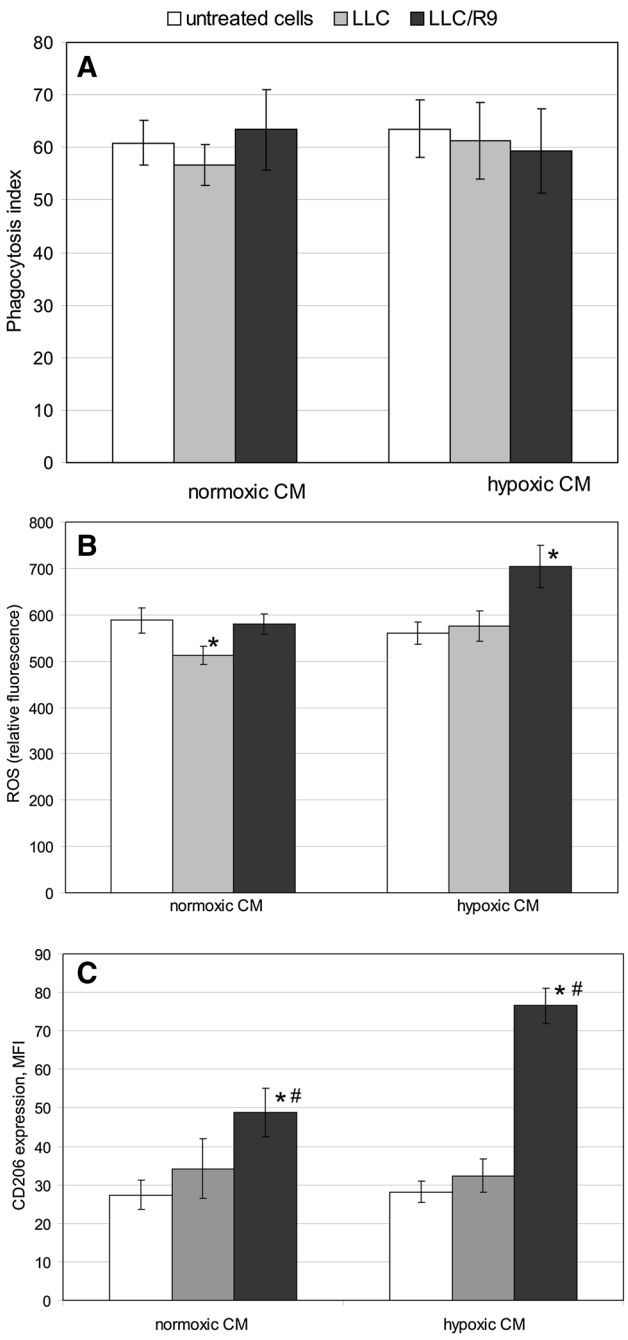

Fig. 1 The effect of conditioned media from LLC/R9 cells cultured in normoxic and hypoxic conditions on phagocytic activity (**a**), ROS generation (**b**) and CD206 expression (**c**) of non-sensitized peritoneal macrophages. Phagocyte endocytosis and oxidative metabolism were analyzed by flow cytometry. Data are presented as means ± SD as average of mean of triplicate in three independent experiments. $^{*}P < 0.05$ compared to untreated phagocytes, $^{\#}P < 0.05$ compared to phagocytes, treated with CM from hypoxic LLC cells

Effects of conditioned media from normoxic and hypoxic LLC/R9 cells on non-sensitized macrophage functional profile

The effects of CM from LLC and LLC-R9 cells cultured in vitro in normoxic conditions on the phagocytic activity, oxidative metabolism and nonopsonic scavenger receptor CD206 expression of non-sensitized PMs from normal adult C57Bl/6 were investigated. FITC-labelled *S. aureus* Cowan was used as a phagocytosis object. CM from tumor cells didn't influence macrophage phagocytosis (Fig. 1a). CM from normoxic chemoresistant tumor cells had no effect on peritoneal phagocyte ROS-generation. Whereas treatment with CM from normoxic wild-type LLC cells slightly suppressed macrophage oxidative metabolism (Fig. 1b). In contrast, CM from hypoxic LLC/R9 cells moderately stimulated phagocyte oxidative metabolism, whereas CM from hypoxic wild cells did not affect phagocyte ROS generation (Fig. 1b). In addition, CM from hypoxic chemoresistant tumor cells up-regulated CD206 expression in non-sensitized phagocytes (Fig. 1c).

Susceptibility of LLC/R9 cells to contact non-sensitized macrophage-mediated cytotoxicity

The susceptibility of LLC/R9 cells to non-sensitized PMs from normal adult C57Bl/6 mice at an *E:T* ratio of 40:1 and 20:1 was analyzed after the co-culturing for 18 h, and was compared to that of wild-type LLC. Sensitivity of LLC/R9 cells to macrophage contact tumoricidal activity depended on *E:T* ratio, and was higher at an *E:T* ratio of 40:1 (Fig. 2a). At this *E:T* ratio, sensitivity of LLC/R9 cells to macrophage-mediated cytolysis was lower than that in wild-type cells. At *E:T* ratio of 20:1 sensitivity of LLC/R9 was also significantly lower than that in LLC.

Susceptibility of LLC/R9 cells to TNFα-mediated apoptosis

To examine the susceptibility to TNFα-mediated apoptosis, LLC/R9 cells, as well as wild-type LLC cells as a control, were treated with TNFα at the growing concentrations for 24 and 48 h. Flow cytometry analysis revealed that chemoresistant LLC/R9 cells were slightly less susceptible to TNFα-mediated apoptosis as compared with wild-type cells only after 24 h exposition (Fig. 2b). After more prolonged treatment (Fig. 2c), both cell variants had equal sensitivity to TNFα action. It is necessary to note that LLC/R9 cells produced higher level of apoptosis than wild-type cells when were treated with TNFα at the highest concentration regardless of the exposure time.

Susceptibility of LLC/R9 cells to natural killer cell-mediated cytotoxicity

LLC/R9 cells were tested in 4 h MTT-colorimetric assay using nucleated spleen cells from normal adult C57Bl/6 mice as effectors in *E:T* ratios 40:1, 20:1 and 10:1. Wild-type cells LLC and Yac-1 cells were used as a control. Sensitivity of all cancer cells to splenic natural killer cell-mediated cytotoxicity depended on *E:T* ratio and was the highest at a *E:T* ration of 40:1 (Fig. 3a). Susceptibility of LLC/R9 cells to natural killer-mediated cytotoxic effect was higher than that in Yac-1 cells and in wild-type LLC cells at all *E:T* ratio.

High susceptibility of LLC/R9 cells to natural killer cell-mediated cytotoxicity was confirmed in in vivo experiments with the removal of spleen—one of the main sources of natural killer cells, especially in tumor-bearing mice. In these experiments, mice were splenectomized 14 days before the tumor cells inoculation. Animals from control group were laparotomized without splenectomy. Splenectomy did not affect the average volume of primary tumor focus in both LLC- and LLC/R9-bearing mice (data are not presented). Meanwhile metastasizing of LLC/R9 was substantially stimulated in splenectomized animals (Fig. 3b, c). The number of metastatic foci in the lungs of splenectomized mice with chemoresistant tumor was 2.5 times higher than in their laparotomized counterparts (Fig. 3b), and the volume of metastatic lesion was also 1.7 times higher (Fig. 3c). Splenectomy in mice bearing wild-type LLC tumor didn't affect metastasis rate.

Effect of LLC/R9 cells on dendritic cell properties

To evaluate the effect of LLC/R9 cells on DC properties two different experiments were performed. In the first experiment, immature DCs generated from murine spleen were loaded with LLC/R9 cell lysates, following which the expression of accessory molecules CD54 and CD86 as a phenotypic markers of DC maturity were examined. In addition, it was investigated whether pulsing of DC with LLC/R9 cell lysate changes their ability to phagocytize antigen (fluorescein-labelled bacterial cells). Generated from spleen, non-adherent cells developed with dendritic morphology and exhibited a relatively mature phenotype with the expression of CD86 and CD54, that consistent with reports of other research groups [35, 36]. Tumor antigen (both LLC and LLC/R9) pulsing strongly increased the frequency of CD86 + DC from 7.5% in control probes to 33% (Table 2). The loading of DC with cell lysate of both LLC and LLC/R9 was also associated with moderate increase of the frequency of CD54+ cells. Cell-lysate pulsed DC had decreased an uptake of antigen, compared with control cells (by 2.7 times for LLC cell lysate and by 1.8 times for LLC/R9 cell lysate).

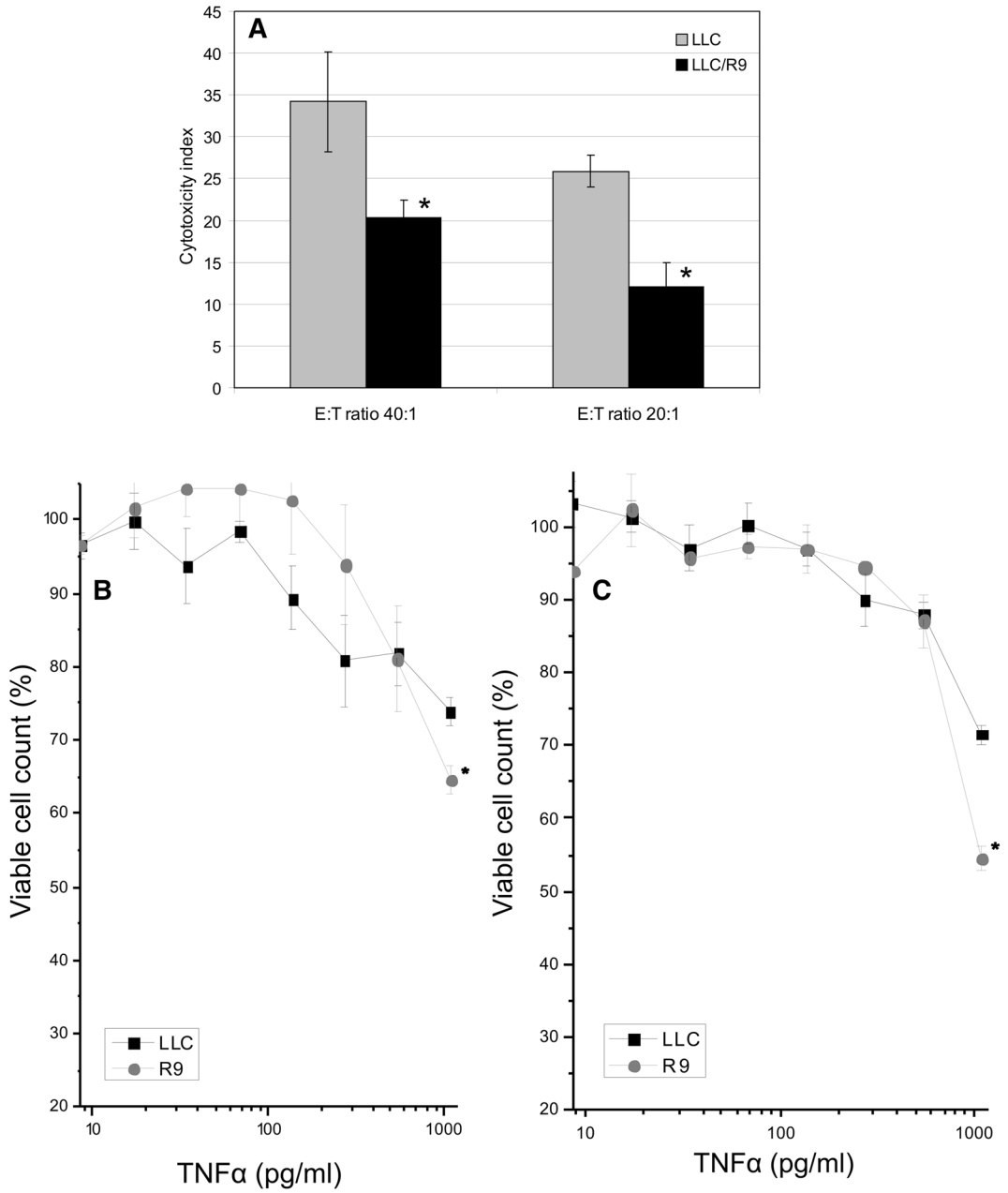

Fig. 2 The susceptibility of LLC/R9 cells to contact macrophage tumoricidal activity (**a**) and TNF-mediated apoptosis (**b, c**). **a** Chemoresistant LLC/R9 cells were incubated with non-sensitized peritoneal macrophages from C57Bl/6 for 18 h, after which cell viability was analyzed by MTT assay. LLC cells were used as a controls. Data shown are means ± SD as average of mean of triplicate in four independent experiments. *$P < 0.05$ compared to LLC cell line. **b, c** Chemoresistant LLC/R9 cells were treated with TNFα for 24 h (**b**) and 48 h (**c**), and analyzed by flow cytometry after propidium iodide staining. LLC cells were used as a control. Data shown are means ± SD as average of mean of triplicate in three independent experiments. *$P < 0.05$ compared to LLC cell line

In the second experiment, the effect of generated ex vivo and loaded with tumor cell lysates DC on growth and metastasizing of LLC and LLC/R9 in vivo was investigated. For this purpose prepared DC vaccines were administered in the retro-orbital sine of tumor-bearing mice three times starting from the 12th day after the tumor cell inoculation. As shown in Fig. 4, treatment of LLC-bearing mice with DC vaccine resulted in the inhibition of the growth of primary tumor focus (tumor volume at the time of the experiment cessation was 1.43 ± 0.2; $P < 0.05$ as compared to untreated animals 2.19 ± 0.09) (Fig. 4a) and in a reduction in the number and total volume of lung metastatic lesions (by 3.4 times as compared to untreated mice) (Fig. 4b, c).

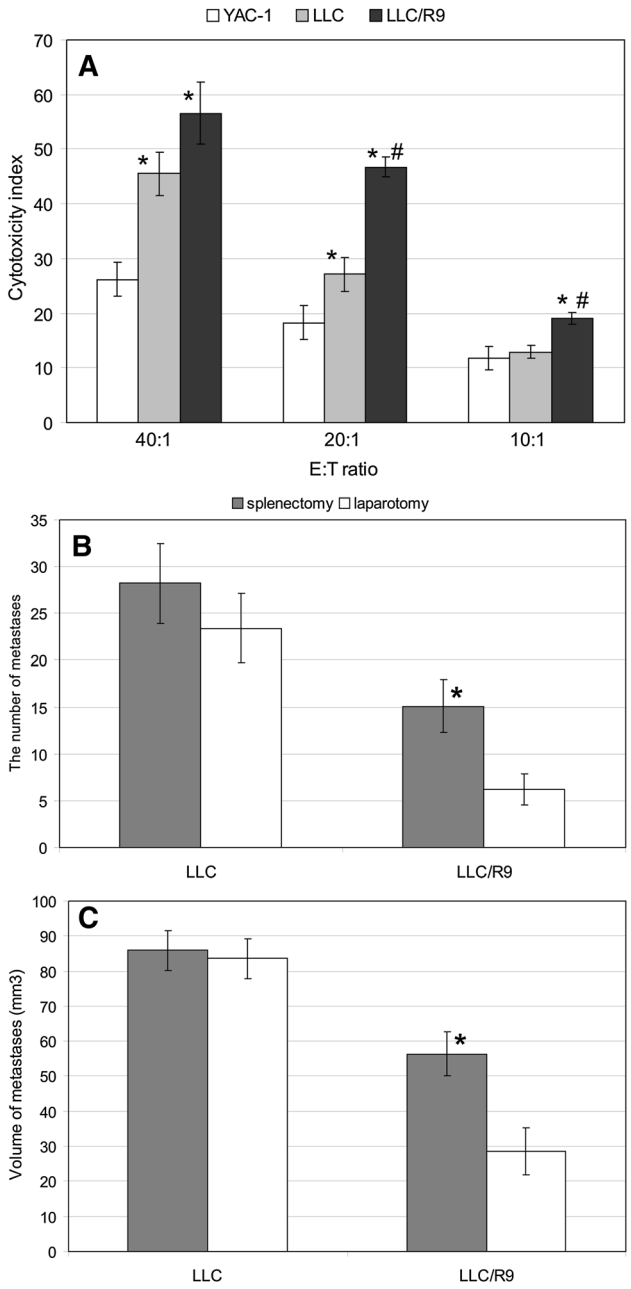

Fig. 3 The susceptibility of LLC/R9 cells to splenic mononuclear cell-mediated cytotoxicity. **a** Chemoresistant LLC/R9 cells were incubated with splenic mononuclear cells from C57Bl/6 for 4 h in vitro, after which cell viability was analyzed by MTT assay. YAC-1 and LLC cells were used as a controls. Data shown are means ± SD as average of mean of triplicate in four independent experiments. $^{*}P < 0.05$ compared to YAC-1 cell line; $^{\#}P < 0.05$ compared to LLC cell line. Mice were splenectomized 14 days before the tumor cells inoculation. Animals from control group were laparotomized without splenectomy ($n = 10$ in each group). The number (**b**) of metastatic nodules and their volume (**c**) on the surface of five lung lobules of each mouse were determined on the 25th day after tumor cell inoculation. LLC-bearing mice were used as a control. Data are presented as means ± SD. $^{*}P < 0.05$ compared to laparotomized mice of corresponding group

Treatment of LLC/R9-bearing mice with DC vaccine resulted in contrasting effects on tumor growth and metastasizing. As shown in Fig. 4, treatment with DC pulsed with LLC/R9 lysates didn't affect significantly the growth of primary tumor focus and dramatically enhanced the number and total volume of tumor nodules in the lungs of animals.

Discussion

Conventional anticancer therapeutics can increase tumor susceptibility to the attack of the host immune system by inducing of immunogenic tumor cell death. However, development of the resistance of tumor cells to these preparations can induce multiple changes in their immunobiological properties, which may enable tumor to escape immune surveillance and cause disease progression [7, 37]. The detection of immunological hallmarks of resistant tumor cells expands our knowledge of tumor biology and may help to develop more effective combined therapeutic approaches targeted towards drug-resistant tumors. So, the objective of our work was to investigate immunological features of *cis*-DDP-resistant LLC cells, primarily concerning their interactions with myeloid cells. Numerous experimental studies evidence that polarized myeloid cells can modulate key cancer-associated activities, including evasion of immune surveillance, and can affect virtually all types of cancer chemotherapy [16, 26, 38, 39]. Immunomodulatory effect of tumor cells is achieved through several mechanisms, including both contact interaction with immunocytes and indirect action through the production of bioactive substances. Development of the chemoresistance is accompanied by drastic genetic and metabolic changes in tumor cells, that are closely related to the acquisition of new immunomodulatory properties [40, 41]. The expression of some immunologically relevant genes, alterations of which are often associated with the drug resistance acquisition, in *cis*-DDP-resistant LLC/R9 cells grown in vitro and in vivo was evaluated: immunosuppressive and pro-angiogenic cytokines and matrix metalloproteinases, as well as transporters associated with antigen processing 1 and 2. Tumor-derived TGFβ and IL10 are known to promote macrophage and DC pro-tumoral alternative polarization. At the same time, IL10 can increase NK-cell cytotoxicity and migration [42]. VEGF inhibits the activation of the transcription factor NFκB, and thereby prevents DC maturation. It also favors the accumulation of myeloid-derived suppressor cells, tumor-infiltrating macrophages with M2 phenotype in tumor growth area [43, 44]. MMPs are important players in local tumor growth and metastasizing. A growing number of findings also indicates powerful immunomodulatory effects of these enzymes [45, 46]. When growing in vitro LLC/R9 were characterized by slightly increased immunosuppressive

Table 2 The effect of the pulsing with LLC/R9 cell lysate on the maturation of dendritic cells generated from murine spleen

	Percent CD86+	Percent CD54+	Phagocytosis, %
Unpulsed DC	7.5 ± 2.4	57.0 ± 2.0	27.0 ± 2.4
DC pulsed with LLC cell lysates	33.0 ± 5.0*	82.0 ± 5.0*	10.0 ± 1.3*
DC pulsed with LLC/R9 lysates	33.0 ± 7.0*	84.5 ± 9.5*	15.0 ± 2.0*

DC were isolated from murine spleen and loaded with tumor cell lysates as described in "Materials and methods", and expression of surface molecules as well as phagocytosis were analyzed by flow cytometry. Results are shown as mean ± SD ($n = 8$) and are representative of three separate experiments

*$P < 0.05$ compared to unpulsed cells

cytokine TGFβ and VEGF mRNA levels as compared to wild-type cells. mRNA of another immunosuppressive cytokine IL10 in these cells was un-detectable. The level of MMP mRNA in cultured in vitro drug-resistant cells was substantially decreased in comparison with wild-type cells. This is a typical feature of low-metastasizing tumors [47]. The transition to in vivo growth was associated with a moderate decrease in the number of copies of TGFβ mRNA in the chemoresistant cells. TGFβ is involved in the regulation of tumor growth and metastasis. Novitsky et al. [48] demonstrated a negative correlation between TGFβ signaling and VEGF gene expression, as well as between TGFβ signaling and metastatic potential of tumor cells. Such correlation may serve as one of explanations of increased VEGF expression along with decreased metastatic potential in chemoresistant LLC/R9 cells in comparison with wild-type cells. The transition to in vivo growth was also accompanied by the appearance of IL10 mRNA and by the increase in the number of VEGF mRNA copies at the early stage (10 days after the tumor cell transplantation) in chemoresistant cells. It evidences stimulatory effect of tumor microenvironment on tumor cell transcriptome.

Revealed changes in drug-resistant tumor cell transcriptome was associated with the alteration in their secretome that was demonstrated by the results of comparative investigation of the effect of CM from LLC/R9 and LLC cells on non-sensitized phagocyte functions. Taking into account that tumor cell transcriptome and secretome are significantly activated by hypoxia [49], we have used CM from normoxic and hypoxic LLC and LLC/R9 cells. CM from LLC/R9 cells cultured in normoxic conditions didn't affect macrophage functions, whereas CM from hypoxic LLC/R9 cells up-regulated ROS generation as well as expression of one of the phenotypic markers of alternative phagocyte polarization—CD206—by these cells. It indicates the ability of drug-resistant cells to shift macrophage metabolic profile to M2 phenotype and to promote tumor-associated macrophages occurrence [50]. It is well documented, that tumor-associated hypoxia indirectly influences phagocyte functional polarization by stimulating cytokine secretion by tumor cells. Cytokine milieu of hypoxic tumor cells is known to stimulate ROS generation and macrophage M2 polarization [51–53]. In addition to polarizing effect on macrophage metabolism, drug-resistant LLC/R9 cells were characterized by lowered sensitivity to contact-dependent macrophage-mediated cytotoxicity. Macrophages also have capacity to destroy tumor cells through the secretion of TNFα. Our in vitro experiments revealed slightly decreased sensitivity of LLC/R9 cells to TNFα-mediated apoptosis. Taken together these results suggest that alteration of macrophage functions can be considered as one of the possible mechanisms responsible for the increased local growth rate of drug-resistant LLC/R9 cells.

Acquisition of drug resistance by LLC/R9 cells was associated with their increased sensitivity to NK-cell-mediated cytolysis. It is one of the facts that explains low metastatic potential of drug-resistant cells. Recently, Arsenijevic et al. [54] have shown that decreased expansion of malignant tissue in the lungs of LLC1-bearing animals treated with platinum-based drug was associated with sharp increase of the fraction of splenic NK cells. In our experiments, the removal of the spleen—main source of NK cells—was accompanied by the increased metastasizing in LLC/R9-bearing animals. One of the reasons of increased NK sensitivity of LLC/R9 cells can be progressive down-regulation of TAP mRNA expression in the course of in vivo growth along with constitutive IL10 mRNA expression. IL10 is known to inhibit MHC I expression and negatively effect TAP functions. TAP deficiency sensitizes cells to NK-mediated immune attack [55, 56]. In addition, Xu et al. [57] reported that some chemotherapeutics can up-regulate the surface protein expression of the NKG2DL and MICA in tumor cells, sensitizing them to NK-cell killing.

NK-cell functions substantially depend on the cooperation with DCs. The ability of mature myeloid DCs to enhance NK-cell proliferation, interferon secretion and cytotoxic activity is widely described [58]. Nowadays, the interaction between DC and NK cells is considered as a complex network of cell subset cooperation [59], and the consequences of the crosstalk between DCs and NK cells from different subpopulations in vivo have not been extensively investigated. In our experiment, DCs generated ex vivo and loaded with both LLC and LLC/R9 cell lysates

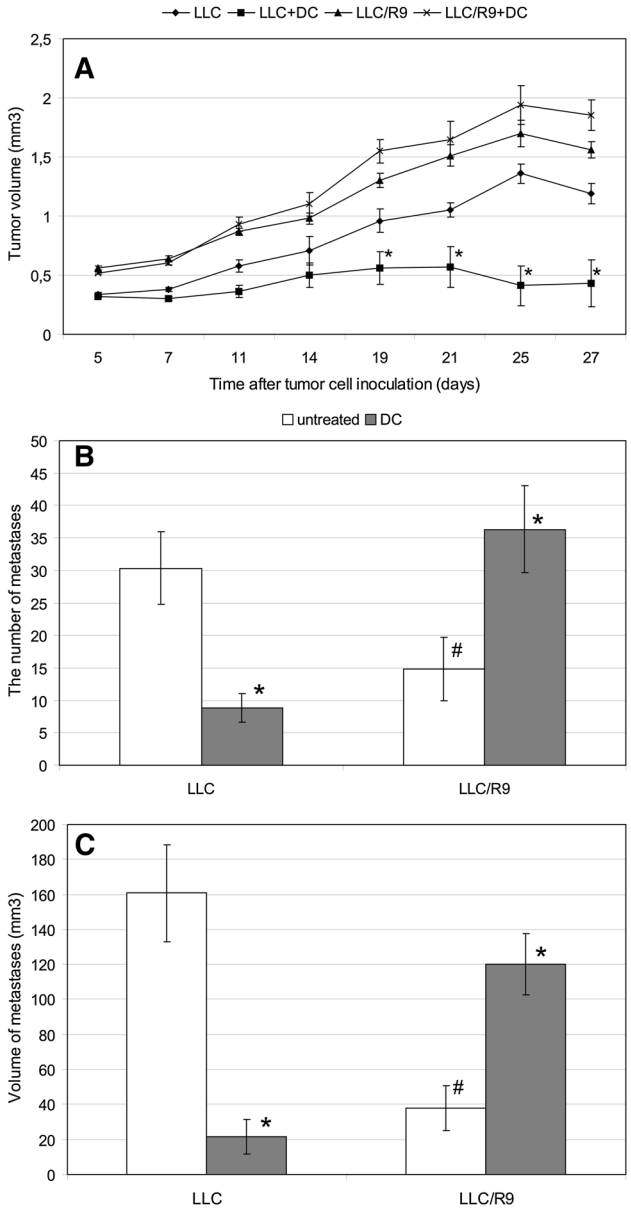

Fig. 4 The effect of DC pulsed with LLC/R9 cell lysates on the tumor growth (**a**) and metastasizing (**b, c**) in vivo. Antigen-pulsed DC were administered in the retro-orbital sine tree times at 3 days interval starting from the 12th day after tumor cell inoculation. Tumor growth and metastasizing were evaluated as described in "Material and methods". Results are shown as mean ± SD ($n = 10$) and are representative of three separate experiments. $^{*}P < 0.05$ compared to untreated animals of corresponding group; $^{\#}P < 0.05$ compared to untreated LLC-bearing animals

notably enhanced surface expression of the costimulatory molecules and decreased phagocytic activity that indicates mature state of these cells. Meanwhile, DCs loaded with drug-resistant cell lysates sharply enhanced metastasizing in tumor-bearing mice, whereas DCs loaded with wide-type cell lysates significantly decreased local tumor growth and metastasizing in treated animals. There is an opinion that DC vaccines function primarily only as vehicles for transferring antigens to endogenous antigen presenting cells, which are responsible for the activation of T cells and NK cells for antitumor immunity [60]. If so, enhanced metastasizing in LLC/R9-bearing mice after the treatment with syngeneic DC vaccine can be the consequence of antigenic stimulation of polarized by tumor-derived immunosuppressive cytokines endogenous DCs followed by the tolerogenic shift in the NK-cell functions. However, this assumption, as well as revealed phenomenon, warrant further study.

Taken together, our data demonstrate that the acquisition of resistance to *cis*-DDP by LLC/R9 cells resulted in the alteration of their immunological hallmarks: decrease the sensitivity to macrophage cytolysis and increase the susceptibility to NK-cell cytotoxic action. These immunobiological properties promote intensive local growth of chemoresistant tumors in vivo, but hamper their metastasizing. If so, therapeutic approaches targeting macrophage re-education along with the amplification of NK-cell functions could be perspective in the treatment of *cis*-DDP-resistant tumors, whereas the use of DC vaccination requires thorough investigation.

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Compliance with ethical standards

Conflict of interest The authors state that they have no conflict of interest.

Ethical approval Animal protocol was reviewed and approved by the IEPOR NASU and Taras Shevchenko National University animal welfare committee according to the Animal Welfare Act guidelines. Study was conducted in compliance with the standards of the Convention on Bioethics of the Council of Europe 'Europe Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes' (1997), the general ethical principles of animal experiments, approved by the First National Congress on Bioethics in Ukraine (September 2001) and national law (LAW OF UKRAINE no. 3447-IV) issued by the Cabinet of Ministers of Ukraine (2006).

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