ORIGINAL CONTRIBUTION

EXPERIMENTAL ONCOLOGY

https://doi.org/10.15407/exp-oncology.2025.03.332

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NON-PROFESSIONAL PHAGOCYTOSIS OF LEWIS LUNG CARCINOMA CELLS UNDER DIFFERENT GROWTH CONDITIONS

Background. Phagocytosis occurs in almost all cell types of multicellular organisms. Based on their efficiency, cells are classified as professional or non-professional phagocytes, with cancer cells belonging to the latter. This property of cancer cells underlies the formation of "cell-in-cell" structures, the high frequency of which is often associated with invasion and metastasis of malignant tumors. **Aim.** To investigate the ability of Lewis lung carcinoma (LLC) cells to perform non-professional phagocytosis and to analyze how this process depends on cancer cell growth conditions. **Materials and Methods.** A low-metastatic variant of LLC cells (LLC/R9) was used. Phagocytic activity was examined under anchorage-dependent and anchorage-independent growth conditions, in both standard and glucose-free culture media, using fluorescent latex beads (1.0 μm in diameter). **Results.** LLC/R9 cells demonstrated phagocytic activity, which increased nearly fourfold under anchorage-independent conditions, irrespective of E-cadherin expression. Glucose deprivation reduced the percentage of bead-engulfing cells by more than twofold under both growth conditions, while increasing the number of beads internalized per cell. This indicates a pronounced heterogeneity within the cancer cell population in **their** sensitivity to phagocytic activation under glucose deficiency. **Conclusions.** Non-small cell lung cancer LLC/R9 cells are capable of phagocytosis, which is markedly enhanced under anchorage-independent growth and only weakly influenced by glucose deprivation.

Keywords: non-professional phagocytosis, Lewis lung carcinoma cells, anchorage-independent growth, glucose deprivation.

Phagocytosis is defined as the uptake of particles larger than 0.5 μm in diameter. It is the primary feeding mechanism of unicellular organisms, but is also found in nearly all cell types of multicellular organisms [1]. Only a specialized group of cells that perform phagocytosis with high efficiency are termed professional phagocytes, including macrophages, neutrophils, monocytes, dendritic cells, and osteoclasts. Other cells, such as fibroblasts, epithelial cells, endothelial cells, and cancer cells, can also perform phagocytosis, albeit far less efficiently, and are classified as non-professional phagocytes [2].

During phagocytosis, cells engulf microorganisms, large particles, cellular debris, or even intact viable cells. In recent years, the ability of non-professional phagocytes, particularly cancer cells, to internalize other intact cells has been referred to as the cell-in-cell phenomenon. "Cell-in-cell"

Citation: Kolesnik D, Lykhova O, Stepanov Y, Solyanik G. Non-professional phagocytosis of Lewis lung carcinoma cells under different growth conditions. *Exp Oncol.* 2025; 47(3): 332-337. https://doi.org/10.15407/exp-oncology.2025.03.332

structures were first described in malignant tumors more than a century ago, but detailed studies of the underlying mechanisms have intensified only over the past two to three decades [3, 4]. This process is typically categorized into three main types: emperipolesis, entosis, and cell cannibalism. These phenomena are closely related, with subtle distinctions that remain to be fully elucidated. Non-professional phagocytosis in cancer cells may contribute to cancer progression by providing nutrients, enabling immune evasion, and increasing genetic instability. Conversely, it may suppress tumor growth through mutual engulfment, leading to the death of internalized cells.

The occurrence of "cell-in-cell" structures is considered a promising prognostic marker across different cancers [5—7]. Most studies have focused on breast cancer cells, which exhibit the highest frequency of such structures among the investigated tumor types [8]. In contrast, the ability of non-small cell lung cancer (NSCLC) cells to perform non-professional phagocytosis remains poorly studied, and the role of "cell-in-cell" structures in NSCLC progression is unclear. Nonetheless, recent findings suggest that their frequency has independent prognostic significance for the overall and progression-free survival in NSCLC patients [9, 10]. Moreover, a high frequency of these structures at the invasive tumor front indicates a strong association between the cell-in-cell phenomenon and processes of invasion and metastasis.

Therefore, the aim of this study was to determine the ability of Lewis lung carcinoma (LLC) cells (histologically classified as NSCLC) to perform non-professional phagocytosis and to analyze how this process is influenced by growth conditions. Specifically, we assessed phagocytic activity under anchorage-dependent (adhesive cells) and anchorage-independent (deadhesive cells) growth, as well as under glucose deprivation.

Materials and Methods

Cell lines. A low-metastatic variant of Lewis lung carcinoma cells (LLC/R9), derived from the parental LLC strain during the development of cisplatin resistance [11], was used in this study. The cells were maintained in vitro at 37 °C in RPMI-1640 medium (Biowest, France) supplemented with 10% fetal bovine serum (Biowest, France), 40 μg/mL gentamicin, and 2 mM L-glutamine in a humidified atmosphere containing 5% CO_2.

Non-professional phagocytosis assay. Non-professional phagocytosis of LLC/R9 cells was evaluated under both anchorage-dependent and anchorage-independent growth conditions, in either standard culture medium or glucose-free medium. Fluorescent latex beads (1.0 μm diameter; Sigma, USA) were used for the assay [12]. The activity was assessed by determining (i) the percentage of cells that internalized fluorescent beads and (ii) the fluorescence intensity of bead-containing cells using a FACSCalibur flow cytometer (Becton Dickinson, USA) equipped with a 488 nm argon laser and a 614/20 nm filter. A minimum of 10,000 events per sample were analyzed.

Experimental design. To assess the impact of deadhesive growth on non-professional phagocytosis, cells were seeded either in standard 60 mm Petri dishes (anchorage-dependent growth) or in Petri dishes coated with polyHEMA (Sigma, USA) (anchorage-independent growth) in standard RPMI-1640 cell culture medium. After 24 h of incubation, the number of viable cells was determined under both adhesive and deadhesive conditions. Fluorescent beads were then added at a ratio of 100 beads per viable cell, and incubation continued for an additional 24 h (Fig. 1). Cells were subsequently har-

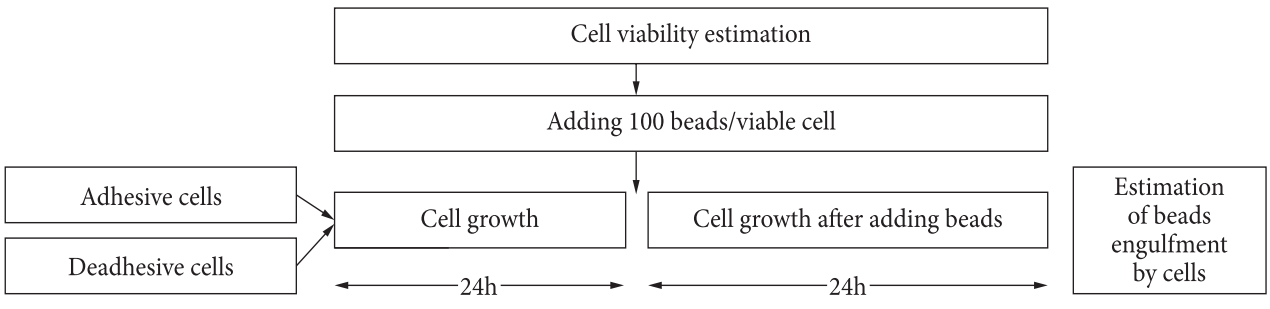

Fig. 1. Experimental design for studying non-professional phagocytosis of LLC/R9 cells under adhesive and deadhesive growth conditions

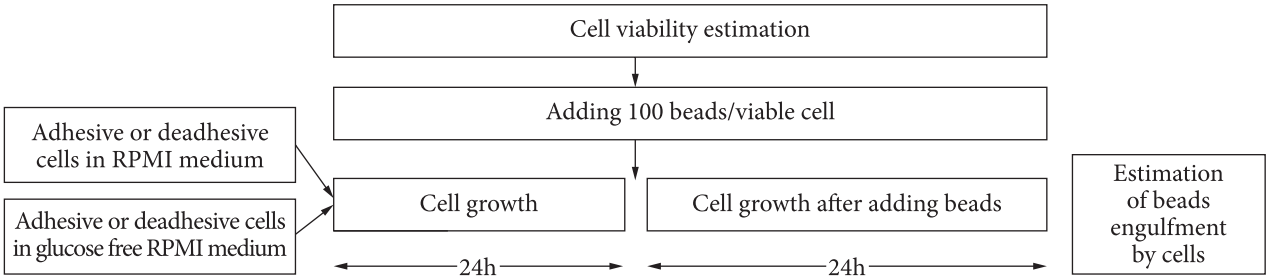

Fig. 2. Experimental design for studying the effect of glucose deprivation on non-professional phagocytosis of LLC/R9 cells under adhesive and deadhesive growth conditions

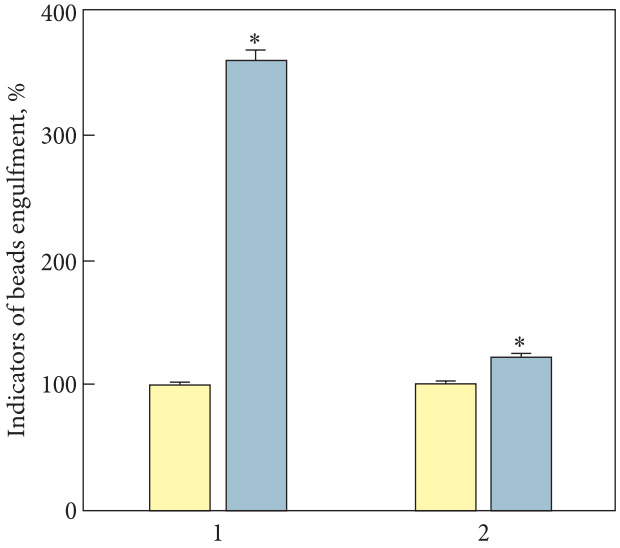

Fig. 3. Percentage of LLC/R9 cells that engulfed beads (1) and the number of beads (by fluorescence intensity) engulfed per cell (2) under adhesive (yellow columns) and deadhesive (blue columns) growth conditions. Phagocytic activity of adhesive LLC/R9 cells was taken as 100%. * $p < 0.05$

vested, washed twice with phosphate-buffered saline (PBS), and analyzed by flow cytometry.

The experimental design for evaluating the effect of glucose deprivation was identical, except that cells were cultured either in standard Petri dishes (anchorage-dependent growth) or in polyHEMA-coated dishes (anchorage-independent growth) (Fig. 2). Cells were seeded under the respective conditions and incubated for 24 h either in standard medium or in glucose-free RPMI 1640 medium (Biowest, France). After determining the number of viable cells, fluorescent beads were added at a ratio of 100 beads per viable cell, followed by a further 24 h incubation. The cells were then harvested, washed twice with PBS, and analyzed by flow cytometry.

E-cadherin expression analysis. Quantitative assessment of E-cadherin expression in malignant cells under adhesive and deadhesive conditions was performed using anti-E-cadherin antibodies (Invitrogen, USA). Cells were fixed and permeabilized in 0.5 mL of 0.1% Triton X-100 in PBS at RT for 30 min, followed by washing. After centrifugation at 1500 rpm for 10 min, 100 µL of 10% fetal calf serum in PBS containing anti-E-cadherin antibodies (1:150 dilution) was added to the cell pellet, and staining was performed at RT for 30 min. The stained cells were analyzed using a Navios EX flow cytometer (Beckman Coulter, USA). E-cadherin expression was detected using a 488 nm blue laser in the FITC channel (525/40 nm BP filter). Data were processed with Navios EX Tetra software.

Statistical analysis. Statistical analysis included descriptive statistics, Student's *t*-test, and the Mann–Whitney *U*-test. Data are presented as M ± SE, where M is the mean and SE is the standard error.

Results and Discussion

Until recently, the main trigger of the cell-in-cell phenomenon was considered to be the loss of attachment to the extracellular matrix [13]. However, more recent studies have shown that this phenomenon may also occur among adherent epithelial cells, induced either by mitosis or by glucose starvation [14]. Therefore, we investigated the intensity of non-professional phagocytosis in metastatic LLC/R9 cells under anchorage-independent growth conditions (in comparison with anchorage-dependent growth) and assessed the effect of glucose deprivation on this process.

Non-professional phagocytosis of LLC/R9 cells under anchorage-dependent and anchorage-independent growth. Our experiments showed that 2.34 ± 0.07% of adhesive cells exhibited a non-professional phagocytic activity. Under deadhesive growth conditions, the proportion of such cells increased significantly, nearly fourfold (Fig. 3). The number of beads engulfed per cell (estimated by fluorescence intensity) was also significantly higher

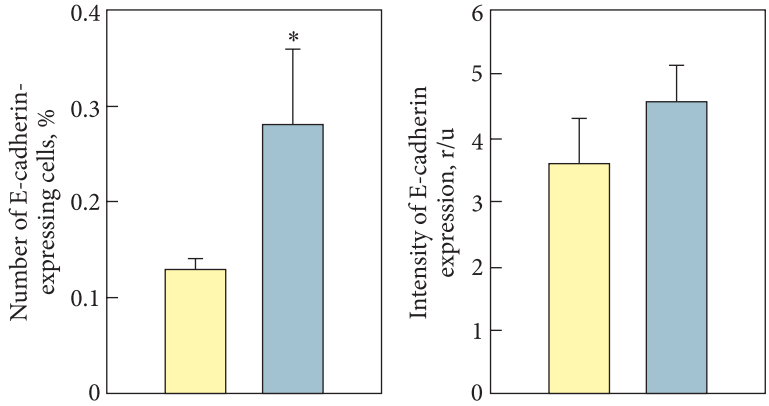

Fig. 4. Percentage of E-cadherin–expressing LLC/R9 cells and intensity of E-cadherin expression under adhesive (yellow columns) and deadhesive (blue columns) growth conditions . * $p < 0.05$

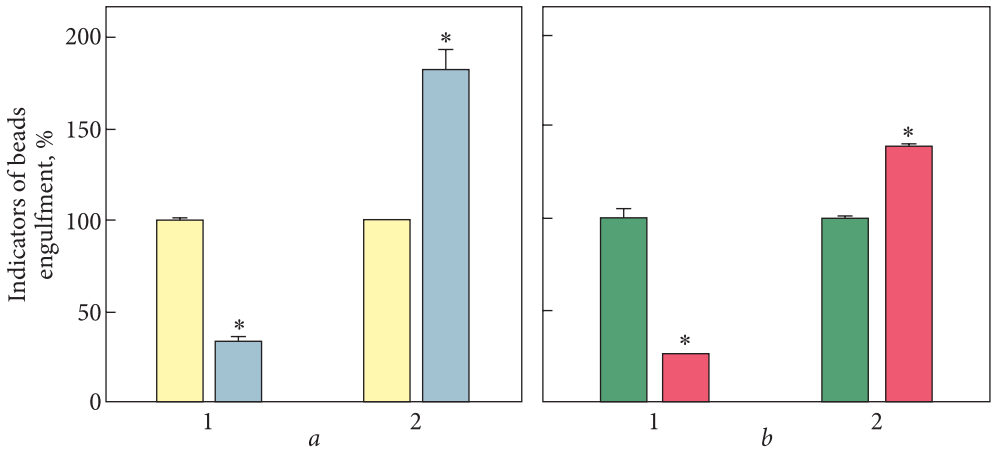

Fig. 5. Percentage of LLC/R9 cells that engulfed beads (1) and the number of beads (by fluorescence intensity) engulfed per cell (2) under adhesive growth (*a*) and deadhesive growth (*b*). Phagocytic activity of adhesive cells (*a*) in a glucose-rich medium (yellow columns) and in a glucose-free medium (blue columns). Phagocytic activity of deadhesive cells (*b*) in a glucose-rich medium (green columns) and in a glucose-free medium (red columns). The indicators of bead engulfment by LLC/R9 cells in a glucose-rich medium are taken as 100%. * $p < 0.05$

under deadhesive conditions compared to adhesive ones, but only by 23% ($p < 0.005$). Since the initial bead-to-cell ratio was identical in both conditions, and the proliferation rate of deadhesive LLC/R9 cells was not higher than that of adhesive cells, these findings indicate that deadhesive cells are able to engulf substantially more beads per cell compared with adhesive cells.

It is generally believed that cell adhesion plays an important role in phagocytosis. Some studies have demonstrated a correlation between the expression level of the adhesion protein E-cadherin and the frequency of the cell-in-cell phenomenon, although this correlation depends on the cell type and growth conditions. For example, in breast cancer cells, the expression of the epithelial E- or P-cadherins was sufficient to activate cell cannibalism under both adhesive and deadhesive conditions [15]. In contrast, in melanoma cells, although both adhesive and suspension cells were capable of forming cell-in-cell structures, the correlation between E-cadherin expression and the rate of cell-in-cell formation was observed only under adherent conditions and was absent during deadhesive growth [16].

It is well known that metastasis is often accompanied by loss of E-cadherin expression due to epithelial–mesenchymal transition (EMT) [17]. However, several studies have shown that many metastases continue to express E-cadherin, suggesting an ambiguous, and possibly even positive, role of this protein in the metastatic process [18—20].

In our study, we used highly metastatic NSCLC cells. We found that E-cadherin expression in LLC/R9 cells was extremely low (against the background of high vimentin expression) and showed no correlation with their ability to perform non-professional phagocytosis. As shown in Fig. 4, only 0.1% of adhesive LLC/R9 cells expressed E-cadherin, which was 20 times lower than the proportion of

bead-engulfing cells, and the overall intensity of E-cadherin expression was also very low.

Although a tendency toward increased E-cadherin expression was observed under deadhesive conditions, the proportion of E-cadherin–positive cells remained very low and did not correlate with non-professional phagocytosis events.

Effect of glucose deprivation on non-professional phagocytosis of LLC/R9 cells under anchorage-dependent and anchorage-independent growth conditions. For a long time, it was thought that the only trigger of non-professional phagocytosis in cancer cells was the loss of attachment to the extracellular matrix. This distinguished them from professional phagocytes, in which glucose starvation enhances phagocytic activity [21]. However, numerous studies of entosis and cannibalism have demonstrated that glucose starvation can activate these processes in adhesive epithelial cells [14, 22]. This suggests that certain intrinsic features of cancer cells — anchorage-independence, aberrant proliferation, and metabolic stress — may promote the induction of cell cannibalism, a phenomenon frequently observed in tumors.

In our study, glucose deprivation of adhesive LLC/R9 cells led to a significant 65% decrease ($p < 0.001$) in the proportion of bead-engulfing cells. At the same time, bead uptake intensity increased by nearly 80% ($p < 0.0001$) (Fig. 5, *a*). This points to pronounced heterogeneity within the LLC/R9 population regarding their sensitivity to phagocytic activation under glucose deficiency: only about 0.5% of the cells showed marked activation of phagocytic activity in response to glucose deprivation. A likely explanation is that the majority of metastatic LLC/R9 cells exhibit metabolic plasticity. In glucose-deficient conditions, they are able to switch energy metabolism from glycolysis to oxidative phosphorylation (OXPHOS), thereby avoiding the need to activate cannibalism [23].

Interestingly, similar patterns were observed in deadhesive cells (Fig. 5, *b*). Under glucose deprivation, the percentage of the bead-engulfing cells decreased by 76% ($p < 0.001$), yet remained almost threefold higher than in adhesive cells. This suggests that the activation of non-professional phagocytosis in LLC/R9 cells under anchorage-independent conditions is largely independent of glucose availability and persists even in glucose-free medium. The intensity of bead uptake was also significantly higher (by about 40%, $p < 0.001$), highlighting that only a small fraction of cancer cells is sensitive to the phagocytic activation by the glucose starvation.

Taken together, these results indicate that non-small cell lung carcinoma LLC/R9 cells are capable of non-professional phagocytosis, which is markedly enhanced under anchorage-independent growth conditions and only weakly affected by glucose deprivation.

Funding

This work was supported by the National Research Foundation of Ukraine (2021.01/0229).

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Submitted: September 04, 2025

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НЕПРОФЕСІЙНИЙ ФАГОЦИТОЗ КЛІТИН КАРЦИНОМИ ЛЕГЕНІ ЛЬЮЇС ЗА РІЗНИХ УМОВ ЇХ РОСТУ

Стан питання. Відомо, що фагоцитоз зустрічається майже у всіх типах клітин багатоклітинних організмів. За ефективністю фагоцитозу клітини ділять на професійні та непрофесійні фагоцити. До останніх належать і пухлинні клітини. Така здатність пухлинних клітин обумовлює появу структур «клітина в клітині», високу частоту виникнення яких часто пов'язують з процесами інвазії та метастазування злоякісних новоутворень. **Мета** роботи — дослідити здатність клітин карциноми легені Льюїс до непрофесійного фагоцитозу та аналіз залежності цього процесу від умов росту пухлинних клітин. **Матеріали та методи**. Дослідження проводили з використанням низькометастатичного варіанту клітин карциноми легені Льюїс. Фагоцитарну активність клітин досліджували за умов як адгезивного, так і деадгезивного росту в стандартному культуральному середовищі та в безглюкозному. Дослідження фагоцитарної активності клітин проводили з використанням флуоресцентних латексних кульок розміром 1,0 мкм. **Результати**. Доведена здатність пухлинних клітин недрібноклітинного раку легені до фагоцитарної активності, інтенсифікація якої суттєво збільшувалась майже в 4 рази за умов деадгезивного росту незалежно від рівня експресії Е-кадгерину. Глюкозне голодування пухлинних клітин за умов як адгезивного, так і деадгезивного росту обумовлювало більш ніж 2-разове зниження відсотка клітин, що поглинають латексні кульки на фоні збільшення кількості поглинутих кульок. Це вказує на суттєву гетерогенність популяції пухлинних клітин щодо їх чутливості до активації фагоцитарної активності дефіцитом глюкози. **Висновки.** Результати вказують на здатність пухлинних клітин недрібноклітинного раку легені до фагоцитарної активності, інтенсифікація якої суттєво збільшується за умов деадгезивного росту і слабо залежить від дефіциту глюкози.

Ключові слова: непрофесійний фагоцитоз, карцинома легені Льюїс, деадгезивний ріст, глюкозне голодування.