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Preliminary flagellin gene transfection into tumor cells - attempts of generating anti-tumor response in experimental model

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Abstract

The development of new research techniques, especially molecular ones, creates hopes for improved treatment efficacy and a better prognosis in lung cancer. The starting point for very important experiments in the field of immunotherapy is the sensitization of the immune system to the tumor antigen.

The aim of the study was to determine whether using flagellin can induce innate antitumor effective, and thus, increase the immunogenicity of the tumor.

The test material was the established mouse cell line LLC, derived from Lewis lung cancer. Vectors were constructed by cloning FliC inserts into the pCDH-MSC-T2A Puro plasmid. The cells were transfected directly and indirectly (with pseudoviruses, produced by previously transfected packaging cells HEK 293T). The transfection efficiency was confirmed by RT-PCR. The cells thus prepared were implanted subcutaneously in mice. The control group received non-transfected LLC cells. Then, after 6 weeks, the mice were scarified. The animals were sectioned to isolate the tumor and lungs in which the presence or absence of metastases was assessed.

In mice that received immunotherapeutic vaccines containing transfected LLC cells, less tumor mass was found or a complete lack of it, and the lifespan was noticeably prolonged. In addition, no metastases occurred in the group vaccinated with cells transfected with direct technique.

It can be concluded that flagellin is effective as an adjuvant in the process of presenting tumor antigens to T cells Thus, in the light of recent studies and reports, it is likely that in this experiment effectively increased immunogenicity of the tumor. Activating anti-tumor cellular responses using flagellin is a promising target for lung cancer immunotherapy.

Key words: flagellin, lung cancer, dendritic cells, transfection, anti-tumor response

Introduction

The creation of an effective response to pathogens, including tumor antigens (neoantigens) is possible due to dendritic cells (DC). Their main role is based on switching non-specific innate immunity to the acquired specific immune response, enforced by antigen-specific T and B lymphocytes. Presentation of specific antigenic determinants to lymphocytes proceeds more efficiently when DCs are previously unspecifically stimulated. An important mechanism of stimulation is the activation of cellular PRR receptors (pathogen recognition receptors) by their ligands. These are conservatively evolutionary exogenous components of bacterial origin (such as lipopolysaccharide, LPS, peptidoglycan, or heat shock proteins (HSP) and endogenous structures resulting from the breakdown of dead cells.) PRR series receptor ligands are collectively referred to as molecular patterns associated with pathogens (pathogen associated molecular patterns, PAMP) [1].

The DP family includes the Toll-like receptor (TLR) family. Cells that recognize pathogens with the help of TLRs are DC, but also other antigen presenting cells (APC), such as macrophages, monocytes and T cells and other white blood cells, epithelial cells, cardiomyocytes and adipocytes [2,3]. Excitation of TLR to APC leads to enhanced synthesis of proinflammatory cytokines (TNFα, IL-1, -6, -8, -12), chemokines and nitric oxide; the expression of adhesion molecules and costimulatory molecules increases (CD40, CD80, CD86). These changes facilitate the conversion of non-specific inflammation into an antigenically specific, precise immune response. In its formation, TLR ligands (especially of bacterial origin) play a key role as they play the role of immunological adjuvant [4].

There are many studies carried out using the mechanism of activation of a non-specific response directed against cancer cells. This response can be activated through PAMP structures recognized by TLR receptors, located on cells of the immune system, including among others on dendritic cells [2].

Dendritic cells in cancer immunotherapy. Inadequate activation of the immune system is responsible for the progression of malignant tumors (the so-called tumor escape from immune surveillance). One of the reasons is the lack of stimulation of dendritic cells (DCs) by tumor antigens. As a result, the cancer is not recognized as a threat or even the tolerance of the immune system to the tumor antigens develops. This is an extremely unfavorable phenomenon; numerous attempts of specific immunotherapy of malignant tumors, using dendritic cells, are ineffective [5,6].

Meanwhile, according to theoretical assumptions, DC cells should be a promising tool for oncological immunotherapy. They induce both specific destruction of primary tumors as well as immune system response to single tumor cell dissemination, including metastases [3]. In order to generate cancer vaccines, tumor antigens are introduced in the form of a charge. Constructed therefore, vaccines containing antigens and adjuvants; the adjuvant is intended to switch the maturing mDC cells to stimulate cytotoxic T cells or / and NK cells to obtain a cellular response and antibody production, and thus a humoral response [7].

Limited efficacy of anticancer vaccines, including preclinical and clinical studies using dendritic cells, gave rise to the assumption that for the effective immunization of the body with tumor antigens may be the continued use of additional stimulants (immune adjuvants). This could involve introducing into the cells of the immune system vectors encoding transgenes for TLRs or their ligands [8].

Such a substance may be flagellin (FliC) - it is highly immunogenic and at the same time acts as an immunological adjuvant. Flagellin was isolated from Salmonella. It is a monomeric protein that polymerizes when the bacteria forms flagella or cilia. Exposure to FliC on mucous membranes stimulates the production of pro-inflammatory cytokines (eg, TNFα by monocytes) and neutrophil recruitment. Especially in DC cells, the response to flagellin allows conversion of innate (non-specific) immune response into acquired (specific). FliC attaches to TLR5, which triggers a signal cascade in the cells, involving the MyD88 adapter protein and the phosphorylated IRAK kinase. Further stages include activation of MEK kinase and NF-κB transcription factor [8,9,10]. FliC thus facilitates the maturation of human myeloid DCs and enhances cytokine production (IL-6, IL-10, IL12p70, TNFα i IFN–γ), reduces the secretion of IL-5 and IL-3 [8,11,12,13,14].

In the mice administered subcutaneously vector with the gene of flagellin, there was a severe inflammatory reaction which also included a strong antigen specific cellular response subject to MHC restriction I. When given to animals in parallel vector encoding the nucleoprotein fatal to mice influenza A virus, there was an effective, specific resistance on this infection [8]. It is possible, therefore, that flagellin could also be useful in immunotherapy of cancerous diseases.

On the other hand, Flagellin combines with TLR5 on Treg regulatory lymphocytes (CD4 + CD25 + ^{high}), so it can add immunosuppression [10]. This is the only way to get more information.

In total, the response to flagellin involves the development of an increased specific response, accelerated DC maturation and sensitization of antigen-specific CD4 + helper cells and secretion of both cellular (IFN-γ) and humoral (IL-4, -13) mediator responses. In total, FliC is an activator of both Th cell secretion patterns, however with the majority of Th2 activity [8].

Then finally, flagellina activates the early stages of inflammation as it causes white blood cell chemotaxis to enter the infection and lymph node glands. It induces the expression of chemokines mobilizing neutrophils (GRO−α, −β, −γ i IL-8), monocytes, macrophages and NK cells (MCP-1, MIP-1α, MIP-1β) [15].

Vaccines with the use of flagellin. Providing naked DNA encoding the antigen in the form of a vaccine makes it possible to induce an immune response directed to a defined antigen. The vaccine is easy to prepare and, above all, stable. Furthermore, using DNA vaccines, there is no need to grow virulent microorganisms in vitro, purify and modify proteins, and immunization by the antigen transporting organism may be omitted. The emergence of a stronger immune response and the induction of both humoral and cellular responses depend on appropriately selected adjuvants [8].

Increasing the effectiveness of vaccines using DNA molecules may theoretically involve introducing into the cells DNA molecules encoding either TLR or its ligands, which would imitate the inflammatory response generated by the infection [8].

Studies have also shown that the expression of TLR-agonist-encoded DNA molecules in mammalian cells significantly increases the immune response, making new possibilities available in the field of DNA vaccines directed against infective antigens and tumors [8].

Flagellina as an adjuvant. FliC can, however, not only induce Th2 response, but also Th1 module response. Bearing in mind all these properties of flagellin, one can suppose

that a TLR-agonist encoded with DNA has the potential for immunostimulation as a molecular adjuvant in combination with a DNA vaccine [8].

Purpose of work

The aim of the study was to confirm the hypothesis that forcing the maturation of dendritic cells by transfection with the genes of proteins stimulating their activation may lead to the efficient recognition of cancer cells and their destruction by the patient's immune system.

The validity of the hypothesis was verified using the FliC gene (encoding flagellin) as an adjuvant stimulating the immune response of mice vaccinated with LLC (*Lewis Lung Carcinoma*) cancer cells. Indirectly the potential for gene immunotherapy of non-small cell lung cancer, currently fatal to humans in approximately 90% of cases, was investigated.

Materials and methods

Animal Model

Male C57BL/6N mice (12-weeks old) were obtained from Tri-City Academic Laboratory Animal Centre, Poland. Mice were given free access to water and standard rodent chow and were housed in pathogen-free cages. The animals were acclimated for a week before the experiments. Animal welfare and experimental procedures complied with national guidelines and were approved by the local Animal Ethics Committee.

Medium: E. coli grown in LB broth, 15g/L.

Cell lines

Lewis Lung Cancer, LLC2 were obtained from ATCC and cultured in RPMI 1640 (*Biochrom AG, cat.no. F1295*) and Opti-MEM (*Gibco, cat.no. 31985047)* containing 5% FBS (*Gibco, nr kat. 10106169),* 1% penicillin/streptomycin and 1% glucose (*Polskie Odczynniki Chemiczne, cat. no. 459560117)* at 37°C and 5% CO_2.

Human Embryonal Kidney Cells, HEK 293T were used as a packaging cells. HEK 293T were also obtained from ATCC and cultured in DMEM 90% (*Gibco, nr kat 41966029)* containing 10% FBS.

Plasmids

Plasmid used to transfection of LLC cells was constructed on te basis of two vectors:

1. *pCDNA3.1(Zeo)-Flic(-gly),* kindly provided by Dr. Steven Applequist of the Karolinska Institutet, formed by cloning into the polylinker the plasmid pCDNA3.1Zeo

(+) a fragment containing the following elements in the open reading frame: a) human immunoglobulin leader IgG leader sequence (allows secretion of the protein outside the cell); b) the sequence of the flagellin gene (*Salmonella enterica serovar Typhimurium*, *cat. No. ATCC 14028*), the sequence was modified using a site-specific mutagenesis technique to remove predicted N-glycosylation sites); c) the sequence of the transmembrane domain of the human platelet-derived growth factor receptor, PDGFR, enabling the anchorage of the protein in the cell membrane [7];

2. *pCDH-MCS-T2A-Puro-MSCV* (from a set pPACK H1 Lentiviral Expression Kit, *System Biosciences*), that includes an expression cassette under the control of the MSCV murine stem cell promoter, puromycin resistance gene, and encapsidation signal sequences to allow packaging of the expression cassette into the pseudovirus capsid**;**

3. *pJET* – a cloning vector, comprising: a) a replicon rep responsible for replicating the plasmid; b) bla gene, coding for beta-lactamase responsible for ampicillin resistance; c) modified eco47IR gene encoding restriction endonuclease.

The plasmid used to transfect LLC cells was obtained as follows:

A PCR reaction was performed on the plasmid pCDNA.3.1Zeo-pFliC (-gly) [8] with the following primers:

forward: GCTCGAATCCACTAGTAACGGCCGCCAG

reverse: GCGGCCGCTTTTTGTTCGTCGACCTGCAG

The resulting product, i.e. a sequence comprising the peptide sequence of letters and IgG, and the flagellin gene sequence of the domain PDGFR, was cloned into the vector pJET (set *CloneJet, Fermentas, cat. K1232*) according to the manufacturer's protocol. The FliC insert was then excised from the plasmid pJET-FliC using the enzymes EcoRI and NotI (*Fermentas, cat. No. ER0275 and ER0595, respectively*). Fragments were separated by electrophoresis. At this stage, the signal peptide and PDGFR domains have been deleted. In the next step, the section obtained after cutting the pJET plasmid into the pCDH-MCS-T2A-Puro-MSCV vector (*CD522A-1*) pasted with enzymes previously used (EcoRI and NotI) was pasted. An expression cassette was obtained that allows the expression and secretion of free flagellin to the microenvironment of the transfected cells. The resulting plasmid (pCDH-FliC) was used to transform *E. coli* bacteria.

Transfection of LLC cells using the pCDH-FliC plasmid.

For transfection, TransFectin lipid reagent (*Bio-Rad, Cat. No. 170-3350*) and the previously crafted pCDH-FliC vector were used. The transfection procedure was based on the Bio-Rad protocol (*TransFectin Protocol for 293 Cells*) [16].

Indirect transfection.

In brief, plasmid-TransFectin complexes, 400 µl / bottle was added to the Hek293T cell culture. After 4 hours, serum was added to the bottles in order to reach a final concentration of 10%. The cells were incubated at 37 ° C with 5% CO2 saturation for 48 hours. Medium was collected, centrifuged (5 min., 3000 rpm) and the supernatant was then added to the cell culture LLC for the infection of these cells produced pseudowirusami containing the flagellin gene. The addition of polybrene (*Heksadimethrine Bromide, Sigma-Aldrich,* cat. No. H9268-5G) at a concentration of 5µg / ml for culturing is intended to facilitate adherence of pseudoviruses to LLC cells. After 10 hours, the medium was changed to fresh culture medium.

The selection of transfected cells in the presence of puromycin (1 µg / ml) was carried out for 2 weeks.

Direct transfection of LLC cells.

Plasmid-TransFectin complexes were added to LLC cell culture and incubated for 4 hours (37 ° C, 5% C02.). FBS was then added to a final concentration of 10%. After 48 hours, the medium was changed. Cells were grown to confluence condition.

Cell application to experimental animals.

Mice were randomly divided into three groups: 1) control group (mice implanted with LLC, n=5); 2) group implanted with indirectly transfected cells (n=10); 3) group implanted with directly transfected cells (n=5). Each mouse was injected subcutaneously in the left side with the cells suspended in phosphate buffered saline; 1x106 komórek/100ul (PBS, Gibco, nr kat. 14190-094).

And then mice were sacrificed on day 7; the subcutaneous tumors were removed, and the tumor volume was calculated.

Results

The expression of the FliC gene was observed on the resulting electrophoresies. The presence of a 1500bp band confirmed the expression of flagellin.

Post mortem results

During the isolation of tumors, in some cases, infiltration of tumors on nearby tissues was found. In mice from the control group, evident disease progression was observed: weight loss, significant tumor growth, numerous lung metastases, general body weakness, and increased mortality before the scheduled time to end the experiment.

W płucach wyraźnie widoczne były liczne przerzuty, natomiast wewnątrz guzów występowały ogniska martwicze. Większe guzy (>3 cm) są zwykle niejednorodne (wskutek zmian martwiczych, ognisk krwawienia, obszarów tłuszczowych) - jak to wykazano podczas doświadczenia.

In lungs metastases were clearly visible, whereas necrotic foci were present inside the lungs. Larger tumors (> 3 cm) are usually heterogeneous (due to necrotic lesions, bleeding foci, fatty areas) - as demonstrated during the experiment.

The following tables and graphs show the results of the mice post mortem examination from three individual groups.

Table 1. Results from the administration of untransfected LLC cells to control group mice.

Number of mice	Numberof lived days since tumor implantation	Body weight (g)	Tumor mass (g)	Presence of metastases in the lungs	The percentage of tumor in relation to body weight
1	44	36	0	-	0%
2	44	25,9	6,35	+	25%
3	34	27,9	7,8	+	28%
4	33	30,5	13,3	+	44%
5	33	33,5	11,5	+	34%
Average	37,6	30,76	7,79	80%	25%

Table 2. Results of administration of LLC cells transfected directly to mice.

Ordinal numer of mice	Numberof lived days since tumor implantation	Body weight (g)	Tumor mass (g)	Presence of metastases in the lungs	The percentage of tumor in relation to body weight
1	44	31	0	-	0%
2	44	27,7	0	-	0%
3	44	30,8	0	-	0%
4	44	27,3	0	-	0%
5	44	29,1	5,1	-	18%
Average	44	29,18	1,02	0	4%

Table 3. Results of mice treated with LLC cells transfected with use of pseudoviruses.

Number of mice	Numberof lived days since tumor implantation	Body weight (g)	Tumor mass (g)	Presence of metastases in the lungs	The percentage of tumor in relation to body weight
1	23	24,9	3,5	+	14%
2	33	21,3	2,7	+	13%
3	34	19,6	3,1	+	16%
4	40	31	4,8	+	15%
5	44	22,7	4,4	+	19%
6	44	29,6	0	-	0%
7	44	24,5	0	-	0%
8	44	25,7	0	-	0%
9	44	26,2	0	-	0%
10	44	27,8	0	-	0%
Average	39,4	25,33	1,85	50%	8%

In the above comparison, a smaller amount of mass of the tumor in relation to the body weight, the significantly reduced number of lung metastases, and the noticeably longer survival time of mice vaccinated transfected (directly and indirectly) are noticed.

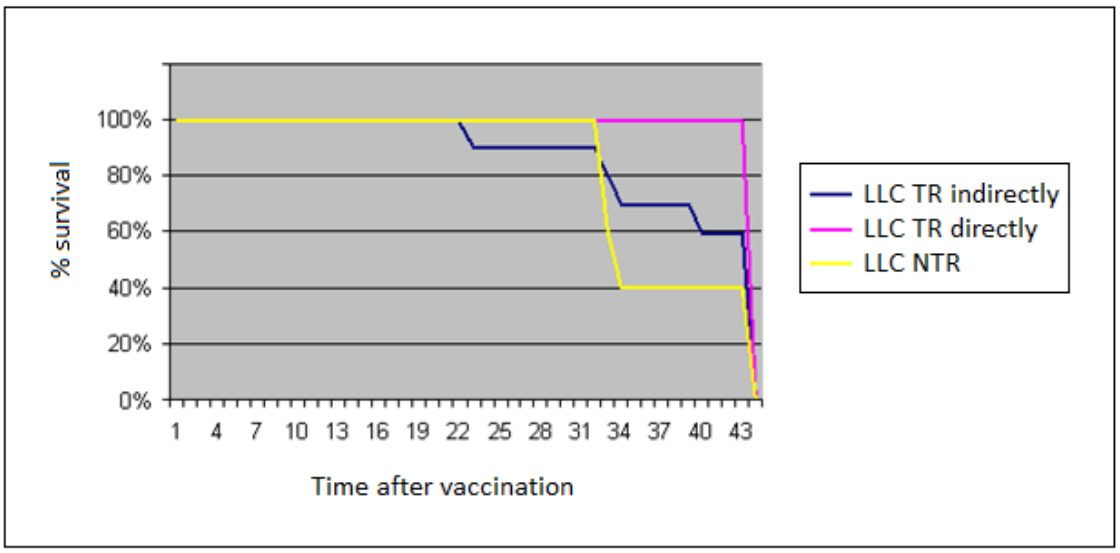

Fig. 1. Average survival time of animals vaccinated with LLC TR and LLC NTR cells. Observations have been made since the inoculation of initial and genetically modified LLC cells (LLC TR - mice vaccinated with LLC-transfected LLC cells, LLC NTR - mice vaccinated with untransfected LLC cells)

The average survival time of animals vaccinated with the original LLC cells was 37.6 days. Administration of transfected cells resulted in an increase in the average survival time to 41.7. The most prolonged survival time was observed in animals injected with LLC directly transfected.

The highest dynamics of tumor growth was observed in the group of control animals. In mice that were injected with LLC cells indirectly transfected, tumor growth was weaker, while in LLC-injected animals that were directly transfected, tumor growth was observed in only one case, which was seen as a promising research success. It is also noteworthy that metastases were very rare in animals vaccinated with cells transfected with both techniques.

Discussion

The immunological tolerance of tumors depends to a certain extent on the phenomenon of inhibiting the differentiation and maturation of dendritic cells under the influence of factors secreted by cancer cells. Therefore, it seems reasonable to assume that providing additional factors stimulating DC maturation could restore the proper functioning of dendritic cells.

Activation of immune system cells by ligation of TLR receptors is an effective way to induce a strong adaptive immune response. Once activated, a cell expressing a TLR receptor stimulates many arms of the immune system, including: effector molecules, interferons, cytokines, chemokines, costimulatory molecules, and also promotes the activation of T and B lymphocytes through APC cells [17]. The experiment attempts to obtain tumor cells secreting the TLR receptor ligand. The choice fell on flagellin, which is the only protein, which determines the ease of expression in cancer cells.

For this purpose, a plasmid vector carrying the cDNA of the Salmonella enterica flagellin gene was constructed using PCR methods and molecular cloning. This vector was used to obtain lentiviruses that were infected with mouse lung cancer cells. In a parallel plasmid experiment, LLC cells were used for direct transfection. The efficiency of DNA transfer was confirmed by performing RT-PCR reactions on the mRNA template from LLC cells. The RT-PCR reaction confirmed the presence of the flagellin transcript in LLC cells [17].

Literature reports confirm the stimulating effect of flagellin on the immune system by stimulating Th1-type responses [17]. Flagellin activates mouse macrophages and osteoblasts for the production of inflammatory mediators, and human monocytes for the production of TNF-α. It is also capable of stimulating mDC for the maturation and production of IL-10, IL-12p70, IL-6, TNF-α and IFNγ. Agrawal et al. Demonstrated that flagellin by combining TLR5 stimulates the Th1 cell response by producing IL-12p70 [18]. Renshaw's studies have shown that mouse peritoneal macrophages express TLR-5 and that the administration of flagellin induces IL-6 and TNFα production. Furthermore, McSorley demonstrated in his experiment that flagellin is capable of inducing a CD4 + T cell response in vivo. It has also been proved that the murine osteoblasts are activated flagellin after stimulation [19]. On the other hand, other reports indicate that intravenous injections of flagellin in C57BL / 6 mice resulted in a rapid increase in IL-6 production and increased expression of activation markers in spleen dendritic cells [20]. Flagellin causes rapid redistribution of DC cells from the red pulp and sphenoid peripheral zones to the T-lymphocyte deposition zones in white pulp [20]. All these reports indicate the fact that TLR5 is expressed in various tissues and cell types, which are thus able to interact with flagellin and induce various elements of the immune response [21].

It was therefore assumed that flagellin secreted by LLC lung cancer cells would affect the phenotype and functioning of dendritic cells. As a measure of the immunomodulatory effect of flegellin, clinical criteria were adopted, including the size of the tumor mass, the number of metastases and survival, because, in the final analysis, they determine the usefulness of the therapeutic method. For this purpose, genetically engineered lung cancer cells were subcutaneously implanted into syngeneic mice. The control group was administered unmodified LLC cells. Then, after 6 weeks, the animals were subjected to autopsy examination, which allowed to assess the severity of the cancer in both groups. In mice treated with flagellin secreting tumor cells, a smaller mean tumor weight was observed. Moreover, in these mice no lung metastases were observed, which were observed in the lungs of mice in the control group. The survival time of mice in the therapeutic group was noticeably prolonged compared to the control group.

Possible explanation obtained in this work brings the results of the recent publication of Vicente-Suares et al. [22]. In this study, it was shown that under the influence of flagellin, tolerant dendritic cells secreting IL-10 change their phenotype and secretory profile, secreting mainly IL-12, which stimulates, among others, cytotoxic lymphocytes. Since, in the lung cancer microenvironment, increased expression of IL-10 is observed and the DC's ability to stimulate lymphocytes is attenuated, one could speculate that the flagellin tumor present in tissue has led to activation of DC cells and stimulation of cytotoxic T lymphocytes, which ultimately translated into suppression of disease progression cancerous disease.

On the other hand, the results of the experiment performed contrast with the results of a breast cancer immunotherapy test carried out by Sfondrini et al. in this study, flagellin was injected into the tumor or synchronously with the injection of tumor cells, or after about 8-10 days. In the study by Sfondrini et al. synchronous administration of flagellin resulted in the induction of lymphocyte polarization towards the regulatory profile (CD4 + CD25 +) and accelerated tumor development, and only in the case of methachronous injection flagellin inhibited the development of neoplastic disease [13]. Moreover, the adopted explanation of the mechanism of flagellin action is also in contradiction with the results of Meansa et al. who failed to observe the expression of TLR5 on the surface of DC cells isolated from C57BL / 6 mice [15]. Observed discrepancies require verification.

Conclusions

1. With the use of transcription techniques using lentiviral vectors and direct administration of plasmids, Lewis lung carcinoma cells (LLC) were genetically modified as confirmed by RT-PCR techniques.
2. In both experiments in which C57BL / 6 mice were vaccinated with genetically engineered LLC cells, no tumor growth or slower growth, less tumor mass, and prolonged survival of experimental animals were observed. Statistical significance was observed especially in the group of animals transfected with direct technique. In this group, the total lack of tumor metastasis was noticed.
3. It can be assumed that the transfection of LLC cells with the gene encoding flagellin (FliC) led to the secretion of flagellin into the tumor environment. Through the TLR5 receptor on DC cells, it could support the antigen presentation by these cells and increase the immunogenicity of the tumor.
4. In the light of the experiments and previous reports, flagellin seems to be an effective adjuvant in the presentation of tumor antigens to T lymphocytes. It is thus a promising target for lung cancer immunotherapy.

The difference in survival of mice observed in the experiment as well as the differences in the remaining parameters of tumor severity testify the modulating effect of flagellin on the development of mouse lung cancer in vivo. Correct, the methodological point of view, the evaluation results obtained make it possible to only compare the phenotype of dendritic cells from mouse research groups and the control group. The revealed discrepancies with the results of other research groups also need to be clarified (Sfondrini) However, given the current knowledge of the mechanism of flagellin action and the similarity between human and mouse immune systems, it seems reasonable to undertake further research this time using human lung cancer cells - diseases still at 90 % deadly.

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