RESEARCH ARTICLE

5-Fluorouracil and Interleukin-2 Immunochemotherapy Enhances Immunogenicity of Non-Small Cell Lung Cancer A549 Cells through Upregulation of NKG2D Ligands

Lei Zhao[1], Wen-Jia Wang[2], Jin-Nan Zhang[3], Xing-Yi Zhang[4]*

Abstract

Background: The aim of this study was to investigate the anti-cancer effects and mechanisms of immunochemotherapy of 5-fluorouracil (5-FU) and interleukin-2 (IL-2) on non-small cell lung cancer (NSCLC) A549 cells. Materials and Methods: In order to detect whether 5-FU+IL-2 could effectively inhibit tumor growth *in vivo*, we established an A549-bearing nude mouse model. The cytotoxicity of natural killer (NK) cells was evaluated using a standard chromium release assay. To evaluate the relevance of NK cells in 5-FU+IL-2-mediated tumor inhibitory effects, we depleted NK cells in A549-bearing mice by injecting anti-asialo-GM-1 antibodies. Effects of 5-FU+IL-2 on the expression and promoter activity of NKG2D ligands (MICA/MICB) in A549 cells *in vitro* were also assessed. Results: In A549-bearing nude mice, combination therapy significantly inhibited tumor growth in comparison with monotherapy with 5-FU or IL-2 and enhanced the recognition and lysis of tumor cells by NK cells. Further study of mechanisms showed that NK cells played a vital role in the anticancer immune response of 5-FU+IL-2 immunochemotherapy. In addition, the combination therapy synergistically stimulated the expression and promoter activity of MICA/MICB. Conclusions: 5-FU and IL-2 immunochemotherapy significantly inhibited tumor growth and activated NK cytotoxicity *in vivo*, and these effects were partly impaired after depleting NK cells in tumor-bearing mice. Combination treatment of 5-FU and IL-2 upregulated the expression and the promoter activity of MICA/MICB in A549 cells, which enhanced the recognition of A549 cells by NK cells. All of the data indicated that immunochemotherapy of 5-FU and IL-2 may provide a new treatment option for patients with lung cancer

Keywords: 5-FU - IL-2 - immunochemotherapy - NK cell - MICA/B

Asian Pac J Cancer Prev, **15 (9)**, 4039-4044

Introduction

Non-small cell lung cancer (NSCLC) is a heterogeneous disease that is difficult to treat, and remains the leading cause of cancer-related mortality worldwide (Holt et al., 2011; Stella et al., 2013). In recent years, despite recent advances in surgery, irradiation, chemotherapy, and targeted therapy, the five-year survival rate of patients with advanced stage NSCLC is very low (Xu et al., 2014). In traditional, chemotherapy has been widely used in the treatment of NSCLC, but their severe side effects and drug resistance limited its use in clinic. Therefore, alternative therapeutic approaches which can effectively treat lung cancer attract the scientists' attentions.

Immunotherapy, which utilizes the immune system to control and eradicate cancer, that are refractory to conventional therapies (Wang et al., 2014). Recently, immunotherapies used in patients with lung cancer has made breakthrough (Zheng et al., 2013; Xu et al., 2014). Notably, one of attractive immunotherapy candidates used in combination with chemotherapy is Interleukin 2 (IL-2). IL-2, a glycoprotein produced by activated T cells (Du et al., 2012), is one of the most successful cytokines applied in tumor immunotherapy for stimulating potent cellular immune response (Ye et al., 2014). In addition, IL-2 enhances the proliferation and cytolytic activities of T and NK cells (Gaffen et al., 2004). All of these properties underly the potential of IL-2 in immnotherapy.

5-Fluorouracil (5-FU) has been widely used in the treatment of various human cancers, including NSCLCs, colorectal cancers, and gastric cancers (Takiuchi et al., 1998; Macdonald et al., 2001). Nevertheless, drug resistance of 5-FU has become a apparent problem in clinical use (Das et al., 2013), which promotes the combination therapy with immunomodulatory factors. It has been reported that continuous infusional 5-FU and subcutaneous IL-2 in treating metastatic renal cancer exhibited good effects (Savage et al., 1997). However, the relative mechanism of this immunochemotherapy has not yet been investigated.

*[1]Institute of Frontier Medical Science, [2]Institute of Pharmaceutical Sciences, [4]Second Hospital, Jilin University, [3]Department of Neurosurgery, Third Hospital, Jilin University, Changchun, Jilin, China *For correspondence: zhang_xingyi28@163.com*

In the present study, the effect and the mechanism of immunochemotherapy of 5-FU and IL-2were investigated. Our results suggested that 5-FU+IL-2 treatment suppressed A549 growth *in vivo* by stimulating NK cytotoxicity, which were based on stimulating the promoter activity and upregulation the expression of NKG2D ligands (MICA and MICB) on A549 cells. These data indicated that the anti-lung cancer activity of 5-FU combined with IL-2 is mediated by NK cytotoxicity .

Materials and Methods

Cell culture

Human NSCLC cells (A549) was obtained from ATCC (Manassas, Virginia, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco, Vienna, NY, USA), 100 U/ml of penicillin and 100 U/ml of streptomycin. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

Experimental animals

Nude mice were purchased from the Academy of Military Medical Science, housed in a rodent facility at 22±1°C with a 12 h light-dark cycle and provided with continuous standard rodent chow and water for acclimatization. All of the procedures, involving animals and their care in this study, were in accordance with protocols approved by the Ethics Committee of Jilin University.

In vivo treatments

A549 cells (0.1 ml, 5×10^7 cells) was transplanted subcutaneously into the right axilla of each nude mouse. When the tumor grew to 100-300 mm3, mice were randomly divided into four groups: a model group administered with normal saline, 5-FU-treated group 35 mg/kg of body weight), IL-2-treated group (500 IU per mouse) and a 5-FU and IL-2 combination therapy treatment group. Each group contained six mice. These solutions were dissolved in saline, filtered through a 0.22 μm Millipore filter and administered by intraperitoneal injection for three times a week. After three weeks of treatment, mice from all the groups were sacrificed by cervical dislocation 24 h after the final administration. The tumor weights of the mice from each group were measured. During the treatment, the tumor volume of each mouse was measured every three days.

In vivo antitumor activity assay

The *in vivo* antitumor activity was expressed as a percent inhibitory rate, which was calculated as follows: [(A–B)/A] ×100%, where A and B are the average weights of the tumors from the control and experimental groups, respectively. The tumor volume (TV) was measured and calculated using the following formula: $TV=1/2\times a\times b^2$, where a and b are the long and short diameters of the tumors in each mouse, respectively.

Depletion of NK cells in vivo

Tumor-bearing nude mice were administered 50 μl of anti-asialo-GM-1 antibody (Wako Pure Chemical Industries)by intraperitoneal injection 3 days prior to drug treatment, followed by repeated injection every four days for three weeks. Control mice received nonimmune antibodies with the corresponding IgG isotype.

Flow cytometry analysis

Single-cell splenic lymphocytes from each group were prepared and then were double stained with anti-DX5 antibodies. After washing twice and resuspending in PBS, the percentage of NK (DX5+) cells were assessed using flow cytometry (BD Biosciences, San Jose, CA, USA) and calculated as a percentage of the total number of splenic lymphocytes.

A549 cells were treated 5-FU (10 μM), IL-2 (50 IU/ml) or 5-FU (10 μM) and IL-2 (50 IU/ml) for 24 h, then the cells were collected, stained with anti-human MICA/MICB PE antibody (eBioscience, San Diego, CA, USA) and analyzed using flow cytometry.

Isolation of NK cells

NK cells were prepared by using DX5 MicroBeads (Miltenyi Biotech, CA, USA), respectively, as previously described (Martinez J, et al., 2010). In brief, single-cell suspensions from the spleens of mice were purified using Mouse CD49b (DX5) MicroBeads. The NK cell population was detected using flow cytometry, and the purity was determined to be greater than 90% (data not shown).

Cytotoxicity Assay

The cytotoxic activity of the NK cells was evaluated using a standard chromium release assay. Target cells (A549 cells) were labeled with 100 μ $Ci^{51}Cr$ radioactive chromium as sodium chromate for 2 hours. After washing, 1×10^4 target cells per well were incubated with effector cells (NK cells isolated from the spleens of mice) at different effector to target cell ratios (E:T ratio) over 4 hours. The supernatant was collected and counts per minute were determined (Packard, Dreieich, Germany). Maximum release was obtained by incubating the target cells with an anionic detergent (0.1% IGEPAL). The negative control (spontaneous release) was represented by target cells without effector cells. Cytotoxicity calculations were performed using the following formula:

Cytotoxicity (%)= (experimental release - spontaneous release of target cells)/ (maximal release - spontaneous release of target cells).

Construction of Recombinant Plasmids (MICA/MICB Promoter and pGL3-Basic Vector)

The primers were designed according to the promoter region of MICA/MICB. The upstream primer and downstream primer of MICA promoter was 5'-ACGGATCCACGCGTTGT CTGT CCTG-3' and 5'-TGCCAGCCAGAAGCAGGAAGACC-3' respectively; The upstream primer and downstream primer of MICB promoter was 5'-ACGGATCCACGCGTTGT CTGT CC-3' and 5'-AGGCGACGGCCAGAAACAGCAG-3' respectively. The primers were synthesized by Meiji Co, Ltd. (Shanghai, China). PCR to amplify MICA/MICB promoter (the complete genome of A549 cell served as the

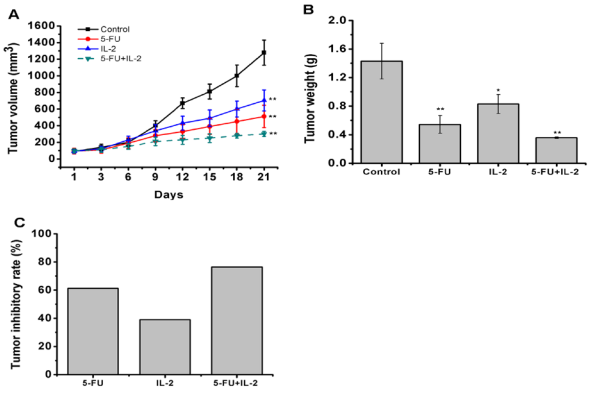

Figure 1. 5-FU+IL-2 Immunochemotherapy Prevents the A549 Tumor Growth In Vivo. A) Tumor volume curve in A549-bearing nude mice. **B)** The tumor weights decreased in in 5-FU+IL-2- treated group. **C)** The tumor inhibitory rate was increased in 5-FU+IL-2-administered A549-bearing nude mice. (n=6; the data represent the means±SD; **p*<0.05 and ***p*<0.01 compared with the model control group)

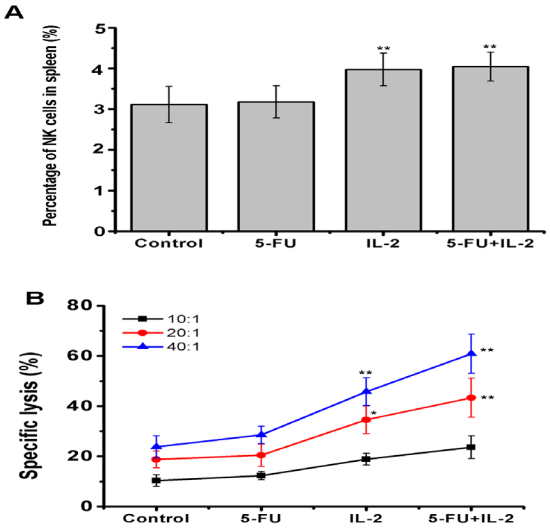

Figure 2. 5-FU+IL-2 Immunochemotherapy Stimulates the NK Cytotoxicity in A549-Bearing Mice. A) The percentage of NK cells in spleens in each group. Spleens were harvested and meshed using a cell strainer, NK cell infiltration in spleens were detected by flow cytometry, the graph showed percentage of NK cells defined as DX5+ cells. The number of NK cells in 5-FU+IL-2 treated group was much higher than other group. **B)** NK cells isolated from 5-FU+IL-2-treated mice showed a highly significant increase in cytotoxicity. NK cells were isolated from the spleens using the magnetic cell separation, then target cells (A549 cells) were labeled with radioactive Chromium51Cr for 2 hours. Then target cells were incubated with effector cells (NK cells isolated from the spleen of mice) at different effector to target cell ratios (E:T) over 4 hours . (n=3; the data represent the means±SD; **p*<0.05 and ***p*<0.01 compared with the model control group)

template). The products of PCR and pGL3-Basic vector were digested with BamH IV and Nco IV (New England Biolabs, American), and then were joined by T4 ligase (Takara, Japan). The products were transformed into BL21 competent cells and the recombinant plasmids were extracted by alkaline lysis method. Nucleotide sequences of the recombinant plasmids were confirmed by Meiji Co, Ltd. (Shanghai, China).

Activity analysis of promoter

1×10^5 A549 cells per well were inoculated in 24-well plate, one day after inoculation A549 cells were transfected with 0.79 μg reporter gene plasmid and 0.01μg internal control plasmid (pRL-TK) per well by Lipofectamine 2000 (Invitrogen, Vienna, NY, USA). The mole ratio of reporter gene plasmid and internal control plasmid was 50: 1. At the same time, the pGL3-Basic and pGL3-Control were transfected as negative and positive control respectively. The cell culture supernatant were abandoned 24 h after transfection, then 100μL passive lysis buffer was added per well to lyse the cells for 15 min. The cell samples were collected respectively and thr relative activity of promoter was measured by Glomax 96 well plate determinator (Promega, WI, USA).

The cell culture medium was changed 24 h after A549 cells were transfected with MICA/ MICB, at the same time, IL-2 (50 IU/ml) alone, 5-FU (10 μM) alone or 5-FU+IL-2 (10 μM+50 IU/ml) was added and incubated for 8 h. Finally, the relative activity of promoter was measured according to the above mentioned method.

Statistical analyses

The data in this article were analyzed using a one-way analysis of variance (ANOVA), followed by Dunnett's test to identify any differences between either the control and drug-treated groups or the isotype antibody control and antibody-treated groups. The results are presented as the means±standard deviation (SD). *P*-values of less than 0.05 were considered statistically significant.

Results

5-FU+IL-2 immunochemotherapy effectively suppresses the growth of A549 in nude mice

As the Figure 1 shown, 5-FU+IL-2 exhibited a significant inhibitory effects in A549-bearing mice. During the treatment, the tumor volume of each mouse was measured every three days. On day 21, mice were killed and tumor weight were measured. The obtained results indicated that the tumor volumes were remarkably decreased in the 5-FU+IL-2 treatment group (Figure 1A). Similar results were obtained in the tumor weights (Figure 1B), and the tumor inhibitory rate was increased to 75.2% in combination therapy group, whereas that in the monotherapy with 5-FU or IL-2 were 60.3% and 36.8%, respectively.

5-FU+IL-2 immunochemotherapy activates cytotoxic activity of NK cells

Considering that NK cells played a predominant role in innate immune system to eliminate cancer cells, and the nude mice were deficient of T cells. Therefore, we suspect that activating NK cells was the main contributor in inhibiting A549 growth *in vivo*. Then we detected both the the percentage of NK cells (defined as DX5+) in spleens and the specific lysis activity of NK cells against A549 cells. As the Figure 2A indicated, the number of NK cells in spleens was 5.28% in the 5-FU+IL-2 treatment group, whereas that in the 5-FU or IL-2 treated alone group were 3.67% and 4.89%, respectively. These data illustrated that the number of NK cells were upregulated

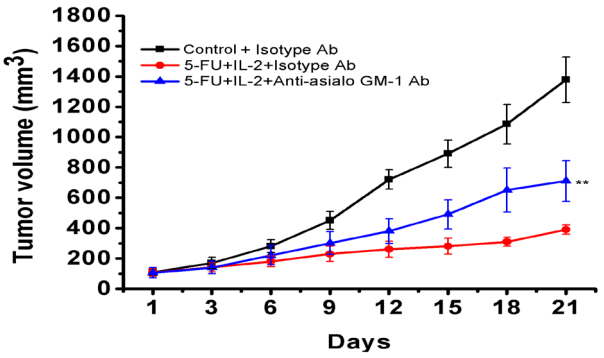

Figure 3. NK Cell Cytotoxicity is Crucial for the Anti-A549 Effects of 5-FU and IL-2 Combination therapy. Selective depletion of NK cells by intraperitoneal injection of an anti-asialo-GM-1 antibody abrogated the 5-FU+IL-2 antitumor effects. (n=6; the values represent the means±SD; **p<0.01 compared with the 5-FU +IL-2 and antibody treated group)

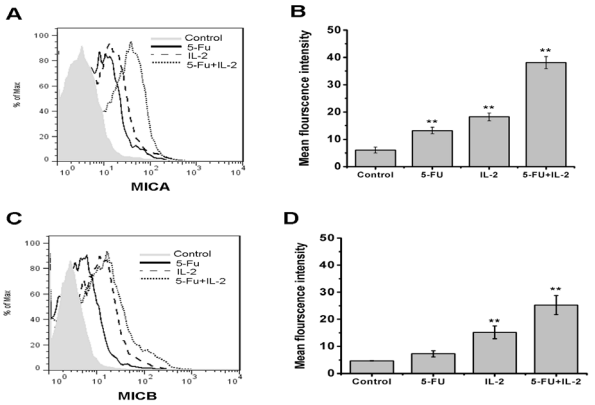

Figure 4. 5-FU+IL-2 Immunochemotherapy Enhances the Expression of MICA/MICB in A549 Cells. A549 cells were cultured without (control) or with 5-FU (10 μM), IL-2 (50 IU/ml) or with 5-FU+IL-2 (10 μM and 50 IU/ml, respectively) for 24 hours.The expression of MHC class I in A549 cells was detected with flow cytometry using Anti-Human MICA/MICB PE and presented as mean fluorescence intensity (MFI). Controls include unstained cells and isotype controls of the corresponding IgG isotype. **A, B)** Peak chart of the flow cytometry analysis (Anti-Human MICA PE) and corresponding MFI (n= 3; **p<0.01). **C, D)** Peak chart of the flow cytometry analysis and corresponding MFI (n= 3; the values represent the means±SD, **p<0.01, compared with the control group)

more in combination therapy than that in the monotherapy group. Simutaniously, the of NK cytotoxicity against A549 cells was also increased in the 5-FU+IL-2-treated group (Figure 2B). Taken together, 5-FU+IL-2 prevented tumor growth *in vivo* through stimulating NK cells.

Depletion of NK cells impairs the antitumor effects of 5-FU+IL-2 immunochemotherapy in A549-bearing nude mice

Both the significant antitumor effects and upregulation of NK cytotoxicity in combination therapy *in vivo* strongly suggested that NK cells were crucial for 5-FU+IL-2-induced lung cancer suppression. To confirm this hypothesis, NK cells were depleted in A549-bearing nude mice via intraperitoneal injection of an anti-asialo-GM1 antibody. As shown in Figure 3, treatment with this

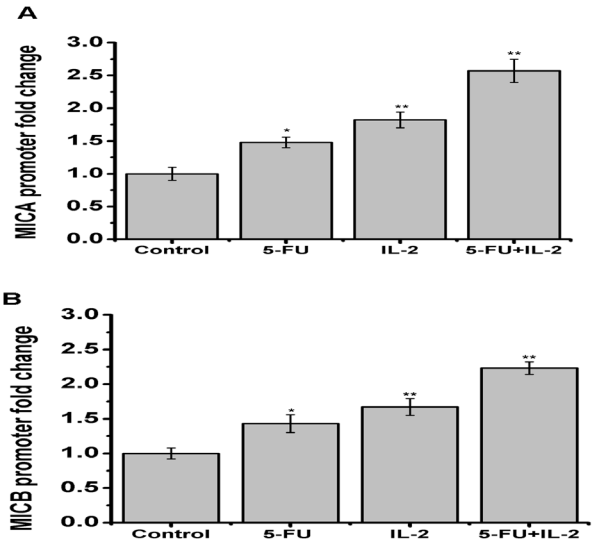

Figure 5. 5-FU+IL-2 Immunochemotherapy Stimulates the Promoter Activity of MICA/MICB. 5-FU alone (10 μM), IL-2 alone (50 IU/ml), or 5-FU+IL-2 (10 μM and 50 IU/ml, respectively) was added after transfection and incubated for 8 h. Finally, the relative activity of promoter was measured.The control referred to groups untreated with 5-FU or IL-2. A The MICA promoter fold change. B The MICB promoter fold change (n= 3; the data represent the means±SD, **p<0.01, *p<0.05, compared with the control group)

antibody almost completely abolished the 5-FU+IL-2-induced suppression of A549 tumor growth. Therefore, these results indicated that NK cytotoxicity is vital to the anti-lung cancer activity of 5-FU and IL-2.

5-FU+IL-2 immunochemotherapy increases the expression of MICA/MICB in A549 cells

The expression of MHC class I-related chain molecule (including MICA and MICB) expressed on tumor cells is essential for recognition by NK cells. Therefore, we evaluated the effects of different treatments on the expression of MICA/MICB in A549 cells *in vitro*. When the cells treated with 5-FU, IL-2, or with 5-FU+IL-2 for 24 hours, the MICA expression was detected using flow cytometry. As the Figure s 4A and C indicated, combination of 5-FU and IL-2 treatment significatly upregulated expression of MICA/MICB. The results of mean flourscence intensity (MFI) showed that the MFI in 5-FU+IL-2 combination therapy was 36.88, whereas that in the 5-FU or IL-2 treatment group was 12.9 and 17.12, respectively. Likewise, MFI showed in Figure 4D indicated the combination therapy significantly increased the expression of MICB in A549 cells.

5-FU+IL-2 activates the promoter activity of MICA/MICB

Since the expressions of both MICA and MICB were upregulated in the 5-FU+IL-2 treatment group, then we detect the promoter activity of MICA and MICB in 5-FU+IL-2 group. As shown in Figure 5A, the MICA promoter activity was increased to 1.45-, 1.79 - and 2.72, -fold at the 5-FU, IL-2 and 5-FU+IL-2 group, respectively, compared with the control group. Similar results obtained in the MICB prompter, which was remarkably increased

in the 5-FU and IL-2 combination threated group (Figure 5B). Hence, combination therapy of 5-FU and IL-2 would stimulated the promoter activity of MICA and MICB firstly, then enhancing the protein expression of these two proteins.

Discussion

Lung cancer is the most common malignant cancer and has been associated with high fatality rates in humans. Chemotherapy in the clinical management of lung cancer cause significant side effects. Interleukins which could stimulate the immune response has been applied to treat cancer, such as IL-7, IL-12 (Ahamed et al., 2014; Yuan et al., 2014). Therefore, combination with immunotherapy was developed to decrease the toxicity of chemotherapy in treating lung cancer and the relative mechanism would lay a foundation in clinic use.

5-FU is widely used in chemotherapeutic regimens. Although 5-FU-based chemotherapy improves the overall survival of patients, the response rate is extremely low. In order to overcome this challenge, 5-FU combined with immunotherapy attracted the scientists' attention. IL-2, secreted by activated T cells, could promote the proliferation and cytolytic activity of CTLs and NK cells to eliminate cancer cells (Fearon et al., 1990; Makedonas et al., 2010). Moreover, it has been reported that immunochemotherapy with IL-2 in combination with 5-FU is superior to 5-FU chemotherapy in enhancing survival rate (Savage et al., 1997). In this study, we found that in the 5-FU+IL-2-treated group, the tumor growth of A549 was significantly inhibited, which is better than 5-FU or IL-2 alone treated group.Considering that IL-2 played a vital role in activating immune system, the relative immunoregulatory mechanisms were studied further and the result would provide a basis for the combination therapy of 5-Fu and IL-2 in clinic.

NK cells and CTLs played crucial roles in cell-mediated immunity in innate and adaptive immunity and eliminated target tumor cells. Notably, tumor cells often exhibit low MHC expression, thereby escaping from CTL-mediated immunologic surveillance (Sandel et al., 2005). Bone marrow-derived NK cells are an important effector population required to eliminate malignant tumor cells in the absence of MHC expression or the presence of mutant MHC molecules (Siddle et al., 2013; Kärre, 2002). Therefore, NK cells may play more important roles than CTLs in the treatment of lung cancer. It has been reported that IL-7 and IL-12 exhibited anti-tumor activity via stimulating CTLs Interleukins which could stimulate the immune response has been applied to treat cancer, such as IL-7, IL-12 (Ahamed et al., 2014; Yuan et al., 2014). Moreover, we think that drugs or interleukins which could activate NK cells would be more potential in treating cancer. Our results further indicated that both the percentage and the specific lysis activity of NK cells were enhanced in the 5-FU+IL-2-treated group, indicating that the activation of NK cells was the main contributor in 5-FU+IL-2-induced tumor suppression. Moreover, NK cell depletion via pretreatment with an anti-asialo-GM-1 antibody in A549-bearing nude mice nearly completely abolished in the 5-FU+IL-2-induced suppression of lung cancer cells. Based on these results, we concluded that enhanced NK cytotoxicity is crucial and that NK cells may be a unique target in A549-bearing mice.

In addition, there are a lot of receptors expressed on NK cells, including activation and inhibitory receptors. Among NK activating receptors, natural killer group 2, member D receptor (NKG2D) is a C-type lectin-like transmembrane glycoprotein recognizing self-molecules (referred as NKG2D ligands; NKG2DLs) that emerged as a pivotal signaling pathway supporting cancer immune surveillance (Diefenbach et al., 1999; Moretta et al., 2001). MHC class I chain-related A and B (MICA and MICB) or UL16 binding proteins are NKG2D Ligands (Ljunggren et al., 2008; Kim et al., 2008; Nausch et al., 2008). To further confirm the role of 5-FU+IL-2 immunochemotherapy played in MICA/MICB, the promoter activity was investigated. We cloned MICA/MICB promoter and detected the promoter activity after incubation with 5-FU+IL-2. Results showed that 5-FU+IL-2 immunochemotherapy could significantly enhance the activity of MICA/MICB promoter. As described in the theory of Gasser (Gasser et al., 2005; Gasser et al., 2006): 5-FU+IL-2 immunochemotherapy induced DNA damage, which activated stress response molecules (ATM and ATR) and a variety of effector molecules downstream. Then some transcription factors were excited, which up-regulated the transcriptional level of MICA/MICB. The fact that 5-FU+IL-2 increased activity of MICA/MICB promoter consistent with the increased expression of MICA/MICB in A549 cells.

In conclusion, our study provides more insight into the mechanism of action of IL-2 in combination with 5-FU. This immunochemotherapy enhances the recognition of A549 cells by NK cells and up-regulates the activity of MICA/MICB at the level of transcription and protein expression. All of the results presage the presage the potential use of immunochemotherapy of 5-FU and IL-2 in treating lung cancer in clinic.

Acknowledgements

The authors would like to thank Prof. dr. Karel Geboes, Prof. dr. Wim Ceelen.

References

Ahmed Ali HA, Di J, Mei W, et al (2014). Antitumor activity of lentivirus-mediated interleukin -12 gene modified dendritic cells in human lung cancer *in vitro*. *Asian Pac J Cancer Prev*, **15**, 611-6.

Das D, Preet R, Mohapatra P, et al (2013). 1, 3-Bis (2-chloroethyl)-1-nitrosourea enhances the inhibitory effect of Resveratrol on 5-fluorouracil sensitive/resistant colon cancer cells. *World J Gastroenterol*, **19**, 7374-88.

Diefenbach A, Raulet DH (1999). Natural killer cells: stress out, turn on, tune in. *Curr Biol*, **9**, 851-3.

Du G, Ye L, Zhang G, et al (2012). Human IL18-IL2 fusion protein as a potential antitumor reagent by enhancing NK cell cytotoxicity and IFN-γ production. *J Cancer Res Clin Oncol*, **138**, 1727-36.

Fearon ER, Pardoll DM, Itaya T, et al (1990). Interleukin-2 production by tumor cells bypasses T helper function in

the generation of an antitumor response. *Cell*, **60**, 397-403.
Gaffen SL, Liu KD (2004). Overview of interleukin-2 function, production and clinical applications. *Cytokine*, **28**, 109-23.
Gasser S, Orsulic S, Brown EJ, et al (2005). The DNA damage pathway regulates innatemimune system ligands for the NKG2D receptor. *Nature*, **436**, 1186-90.
Gasser S, Raulet DH (2006). Activation and self-tolerance of natural killer cells. *Immunol Rev*, **214**, 130-42.
Holt GE, Podack ER, Raez LE (2011). Immunotherapy as a strategy for the treatment of non-small-cell lung cancer. *Therapy*, **8**, 43-54.
Kärre K (2002). NK cells, MHC class I molecules and the missing self. *Scand J Immunol*, **55**, 221-8.
Kim JY, Bae JH, Lee SH, et al (2008). Induction of NKG2D ligands and subsequent enhancement of NK cell-mediated lysis of cancer cells by arsenic trioxide. *J Immunother*, **31**, 475-86.
Savage P, Costelna D, Moore J, et al (1997). A phase II study of continuous infusional 5-fluorouracil and subcutaneous Interleukin-2 (IL-2) in metastatic renal cancer. *Eur J Cancer*, **33**, 1149-51.
Takiuchi H, Ajani JA (1998). Uracil-tegafur in gastric carcinoma: a comprehensive review. *J Clin Oncol*, **16**, 2877-85.
Macdonald JS, Astrow AB (2001). Adjuvant therapy of colon cancer. *Semin Oncol*, **28**, 30-40.
Moretta A, Bottino C, Vitale M, et al (2001). Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol*, **19**, 197-223.
Ljunggren HG (2008). Cancer immunosurveillance: NKG2D breaks cover. Immunity, **28**, 492-4.
Makedonas G, Hutnick N, Haney D, et al (2010). Perforin and IL-2 upregulation define qualitative differences among highly functional virus-specific human CD8 T cells. *PLoS Pathog*, **6**, 1000798.
Martinez J, Huang X, Yang Y (2010). Direct TLR2 signaling is critical for NK cell activation and function in response to vaccinia viral infection. *PLoS Pathog*, **6**, 1000811.
Nausch N, Cerwenka A (2008). NKG2D ligands in tumor immunity. *Oncogene*, **27**, 5944-58.
Sandel MH, Speetjens FM, Menon AG, et al (2005). Natural killer cells infiltrating colorectal cancer and MHC classIexpression. *Mol Immunol*, **42**, 541-6.
Siddle HV, Kreiss A, Tovar C, et al (2013). Reversible epigenetic down-regulation of MHC molecules by devil facial tumour disease illustrates immune escape by a contagious cancer. *Proc Natl Acad Sci USA*, **110**, 5103-8.
Stella GM, Luisetti M, Pozzi E, et al (2013). Oncogenes in non-small-cell lung cancer: emerging connections and novel therapeutic dynamics. *Lancet Respir Med*, **1**, 251-61.
Wang ZX, Cao JX, Liu ZP, et al (2014). Combination of chemotherapy and immnotherapy for colon cancer in China: A meta-analysis. *World J Gastroenterol*, **20**, 1095-106.
Xu Y, Wang M (2014). Progress in immunotherapy for non-small cell lung Cancer. *Zhongguo Fei Ai Za Zhi*, **17**, 34-41.
Ye L, Fan J, Shi X, et al (2014). Tumor necrosis therapy antibody interleukin-2 fusion protein elicits prolonged and targeted antitumor effects *in vivo*. *Appl Microbiol Biotechnol*, **98**, 4053-61.
Yuan CH, Yang XQ, Zhu CL, et al (2014). Interleukin-7 enhances the *in vivo* anti-tumor activity of tumor-reactive CD8+ T cells with induction of IFN-gamma in a murine breast cancer model. *Asian Pac J Cancer Prev*, **15**, 265-71.
Zheng YW, Li RM, Zhang XW, et al (2013). Current adoptive immunotherapy in non-small cell lung cancer and potential influence of therapy outcome. *Cancer Invest*, **31**, 197-205.