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Immunotherapy with intralesional and systemic interleukin-2 of patients with non-small-cell lung cancer

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Abstract. Eight patients affected by non-small-cell lung cancer were treated with intralesional and systemic recombinant IL-2 (rIL-2) injection with the aim of activating both tumour-infiltrating lymphocytes and circulating cytotoxic or killer cells. The schedule of treatment was as follows: a daily fine-needle transparietal intralesional rIL-2 injection (1×10^5 Cetus units) from day 1 to day 5 and systemic rIL-2 infusion (1×10^5 Cetus units kg^{-1} day^{-1}) from day 6 to day 10. One to four cycles of treatment were received by each patient. Clinical and immunological evaluations were performed (a) before treatment, (b) following the intralesional rIL-2 administration, (c) 1 h after the beginning of rIL-2 infusion and (d) at the end of the systemic rIL-2 infusion. No complete remission was achieved, two patients showed a partial remission, three resulted in stable disease and three patients progressed. Natural killer and lymphokine-activated killer cell activity dramatically decreased 1 h after the beginning of rIL-2 infusion and increased at the end of treatment. A progressive increase of circulating $CD8^+$ and HLA class II^+ T cells as well as of $CD8^+$ T cell clones, most of which displayed NK activity, was recorded following rIL-2 infusion. Present data indicate that (a) the local administration of rIL-2 coupled with systemic rIL-2 infusion may be suggested as an alternative approach for the immunotherapy of lung cancer, (b) rIL-2 induces different immunological modifications according to the route and the time of its administration and (c) rIL-2 administration increases the amount of circulating immune cells with potential antitumour activity.

Key words: Interleukin-2 – Lung cancer – Immunotherapy

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Introduction

Interleukin-2 (IL-2), a cytokine produced by activated $CD4^+$ T lymphocytes, is able to overcome the state of immunosuppression and tolerance induced by cancer [13, 20]. Therefore, a large number of clinical trials have been performed in order to evaluate the efficacy of IL-2 administration, either alone or in combination with lymphokine-activated killer (LAK) cells, in the treatment of patients with advanced cancer [8, 19].

It has been shown that IL-2 injected at the tumour growth site may (a) activate non-reactive lymphocytes harbouring the neoplasm to release lymphokines [12], (b) trigger a cytotoxic response towards the tumour [7] and (c) overcome the inhibition of tumour-infiltrating lymphocytes [3]. Using this approach, about 60% complete or partial remissions of recurrent squamous cell carcinoma of the head and neck have been reported [4].

The incidence and mortality of lung cancer are the highest amongs all malignancies [21] and only a small survival benefit can be achieved with conventional combination chemotherapy [9]. In the present pilot trial we treated eight patients affected by non-small-cell lung cancer with intralesional recombinant IL-2 (rIL-2) injection followed by systemic rIL-2 infusion, in order to keep elevated and long-lasting circulating IL-2 levels that would maintain intralesional lymphocyte cytotoxic activity.

This therapeutic strategy was chosen because the role of chemotherapy in the treatment of non-small-cell lung cancer is controversial [9] and lung cancer patients display a marked early immunosuppression which is, at least in part, produced by suppressor cells [16].

Sequential immune evaluations were performed during rIL-2 treatment by analysing T-cell subsets, natural killer (NK) and lymphokine-activated killer LAK activities as well as the clonal distribution of T cell subsets.

In the present paper the clinical and immunological results of this pilot trial utilizing intralesional and systemic IL-2 for treatment of non-small-cell lung cancer patients are reported.

Materials and methods

Patients. Eight men, aged 55 – 64, affected by non-small-cell lung cancer entered into this study. The admission criteria were: (a) histologically proven evidence of non-small-cell lung cancer (stage IIIb or IV); (b) measurable disease (>3 cm in diameter); (c) no prior chemotherapy, radiotherapy or immunotherapy; (d) no evidence of cardiac disease; (e) absence of central nervous system metastases. Informed consent was obtained from all patients.

Schedule of treatment. Days 1 – 5: a daily fine-needle transparietal intralesional computer-aided-tomography-guided injection (FNTII) of rIL-2 (1×10^5 Cetus units), kindly supplied by Cetus Corporation (Emerville, Calif., USA), was carried out. Days 6 – 10: i. v. infusion of rIL-2 (1×10^5 Cetus Units kg^{-1} day^{-1}) was performed at a subcontinuous rate (16 h/day). Treatment was repeated every 21 days.

Immunological evaluation. Immunological evaluation was performed at the following times: (a) before treatment (*T0*); (b) following the five FNTII rIL-2 administrations and before i. v. rIL-2 infusion (*T1*); (c) 1 h after the beginning of the first i. v. rIL-2 infusion (*T2*); (d) at the end of the last systemic rIL-2 infusion (*T3*).

The absolute number of peripheral blood lymphocytes (PBL), their phenotype, the NK activity, the LAK activity generated ex vivo and in vitro and the clonal distribution of T cell subsets were evaluated. The results reported refer to the first rIL-2 treatment.

Cells. PBL were isolated from heparinized peripheral blood by a Ficoll/Hypaque gradient centrifugation. Lymphocytes were washed three times with Hank's balanced salt solution (HBSS; Eurobio, Paris, France) and then resuspended in RPMI-1640 medium (Sigma Chemical Co., St. Louis, Mo., USA).

Monoclonal antibodies. The fluorescein- or phycoerythrin-labelled T11, T3, T4 and T8 monoclonal antibodies (mAb), the mAb IL2-1R and the fluorescein-isothiocyanate(FITC)-conjugated goat anti-(mouse immunoglobulins) antibody (Coulter Immunology, Hialeah, Fla., USA) were purchased from Kontron S. p. A., Milan, Italy. The anti-HLA class II (DR) mAb Q5/13 was kindly supplied by Dr. S. Ferrone, NYMC, Valhalla, N. Y., USA.

Lymphocyte phenotype. Lymphocyte phenotype was assessed by a direct or indirect immune fluorescence method. Briefly, 2×10^5 cells were incubated for 30 min at 4° C with appropriate dilutions of mAb. In indirect immunofluorescence assays cells were washed three times in HBSS and further incubated for 30 min at 4° C with FITC-labelled anti-mouse immunoglobulins antibody. At the end of the incubation time, cells were washed in HBSS twice and resuspended in 0.5 ml HBSS. The percentage of stained cells was determined by flow cytometry utilizing an Epics Profile II cytofluorimeter (Coulter Electronics Inc., Hialeah, Fla., USA).

NK and LAK activity. Cytotoxicity of PBL was tested in a standard in vitro 4-h ^{51}Cr-release assay. LAK cells were also generated in vitro by culturing PBL (1×10^6 cells/ml) with IL-2 (100 U/ml) for 6 days at 37° C in a 5% CO_2 atmosphere.

Target cells utilized in NK and LAK assays were the human-erythroleukaemia-derived K562 cell line and the NK-resistant Burkitt-lymphoma-derived Daudi cell line respectively. Target cells were labelled with 0.1 mCi Na_2CrO_4 (Sorin, Saluggia, Italy) for 45 min at 37° C in a 5% CO_2 atmosphere. After three washings, target cells were resuspended at 5×10^4 cells/ml in RPMI medium supplemented with 10% fetal calf serum (Sigma Chemical Co., St. Louis, Mo., USA), 1% glutamine (Eurobio, Paris, France), 0.1% penicillin and 0.1% streptomycin (complete medium) and plated (5×10^3 cells/well) in round-bottomed 96-well microtitre plates (Falcon-Beckton Dickinson, Lincoln Park, N. J., USA).

Effector cells (2.5×10^6 cells/ml) were then added to target cells at 50 : 1, 25 : 1 and 12 : 1 effector: target cell ratios. Spontaneous and maximum target cell lysis were determined by incubating target cells in complete medium alone or in Triton X-100 0.1% (Sigma Chemical Co., St. Louis, Mo., USA) respectively. Plates were incubated for 4 h at 37° C in a 5% CO_2 atmosphere and then centrifuged for 5 min at 1800 rpm.

^{51}Cr release was evaluated by counting 100 μl supernatant in a gamma counter, and results were expressed as cpm. The percentage maximum target cell lysis was calculated as the average of quadruplicate experiments by the following formula:

$$\text{Maximum lysis (\%)} = 100 \times \frac{\text{sample } ^{51}\text{Cr release} - \text{minimum } ^{51}\text{Cr release}}{\text{maximum } ^{51}\text{Cr release} - \text{minimum } ^{51}\text{Cr release}}$$

T cell clone generation. PBL were cloned by limiting-dilution as described elsewhere [12]. Briefly, PBL were resuspended at 100, 50, 10 and 5 cells/ml in complete medium containing phytohaemagglutinin protein (PHA-P; Difco, Detroit, Mich., USA) 1 : 1000 final dilution. Then 100 μl cell suspension was plated in round-bottomed 96-well microtitre plates (10, 5, 1 and 0.5 cells/well) containing 100 μl (1×10^5 cells/well) autologous irradiated PBL (35 Gy).

After 24 h, 100 μl supernatant was replaced with 100 μl complete medium containing recombinant IL-2 (100 IU/ml). After 6 days T lymphocytes were restimulated by adding allogeneic irradiated feeder cells (1×10^5 cells/well) and recombinant IL-2 (100 IU/ml).

Statistical analysis. Mean values ± SD are reported throughout the study. Statistical analysis was performed by Student's *t*-test for paired samples. A *P* value less than 0.05 was considered as significant.

Table 1. Clinical data of lung cancer patients treated with recombinant interleukin-2 (rIL-2)

Case no.	Age (years)	Sex	Tumour location[a]		Stage	IL-2 cycle no.	Outcome[b]	Duration (months)
			Right	Left				
1	48	M	UA		IIIb	1	NR	–
2	55	M	LLB		IV	2	PR	6
3	51	M		UA	IV	1	SD	6
4	59	M	LPB		IIIb	1	NR	–
5	61	M		LS	IV	2	NR	–
6	64	M		LLB	IV	4	PR	9
7	57	M		LPB	IIIb	3	SD	8
8	46	M		MM	IV	3	SD	6

[a] UA, upper anterior; LLB, lower lateral basal; LPB, lower posterior basal; LS, lower superior; MM, middle medial

[b] NR, no response; PR, partial remission (50% of reduction of all measurable lesions for at least 4 weeks); SD, stable disease (reduction less than 50% or increase of less than 25% of the measurable lesions)

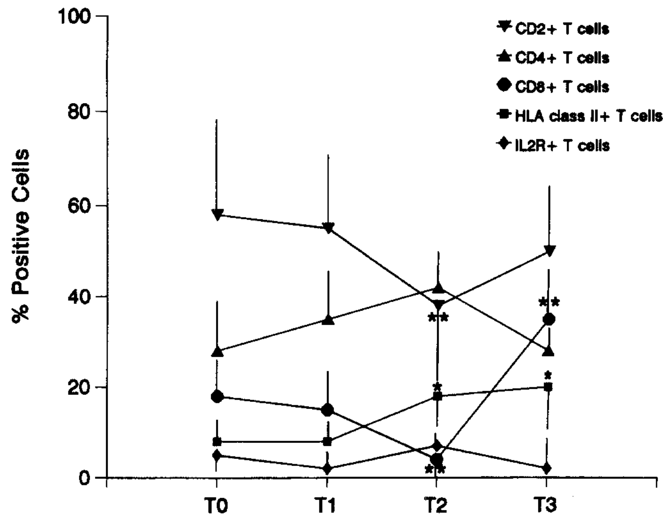

Fig. 1. Percentage of lymphocytes expressing CD2, CD4, CD8, HLA class II and IL-2 receptor antigens before (*T0*) and after intralesional (*T1*) or systemic (*T2* and *T3*) recombinant interleukin-2 (rIL-2) treatment. Values are means ± SD; $P<0.05$ (*) and $P<0.01$ (**) versus baseline are reported

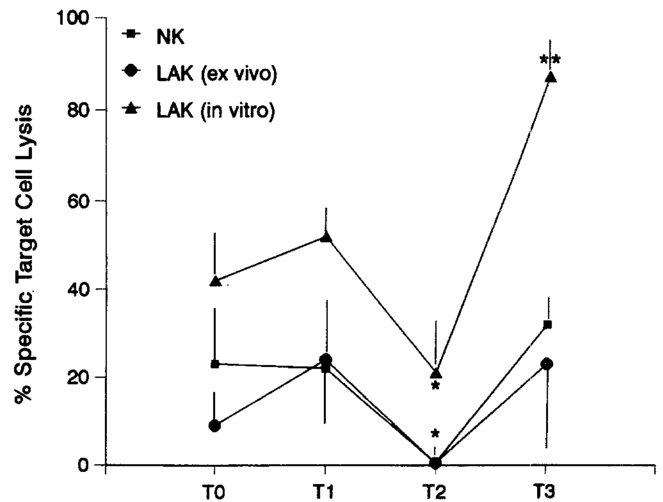

Fig. 2. Cytotoxic activity of natural killer (*NK*) cells and lymphokine-activated killer (*LAK*) cells generated ex vivo and in vitro before (*T0*) and after intralesional (*T1*) or systemic (*T2* and *T3*) rIL-2 treatment. The effector: target ratio 25 : 1 is shown. Values are means ± SD; $P<0.05$ (*) and $P<0.01$ (**) versus baseline are reported

Results

Clinical evaluation

A summary of patients' status and clinical course following rIL-2 treatment is reported in Table 1. No complete remission was achieved, two patients showed a partial remission lasting 6 and 9 months, three resulted in stable disease and three patients progressed. When tumour relapsed or advanced the clinical course rapidly progressed to death a few months later.

Toxicities and side-effects observed after both local and systemic rIL-2 treatment are summarized in Table 2. It is worth noting that FNTII rIL-2 injection involved three episodes of pneumothorax (37%), a complication that makes this procedure somewhat dangerous. During systemic rIL-2 administration all patients complained of some side-effect that spontaneously resolved within 3–6 h following cessation of rIL-2 infusion. Fever and malaise were mitigated by paracetamol, aspirin or indomethacin administration. Four patients completed three rIL-2 treatments and two patients completed two rIL-2 treatments. In two patients systemic rIL-2 infusion was stopped on day 1 because of hypotension and tachyarrythmias.

Table 2. Side-effects of rIL-2 treatment

Effect	Incidence		WHO grading	
	No.	(%)	I–II	III
Pneumothorax[a]	3	37	–	–
Fever (>38° C)	8	100	4	4
Malaise	8	100	5	3
Anorexia	6	75	5	1
Hypotension	8	100	6	2
Arrhythmias	4	50	2	2
Chills	3	37	3	0
Diarrhoea	3	37	3	0
Mucositis	7	87	6	1

[a] After fine-needle transparietal rIL-2 injection

PBL absolute number and phenotype

The mean absolute number of PBL was 1988 ± 960 cells/mm^3 at *t0*, decreased to 660 ± 120 cells/mm^3 and 730 ± 80 cell/mm^3 at *t1* and *t2* respectively, and rose to 3155 ± 1437 cells/mm^3 at *t3*.

The percentage of CD4+ T lymphocytes did not significantly change throughout treatment. The percentage of CD2+ and CD8+ T cells was significantly reduced at *t2* as compared with the baseline value (38 ± 19% and 5 ± 3% versus 58 ± 20% and 18 ± 6% respectively; $P<0.01$). CD8+ T cells significantly increased above the baseline value at *t3* (38 ± 9%; $P<0.01$). A progressive and significant increase of HLA-class-II-antigens-bearing T cells was seen at *t2* and *t3* with respect to the baseline value (18 ± 12% and 22 ± 10% versus 5 ± 3%; $P<0.05$) (Fig. 1).

NK and LAK activity

The results of NK and LAK assays at a 25 : 1 effector: target cell ratio are reported.

The mean NK activity at *t0* and *t1* was 23 ± 16% and 22 ± 16% respectively. NK activity suddenly and significantly decreased at *t2* (0.5 ± 0.5%; $P<0.05$) and returned above the baseline level at *t3* (32 ± 4%) (Fig. 2).

The mean ex vivo LAK activity at *t0* was 9 ± 8%. It increased to 24 ± 17% at *t1*, was practically undetectable at *t2* (0.5 ± 0.5%; $P<0.05$) and increased above the baseline level at *t3* (23 ± 20%) (Fig. 2).

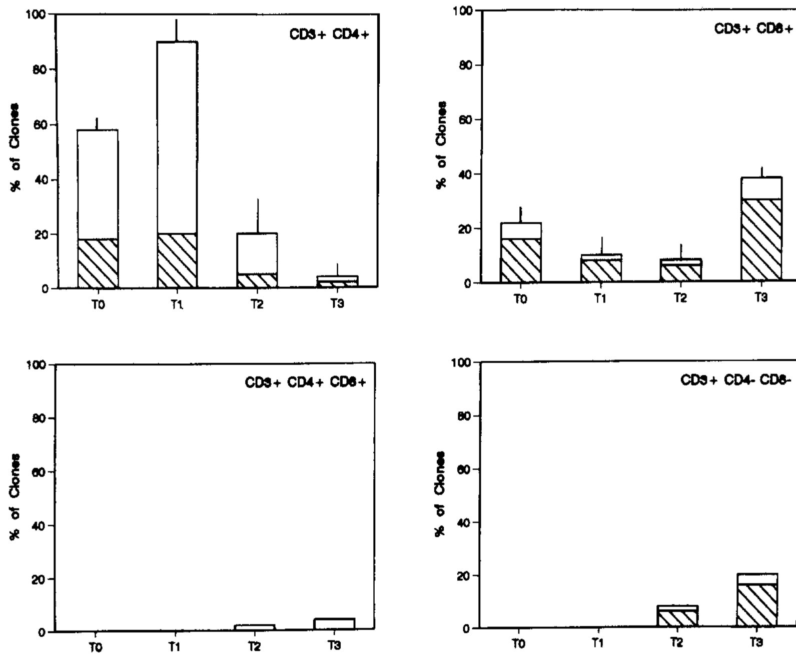

Fig. 3. Percentage of CD3+ CD4+, CD3+ CD8+, CD3+ CD4+ CD8+ and CD3+ CD4− CD8− clones generated before (*T0*) and after intralesional (*T1*) or systemic (*T2* and *T3*) rIL-2 treatment. The percentage of clones displaying NK activity is also indicated by the *shaded areas*. Values are means ± SD

The mean LAK activity generated in vitro was 42±11% and 52±7% at *t*0 and *t*1 respectively. It significantly decreased at *t*2 (21±9%; P <0.05) and then dramatically increased above the baseline level at *t*3 (87±5%; P <0.01).

T cell clones

A total of 392 clones were generated and their phenotype and cytotoxic activity against the K562 cell line were tested. Since no differences in the clonal distribution were found among patients, the global analysis of the results obtained is reported.

The clonal efficiency of CD3+ CD4+ lymphocytes was 58±3% at *t*0. It incresed up to 90±14% at *t*1, and dramatically decreased at *t*2 (20±15%) and *t*3 (4±4%). About 25% of these CD3+ CD4+ clones displayed NK activity. The clonal efficiency of CD3+ CD8+ lymphocytes was 22±4% at *t*0. It decreased to 10±5% and 8±4% at *t*1 and *t*2 respectively, and increased above baseline values at *t*3 (38±3%). About 80% of these CD3+ CD8+ clones demonstrated NK activity (Fig. 3). The clonal efficiency of each T cell subset correlated, at each evaluated time, with the CD4/CD8 ratio.

Notably, during systemic rIL-2 infusion, we also observed the generation of both CD3+ CD4− CD8− double-negative and CD3+ CD4+ CD8+ double-positive clones. Almost all CD3+ CD4− CD8− T cell clones showed NK activity (Fig. 3).

Discussion

Systemic rIL-2 administration is a consolidated therapeutic approach for the treatment of cancer patients [12]. Local or regional therapy with rIL-2 has been proposed as an attractive approach for immunotherapy of cancer aimed at activating tumour-infiltrating lymphocytes to recognize and react against malignant cells. It may also trigger the local secretion of cytokines and/or chemotactic factors leading to the recruitment of circulating lymphocytes, NK cells, macrophages, and granulocytes at the tumour site [12]. A poorly immunogenic tumour would then be transformed into a target of an efficient immune response. In the present study, local and systemic rIL-2 were sequentially administered in an attempt to obtain a better clinical response for non-small-cell lung cancer.

The FNTII is a consolidated method that enables a lung mass to be reached for diagnostic purposes in neoplastic or infectious diseases [24, 25]. In the present trial we adopted this procedure in order to introduce therapeutic substances into the tumour mass. Such a procedure, although performed by an experienced operator, involved a high incidence (37%) of secondary pneumothorax and severe respiratory imbalance. Thus, when considering the frequent poor respiratory conditions of lung cancer patients, we conclude that FNTII for rIL-2 administration is a procedure to be reserved to very skilled operators and must be performed in a hospital setting.

From the clinical point of view, two partial remissions and three cases of stable disease lasting from 6 to 9 months were recorded. These results are similar to those obtained in lung cancer patients, comparable for disease stage, treated with conventional chemotherapy [9]. They are also comparable to those obtained by other authors in patients with advanced non-small-cell lung cancer treated with systemic rIL-2 associated with doxorubicin [18], interferon α [11], or killer cells activated ex vivo [1].

The systemic toxicities recorded following the administration of rIL-2 were in the range reported by others [22]. Moreover, most treatment-related systemic effects spontaneously resolved within 3–6 h following cessation of therapy.

The modifications of PBL subpopulations and functions, analysed both at the polyclonal and at the clonal level, were quite different depending on the bleeding time.

The analysis of PBL subpopulations indicates that intralesional rIL-2 administration does not modify the distribution of T cell subsets. Systemic rIL-2 infusion induced, as previously reported by others [18], an early depletion of CD2+ and CD8+ circulating T cells followed by an increase of CD8+ T cells. Whether the mechanism(s) underlying these changes in T cell subsets have to be attributed to a direct influence of IL-2 on immune cell distribution rather than to its effect(s) on the endocrine homeostasis is not completely clear.

A progressive and significant increase of T lymphocytes expressing HLA class II antigens was observed, in agreement with previous reports, during rIL-2 administration [23]. A defective expression of HLA class II antigens by activated T cells from patients with malignancy has been reported as one of the hallmarks that characterize the immune abnormality of neoplasia [10]. Thus the presence of a significant percentage of HLA-class-II-positive T cells following rIL-2 injection may indicate that this treatment may be able to correct the defect.

After intralesional rIL-2 administration no significant modifications of NK and LAK activities were observed. A significant reduction of circulating T lymphocytes (mostly CD8+ T cells) and a virtual absence of NK and LAK activities were found 1 h after the beginning of rIL-2 infusion. These findings are in agreement with those reported by other authors [14]. They have been attributed to the redistribution of lymphocytes among the body compartments triggered by IL-2-stimulated cortisol production [15]. Nevertheless in our patients no significant variation of serum cortisol and adrenocorticotropic hormone levels were found (data not shown). On the base of the data published by others, which clearly indicate that IL-2 induces an early depletion of cells capable of generating LAK activity [2, 5] one can assume that the present observation may be mostly related to the depletion of NK and/or LAK cell precursors. Accordingly, our data show that the lack of NK activity is concomitant with the decrease of ex vivo and in vitro LAK generation. A striking increase of NK and ex-vivo- as well as in-vitro-generated LAK cell activity was observed at the end of rIL-2 infusion. The data suggest that rIL-2 treatment may activate these cytotoxic cells thus potentiating the immunosurveillance of lung cancer patients against the tumour.

The clonal efficiency of CD4+ T cells transiently increased after intralesional rIL-2 injection and diminished after systemic rIL-2 administration. The clonal efficiency of CD8+ T cells, most of which demonstrated NK activity, increased at the end of rIL-2 treatment. Furthermore, CD3+ CD8+ CD4+ double-positive and CD3+ CD4− CD8− double-negative clones were generated at the end of rIL-2 treatment. In agreement with previous literature [6], these double-negative clones may originate from T lymphocytes bearing the $\gamma\delta$ T cell receptor that display non-MHC-restricted cytolytic activity against tumour cells. We suggest that rIL-2 treatment might have expanded this T cell subpopulation with potential antitumour activity.

The analysis of peripheral blood lymphocytes may not be sufficient to understand the immune modifications triggered at the tumour site by the intralesional injection of rIL-2. A clarifying answer to such a question could come from the analysis of lymphocytes isolated from the neoplastic tissue before and following the local treatment. However, the procedures necessary to obtain a sufficient amount of cells to perform immunological tests are, in our judgment, not applicable in a clinical setting.

Taken together, the data reported here indicate that (a) the local injection of rIL-2 coupled with systemic rIL-2 infusion might be an alternative approach for the immunotherapy of patients with advanced non-small-cell lung cancer, although it does not seem to add therapeutic benefit to other more consolidated therapeutic strategies; (b) rIL-2 administration to lung cancer patients induces different immunological modifications according to the route and the time of its administration; and (c) the intravenous injection of rIL-2 affects subset distribution as well as functional capacities of circulating lymphocytes and increases the amount of circulating immune cells with potential antitumour activity.

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